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Impact of maternal and nutritional factors on the gut microbiome at early age

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„Das Staunen ist der Anfang der Erkenntnis.“

Platon

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Publications

I. Nicole Simone Treichel, Zala Prevoršek, Vesna Mrak, Matea Kostrić, Gisle Vestergaard, Bärbel Fösel, Stefan Pfeiffer, Blaž Stres, Anne Schöler, Michael Schloter; Effect of the nursing mother on the gut microbiome of the offspring during early mouse development. Microb Ecol (2019). https://doi.org/10.1007/s00248-019-01317-7

Contribution:

- sample preparation and 16S rRNA gene sequencing
- analysis and interpretation of sequencing data
- writing of the manuscript

II. Nicole Simone Treichel, Zala Prevoršek, Neža Grgurevič, Alesia Walker, Gisle Vestergaard, Stefan Pfeiffer, Bärbel Fösel, Gregor Majdič, Blaž Stres, Philippe Schmitt-Kopplin, Michael Schloter; Impact of high-fat diet on DCA production by intestinal bacteria in the murine gut. (in preparation)

Contribution:

- contributed to experimental design
- sample preparation and shotgun sequencing
- data analysis and interpretation
- writing of the manuscript

Additional Publications

Following publications have been created during my doctoral thesis, with the help of my expertise in 16S rRNA gene sequencing. Since I was not involved in the development of the experimental design and hypothesis, the data was not used for this thesis.

- Robert Šket, Nicole Treichel, Susanne Kublik, Tadej Debevec, Ola Eiken, Igor Mekjavić, Michael Schloter, Marius Vital, Jenna Chandler, James M. Tiedje, Boštjan Murovec, Zala Prevoršek, Matevž Likar, Blaž Stres; Hypoxia and inactivity related physiological changes precede or take place in absence of significant rearrangements in bacterial community structure: The PlanHab randomized trial pilot study. PLoS ONE 12(12):e0188556. (2017)

- Robert Šket, Nicole Treichel, Tadej Debevec, Ola Eiken, Igor Mekjavic, Michael Schloter, Marius Vital, Jenna Chandler, James M. Tiedje, Boštjan Murovec, Zala Prevoršek, Blaž Stres; Hypoxia and inactivity related physiological changes (constipation, inflammation) are not reflected at the level of gut metabolites and butyrate producing microbial community: The PlanHab study; Frontiers in Physiology 4;8:250. (2017)

Summary

The importance of the gut microbiome for the overall health of its host has been demonstrated by various studies. Therefore, it is of high interest to investigate the factors influencing the gut bacteria community composition during its establishment, as they are assumed to be involved in the development of conditions due to an aberrant microbiome later in life. The first factors shaping the gut microbiome include genetics and the mode of delivery, while post-natal factors at early age include the environmental conditions, breastfeeding and administration of antibiotics.

With this project I aimed to investigate the maternal factors influencing the offspring's gut bacterial composition. As it is unclear to which extend the microbiome is determined by pre-natal factors and delivery in comparison to post-natal factors, it was chosen to conduct a cross-fostering experiment on two genetically different mouse lines, being prone to be obese or lean, respectively. The microbiome of the colon and cecum of three weeks old mice, was analyzed by high-throughput 16S rRNA gene sequencing. I could show that the birth as well as the nursing mother had a distinct impact on the gut microbiome of the offspring, especially on the families *Porphyromonadaceae*, *Rikenellaceae* and *Lactobacillaceae.* Both affected the α- and β-diversity of the offspring's gut microbiome and shaped its composition. As I found indication for the composition of the maternal milk playing a major role, I choose to continue to isolate the impact of a high-fat content of the food at early age.

A high-fat feeding trial showed that young mice, which were on this diet for the same time as they are usually consuming maternal milk, showed similar negative compositional changes as adults on high-fat diet and this independently of obesity.

As accumulating evidence indicates that the increase of bile acids, induced by high-fat diet, mediates changes of the gut microbiome, I further investigated how this impacts murine gut bacteria, which catalyze the transformation of bile acids into secondary bile acids, which are known to play a role in the pathogenesis of colon cancer and several gastrointestinal diseases. I used a metagenomics approach in combination with targeted bile acid profiling and focused on microbes which carry the genes for the bile salt hydrolase (*bsh*) and the bile acid 7α-dehydratase (*baiE*). The first one catalyzes the splitting of the glycine or taurine residue from the primary bile

acids, enabling further downstream modifications. The second one catalyzes the rate-limiting and irreversible step in 7α-dehydroxylation pathway.

I could show that high-fat diet increased the amount of secondary bile acids in the gut and had an impact on the diversity of bacteria capable of secondary bile acid formation. Bacterial families involved in the degradation of bile acids mostly belonged to the phylum *Bacteroidetes,* with the family *Porphyromonadaceae* being predominant. Therefore, high-fat diet and a consequential increase in bile acid levels has a negative impact on both, the diversity of the gut microbiome and the host.

In conclusion the results contributed to the understanding of which factors are involved in shaping the gut microbial community during early age.

Zusammenfassung

Verschiedene Studien belegen inzwischen wie wichtig das Darmmikrobiom für die Gesundheit seines Wirts ist. Da angenommen wird, dass eine Beeinträchtigung des Mikrobioms in jungem Alter an Erkrankungen im späteren Leben beteiligt ist, ist es von großem Interesse, die Faktoren zu untersuchen, die die Entstehung der Darmflora beeinflussen. Das Darmmikrobiom wird als erstes von der Genetik und der Art der Geburt beeinflusst, während anschließend postnatale Faktoren wie die Umweltbedingung, das Stillen und die Gabe von Antibiotika eine Rolle spielen.

Mit diesem Projekt sollen die maternalen Faktoren untersucht werden, die die bakterielle Zusammensetzung des Darmmikrobioms der Nachkommen beeinflussen.

Da unklar ist, inwieweit das Mikrobiom von pränatalen Faktoren im Verglich zu postnatalen Faktoren beeinflusst wird, wurde ein Cross-Foster-Experiment an zwei genetisch unterschiedlichen Mauslinien durchgeführt, wobei eine Linie zur Fettleibigkeit neigte, während die andere schlank blieb. Das Mikrobiom des Kolons und des Blinddarms der drei Wochen alten Mäuse wurde mittels 16S-rRNA-Gensequenzierung analysiert. Es konnte gezeigt werden, dass sowohl die Geburtsmutter als auch die Ziehmutter einen deutlichen Einfluss auf das Darmmikrobiom der Nachkommen, speziell auf die Familien *Porphyromonadaceae*, *Rikenellaceae* und *Lactobacillaceae,* hatten. Beide beeinflussten die α- und β-Diversität des Darmmikrobioms und dessen Zusammensetzung. Da die Zusammensetzung der Muttermilch eine signifikante Rolle zu haben scheint, wurde im folgenden Experiment der Einfluss eines hohen Fettgehalts der Nahrung isoliert untersucht.

Die Hochfettdiät, die die jungen Mäuse für einen Zeitraum bekamen, der dem der üblichen Stillzeit entsprach, hatte negative Auswirkungen auf die Zusammensetzung des Mikrobioms, die denen in adulten Mäusen entsprachen. Dieser Effekt war unabhängig von Adipositas.

Vorhergehende Studien haben gezeigt, dass der Anstieg der Gallensäuren, der durch eine fettreiche Ernährung induziert wird, das Darmmikrobiome beeinflussen kann. Daher wurde untersucht, wie sich dies auf die Darmbakterien auswirkt, die die Umwandlung von Gallensäuren in sekundäre Gallensäuren katalysieren. Sekundäre Gallensäuren spielen bei der Entstehung von Darmkrebs und verschiedenen Magen-Darm-Erkrankungen eine Rolle. Es wurde ein Metagenomik-Ansatz in Kombination mit einer Analyse der Gallensäuren Zusammensetzung verwendet, um die Bakterien

zu untersuchen, die die Gene für die Gallensalz-Hydrolase (*bsh*) und die Gallensäure-7α-Dehydratase (*baiE*) tragen. Das erstere katalysiert die Abspaltung des Glycin- oder Taurinrests von den primären Gallensäuren, wodurch weitere Modifikationen erst möglich werden. Das zweitere katalysiert den Geschwindigkeits-bestimmenden und irreversiblen Schritt der 7α-Dehydroxylierung.

Es konnte gezeigt werden, dass eine fettreiche Ernährung die Menge der sekundären Gallensäuren im Darm erhöht und die Vielfalt der Bakterien beeinflusst, die zur Bildung der sekundären Gallensäure fähig sind. Bakterienfamilien, die am Abbau von Gallensäuren beteiligt sind, gehörten größtenteils zum Stamm *Bacteroidetes,* wobei die Familie *Porphyromonadaceae* am häufigsten ist. Fettreiche Ernährung und die daraus folgende Erhöhung des Gallensäurespiegels haben dementsprechend einen negativen Einfluss auf die Zusammensetzung des Darmmikrobioms und die Gesundheit des Wirts.

Zusammenfassend haben unsere Ergebnisse zum Verständnis der Faktoren beigetragen, die das Darmmikrobiom in jungem Alter beeinflussen.

1 Introduction

- **The gut microbiome**

Within the last decade the importance of the intestinal bacterial community - here also referred to as gut microbiome - for overall human health has been increasingly recognized. It is involved in the development of innate and adaptive immunity, defends its host against pathogens and influences the degradation and uptake of nutrients [1-3]. Moreover, there are accumulating studies on the interplay of the gut microbiome and other organs, this includes the bidirectional interaction within the gut-brain axis [4] and the cross-talk between gut and lung, termed the gut-lung axis [5]. Therefore, the gut microbiome is strongly linked to phenotypic features of the host. In turn the host is also shaping its microbiome. The composition and size of the microbial community varies throughout the gastrointestinal tract, as conditions like pH, transit time, host immunity and nutrient availability change. Whereas the stomach has a very acidic pH (pH = 1.5-5), it increases up to pH = 9 in the small intestine. These differences on the one hand regulate the activity of different digestive enzymes, and on the other hand control the microbial load of the host. Otherwise the host would have to compete with the microbiota for the available nutrients. As bacteria have a favorable surface-to-volume ratio and are closer to the substrates, they would probably have the edge over the host. Other mechanisms inhibiting bacterial growth in the small intestine are the release of bile acids and a strong peristalsis [6]. Additionally, immunoglobulin A and antimicrobial compounds are present in the small intestine. In contrast a pH of 5-7, lower concentrations of bile salts and a longer retention period, is resulting in the largest number and highest metabolic activity of bacteria in the large intestine. Here 10^{11} cells per gram content can be found. The dietary compounds which couldn´t be absorbed in the small intestine, like fibers, are substrate to the bacteria in the large intestine. In the colon these substrates are fermented to short chain fatty acids, particular butyrate, which have beneficial health effects for the host [7]. The bacterial phyla predominant in the gut are Gram positive *Firmicutes* and Gram negative *Bacteroidetes*, which are accompanied by *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* [8].

- **Development of the gut microbiome**

It is still under discussion if the intrauterine fetus is sterile, but it is recognized that the first bacteria colonizing the gut are passed from the mother to the offspring. It was shown that the mode of

delivery has an impact on the development of the gut microbiome during early age. Whereas vaginal birth caused close similarities between the gut microbiome of the infants and the microbiome of the mother`s vagina, the gut microbiome of babies delivered by C-section was more similar to the skin microbiome of the mother [9]. In a mouse experiment Pantoja-Feliciano et *al*. showed, that during the first days after birth the gut microbiome of offspring resembles the ones of the mother's vagina, followed by a phase of high instability the microbiome reaches a stable state and after 21 days a diverse gut microbiota had established [10].
As changes of the gut microbiome have been linked to obesity, inflammatory bowel disease and cancer [3, 11], it is of high interest which factors drive the formation of a healthy gut microbiome.

- **Impact of genetics on the gut microbiome**

The contribution of the genetic make-up of the host has been studied in several settings and experimental conditions [12-14]. The outcome of these studies suggest an impact of genetics on the gut microbiome, as the microbiome of family members is more similar to each other than to unrelated individuals [13] and more similar within monozygotic twins compared to dizygotic twin pairs [12]. Furthermore, genome wide association studies showed bacterial genera to correlate with quantitative trait loci involved in immunity and metabolic characteristics of the host [15, 16]. Nevertheless, the complex interactions involved make it difficult to separate the actual impact of host genetics on the gut microbiome.

- **Impact of the environment on the gut microbiome**

Besides defined conditions like genetics and pre-natal factors, there are post-natal, influenceable factors shaping the gut microbial community, one of them is the environment. During early age the environment of an individual is highly determined by proximity to the mother. Direct transfer of the mothers of oral and skin microbiota to the offspring takes place during care taking [17]. Ferreti et al. showed that the source of the majority of transmitted bacterial strains is the maternal gut microbiome [17]. It also was shown that cohabitation increases the exchange of bacteria through shared objects, surfaces and especially pets [18]. It has been also demonstrated that cohabiting but genetically unrelated parents share a higher proportion of their microbiome with their children than with unrelated children [13]. During further development the environment gains importance as source of microbes colonising the body. This includes transfer of bacteria from

natural environment as well as from surrounding individuals. A study carried out in children residing in Finnish and Russian Karelia emphasises the importance of environmental exposure early in life. These regions are socio-economically distinct but geoclimatically similar. Despite the occurrence of allergies were similar among those born in the 1940s, 30 years later the allergy levels among Finish children were increased [19]. It has been suggested that this is a result of a high content of microorganisms found in environmental dust and drinking water [20].

- **Impact of diet on the gut microbiome**

Diet has a major impact on the gut microbiome as it not only determines the nutrient supply for the host, but also for the bacterial community. The microbial community changes due to the different capability of bacterial species to utilize different substrates [21]. While for example species of the phylum *Bacteroidetes* are capable of degrading a variety of carbohydrates, other groups possess fewer genes encoding for carbohydrate metabolism and are therefore more specialised [22].

Early in life the diet of mammals consists of breast milk. This is a special form of diet, as breast milk not only contains nutrients but also immunoactive molecules, like immunoglobin A and defensins [23]. It is also one of the first post-natal sources of microbiota and has been shown to harbour potentially probiotic bacteria for the infant´s gut [24, 25].
The genera *Streptococcus*, *Staphylococcus*, *Lactobacillus* and *Bifidobacterium* have been found in breast-milk [26]. It has been demonstrated that the latter two are enriched in breast fed infants in comparison to formula fed ones, which leads to a decrease in pH of the intestinal content by the production of short chain fatty acids [27].
Further on in life the diet gets more complex and the intestinal microbiota change accordingly [10, 28]. The currently common 'Western' diet implicates health issues, like diabetes, metabolic disease, and cancer [29, 30]. But not every individual responds to a certain diet in the same way [31, 32]. The contribution of intestinal bacteria and their metabolites contributes to this variation in response. Therefore, it is of high interest to investigate the impact of high-fat diet on the gut microbial composition.

The impact of 'Western' diet on the gut microbiome composition in humans was demonstrated in a seminal study by De Filippo et *al*. who investigated the faecal microbiota of European children

and of children in the African state of Burkina Faso. The plant-rich diet, which had a high in fibre content of the African children lead to lower amount of bacteria of the phylum *Firmicutes* an increase in those of the phylum *Bacteroidetes* in comparison with the 'Western' diet of European children [33]. Therefore, the microbial composition of Burkina Faso children was selected for energy extraction from dietary fibre by their diet.

Another study in humanized gnotobiotic mice showed, that a 'Western' diet, high in fat and sugar, increased the abundance of bacteria belonging to the phylum *Firmicutes*, while decreasing the abundance of those of the phylum *Bacteroidetes*, compared to a low-fat, plant polysaccharide-rich diet [34]. This difference occurred within a single day and has been observed in several high-fat diet studies since [35, 36]. Furthermore high-fat diet was shown to increase the amount of *Proteobacteria* and decrease the diversity of the gut microbial community [35, 36]. The compositional changes may lead to a different metabolic activity of the intestinal microbiota, which further on could induce changes in host response, like inflammatory and immune responses. Moreover, Daniel et al. showed that high-fat diet does not only cause compositional changes, but major alterations in bacterial physiology and metabolite profile [37].

The metabolic pathways affected by high-fat diet included eicosanoid, steroid hormone, macrolide, bile acid and bilirubin metabolism [37].

As high fat diet increases bile acid concentrations in the gut, it is argued that this is a major driver of the alterations of the gut microbiome [38, 39].

- **Bile acids**

Bile acids are amphipathic molecules responsible for emulsifying fat, enabling lipid digestion and uptake. They also have endocrine functions, which target the nuclear receptor, Farnesoid X Receptor and the plasma membrane bile acid receptor TGR5 [40, 41]. Primary bile acids are produced in the liver of vertebrates. 95 % of bile acids secreted during digestion are reabsorbed by high affinity transport in the ileum. The remaining 5 % which enter the colon are subject to modification by bacteria. They are toxic to bacteria primarily by causing membrane damage [42]. However, bacteria modify bile acids, probably as matter of detoxifying or for obtaining energy and nutrients. The products of these modifications are called secondary bile acids and could not be detected in germ-free animals, therefore being solely produced by bacteria [43]. The first crucial step, the splitting of the side chain, is carried out by the enzyme bile salt hydrolase (BSH) (Figure

1A) [44, 45]. The bile acids are either glycine or taurine conjugated. In the mouse the taurine conjugation is predominant [46]. BSH is a generally intracellular enzyme classified as N-terminal nucleophilic hydrolase [47]. Substrates to BSH are Glycocholic acid (GCA), Glycodeoxycholic acid (GDCA), Glycochenodeoxycholic acid (GCDCA), Taurocholic acid (TCA), Taurodeoxycholic acid (TDCA) and Taurochenodeoxycholic acid (TCDCA) [48]. The taurine or glycine side chains of these substrates are removed by BSH via the hydrolysis of the C-24 N-acyl. In the bacterial community occupying the intestine BSH activity has been shown to be present in several Gram-positive genera, like *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus*, as well as in the Gram-negative genus *Bacteroides* [49].

Further conversion of deconjugated bile acids like cholic acid (CA) and chenodeoxycholic acid (CDCA), yielding deoxycholic acid (DCA) or lithocholic acid (LCA), is conducted via a multi-step 7α-dehydroxylation pathway (Figure 1B). This reaction can only occur after deconjugation due to the otherwise inaccessible hydroxyl group. The genes for this pathway are encoded by the bile acid-inducible (*bai*) operon, which has been mainly investigated in species within the genera Clostridium [45, 50, 51]. The proposed 7α-dehydroxylation pathway of CA in *Clostridium scindens* includes the import of primary unconjugated bile acids via a proton-dependent transporter, encoded by the *baiG* gene into the cell [51]. Further on a bile acid-coenzyme A ligase (encoded by *baiB*) [52], a 3α-hydroxysteroid dehydrogenase (*baiA*)[53] , a 7α-hydroxy-3-oxo-Δ^4-cholenoic acid oxidoreductase (*baiCD*) [54] and a bile acid coenzyme A transferase/hydrolase (*baiF*) [55] are involved, before the rate limiting and irreversible step in this pathway is catalyzed by a bile acid 7α-dehydratase (*baiE*) [56]. The genes encoding enzymes for the processes final yielding DCA or LCA and the export of these have not been identified yet.

As secondary bile acids, and especially LCA and DCA, contribute to the pathogenesis of colon cancer, gallstones, and other gastrointestinal diseases [57-59], it is of high interest how high fat diet is influencing the secondary bile acid forming bacterial community and their metabolism.

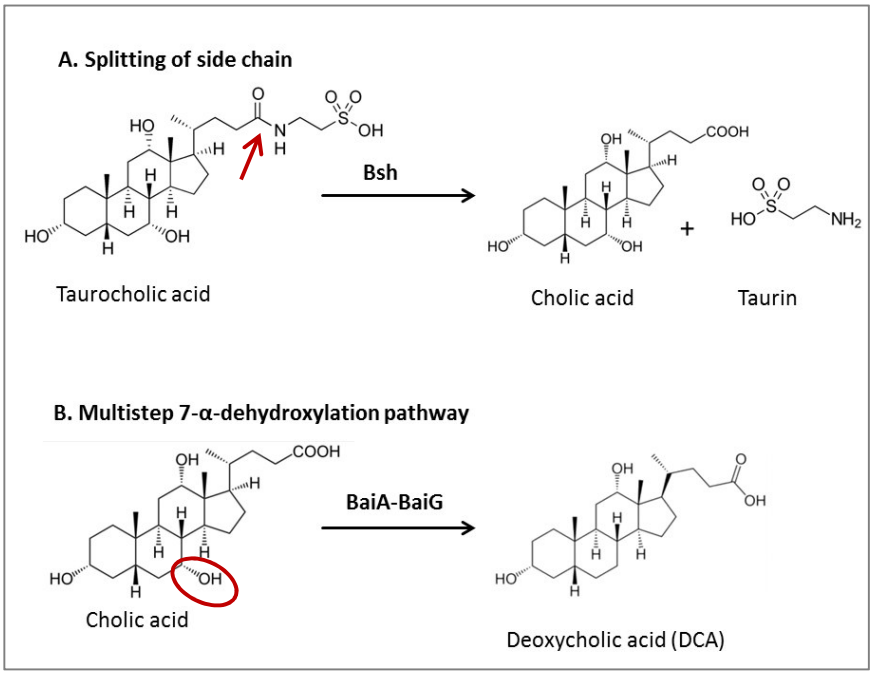


Figure 1: Example of bile acid modification reactions mediated by bacterial enzymes

- **Importance to investigate gut microbiome at early age**

All together the different factors shaping the gut microbiome at early age are of special interest, as a diverse microbial community is supposed to have health beneficial effects for the infant. The exposure to a variety of microbes for example is assumed to protect the infant from developing allergies [19, 60]. Furthermore epidemiological studies demonstrated that factors altering the gut microbial composition during childhood increase the risk for several diseases. And the disturbance of the gut microbial community by the use of antibiotics early in life for has been associated with the development of childhood obesity, type 2 diabetes and inflammatory bowel disease [61-63]. It is thus important to gain further insight into the influences determining the gut microbiome at early age.

2 Scientific questions and hypotheses

My project aimed to contribute to the knowledge of gut microbiome development during early age. From the numerous factors being discussed to influence the gut during early age, I hypothesized maternal factors having the major impact on the offspring´s gut microbial structure and composition. Previous studies have revealed that the first microbes colonizing the gut are transferred from the mother to the offspring during birth. The second factor which has an immediate impact on the assembly of the gut microbiome is the genetics of the host, which also determines the properties of the immune system. Throughout further development maternal effects, like the quality of the breast milk and transfer of bacteria associated with the mother´s body come into effect.

I hypothesize that birth and nursing mother have a distinct impact on the gut microbiome of the offspring.

To separate the factors of the birth mother – which include the influence of the offspring´s genetics – from the impact of the nursing mother, a cross-foster experiment on two genetically different mouse lines was conducted and the changes in the bacterial community of colon and cecum were analyzed.

With this study I intended to answer the following scientific questions:

I. How does the nursing mother influence the gut microbiota of the offspring?
II. How do birth and the genetic background influence the gut microbiota of the offspring?
III. Is the impact of the respective mothers similar in colon and cecum?

As the experiment showed the important impact of the nursing mother on the gut microbiome, I choose to further refine the question of how the gut microbiome is influenced at early age by focusing on food as influential factor. Literature suggests the body weight of the mother has an influence on the fat content and composition of the breast milk, and as increased fat content in diet is known to alter the gut microbial structure, I hypothesized that the fat content of the diet will further on shape the gut microbiota at early age in a distinct way. Thus, I analyzed the impact of high-fat diet on the gut microbiota at early age to answer the question:

IV. How does high-fat diet after weaning impact the development of the gut microbiome during early age?

This was investigated by analyzing samples of a high-fat diet feeding trial, where the mouse cecum samples were taken at the early age of 6 weeks after 3 weeks of high-fat diet, performing shotgun metagenomic sequencing.

The changes induced in the gut microbiome by high-fat diet are supposed to be mediated by an increase of bile acids. I therefore investigated the impact of high-fat diet on the bile acid profile and the cecal bacterial community abled to metabolize bile acids and subsequently produce secondary bile acids, as I hypothesized their abundance would be influenced by the bile acid increase.

The following two questions where stated:

V. How does high-fat diet impact the bile acid profile in the gut at early age?
VI. How does high-fat diet impact bacteria capable of bile acid degradation?

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Substance	Company
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
BSA (bovine serum albumin)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
DEPC	AppliChem GmbH, Darmstadt, Germany
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
dNTPs	Fermentas, Vilnius, Lithuania
Ethanol	Merck KgaA, Darmstadt, Germany
Ethidium bromide solution	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific Inc., MA, USA
DNA Gel Loading Dye (6X)	Thermo Fisher Scientific Inc., MA, USA

3.1.2 DNA fragments

All PCR primers were supplied by Metabion International AG (Planegg, Germany); purified by HPLC, synthesized with scale 0.02 µmol, quality checked by mass spectrometry and shipped in dry conditions.

3.1.3 Kits and master mixes

Kit	Company
Agencourt AMPure XP beads	Beckman Coulter Inc., Brea, California, USA
Agilent DNA 7500 kit	Agilent Technologies Inc., Santa Clara, California, USA
Standard Sensitivity NGS Fragment Analysis Kit	Agilent Technologies Inc., Santa Clara, California, USA
Gel and PCR Clean-up kit	Macherey-Nagel GmbH&Co. KG, Düren, Germany
MiSeq Reagent Kit v3	Illumina Inc., San Diego, California, USA
NEBNext Multiple Oligos for Illumina (Index Primers Set 1)	New England BioLabs LtD., Hitchin, UK
NEBNext High Fidelity Master Mix	New England BioLabs LtD., Hitchin, UK
NEBNext Ultra II DNA Library Prep kit for Illumina	New England BioLabs LtD., Hitchin, UK
Nextera XT Index kit v2 (Set A and C)	Illumina Inc., San Diego, California, USA
Power soil Kit	MO BIO Laboratories Inc, Carlsbad, California, USA
Quant-iT™ PicoGreen® dsDNA Assay Kit	Thermo Fisher Scientific Inc., MA, USA

3.1.4 Instruments and lab equipment

Instrument	Company
Agilent 2100 Bioanalyzer	Agilent Technologies Inc., Santa Clara, California, USA
M220 Focused-ultrasonicator	Covaris Inc., Woburn, Massachusetts, USA
Precellys 24 homogenisator	PEQLAB GmbH, BY, Germany
MiSeq System	Illumina Inc., San Diego, California, USA
Nanodrop 1000 Spectrophotometer	PeqLab Biotechnologie GmbH, Erlangen, Germany
PeqStar 96x PCR cycler	PeqLab Ltd, Fareham, UK
SpectraMax Gemini EM Spectrofluorometer	Molecular Devices GmbH, Ismaning, Germany
Victor Microplate reader	Perkin Elmer Inc. Waltham, Massachusetts, USA
Fragment Analyzer	Agilent Technologies Inc., Santa Clara, California, USA
UHPLC system	Waters, Milford, MA, USA
amaZon ETD Ion Trap	Bruker Daltonics GmbH, Bremen, Germany
polycarbonate mouse cages	Techniplast Inc., VA, Italy
wood chip bedding	Mucedola, Italy
wood chip bedding 2	Lignocel, J. Rettenmaier & Sohne, BW, Germany
Mouse standard chow	4RF21 standard diet for mice and rat reproduction, weaning and growth, Mucedola, Italy
High-fat and control diet	ssniff Spezialdiäten GmbH, Soest, Germany

3.2 Methods

3.2.1 Mice*

The thesis is based on two mouse studies: first a cross fostering experiment including two genetically different mouse lines and second a high-fat diet feeding trial.

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.

* Mice were handled and sacrificed by Vesna Mrak, Zala Prevoršek (Department of Animal Science, University of Ljubljana, Ljubljana, Slovenia) and Neža Grgurevič (Institute for Preclinical Sciences, Veterinary Faculty, University of Ljubljana).

3.2.1.1 Setup of the cross-fostering experiment

Mouse lines used during the study originated from a three-way cross base (two inbred [CBA, JU] and one outbred line [CFLP]). The polygenic mouse model was developed by divergent selective

breeding to study consequences of obesity [64, 65] and was selected for high fat (Fat line) or low fat (Lean line) content. The mouse lines differ more than fivefold in fat content having a body fat content of 4 % (lean line), and 22 % (obese line), respectively and have been shown to be stable for more than 60 generations [66].

To distinguish the impact of the birth mother (BM) - including prenatal effects of the mother, genetic make-up, microbial transfer during birth and first milk - from the post-natal influences of the nurturing mother (NM) on the development of the gut microbiome of the offspring, a cross-fostering experiment was conducted. Therefore, a part of the new-born mice was exchanged between mothers of the lean and the obese mouse lines that recently gave birth and were ready to nurse (Figure 2). A complete replacement of the litter was not possible as foster mothers do not accept a complete litter exchange.

As mice were fed the same sterilized food and were provided with the same sterile wood chip bedding, the main source of microbiota in the environment was the mouse mothers e.g. microbiota from skin, gut, mouth and milk.

Consequently, the analysis of colon and cecum samples from the mice resulted in the following treatments: Mice which were switched to a genetically different mother for nurturing (obeseBM/leanNM; leanBM/obeseNM); mice, which stayed with their birth mother, but got siblings from different birth mothers (leanBM/leanNM, obeseBM/obeseNM) and mice of which the litter was not changed (obeseControl, leanControl). The number of replicates, as well as the number of different nursing mothers, for each treatment and sample type is shown in table 1.

Table 1: settings analyzed in this study:

Treatment	# of replicates cecum	# of replicates colon	# of different nursing mothers
obeseBM/leanNM	13	11	4
leanBM/obeseNM	10	10	4
leanBM/leanNM	9	9	4
obeseBM/obeseNM	13	13	4
obeseControl	9	9	2
leanControl	4	4	1

Mice were housed in individually ventilated polycarbonate cages containing wood chip bedding. Acidified water and standard chow were available ad libitum. The environmental conditions of the facility were set to a temperature of 21 ± 2 °C, 40–70 % humidity and a light dark cycle of twelve hours each during the experiment.

Mice were sacrificed at three weeks of age and intestinal samples with content were divided into four parts (colon, cecum and lower and upper small intestine), immediately snap frozen and stored at -80 °C. For analysis the colon and cecum parts were chosen.

The body weight of exemplary mice was checked and did not show significant differences in the between the different treatments at the time point of sacrifice (Supplementary Table 1).

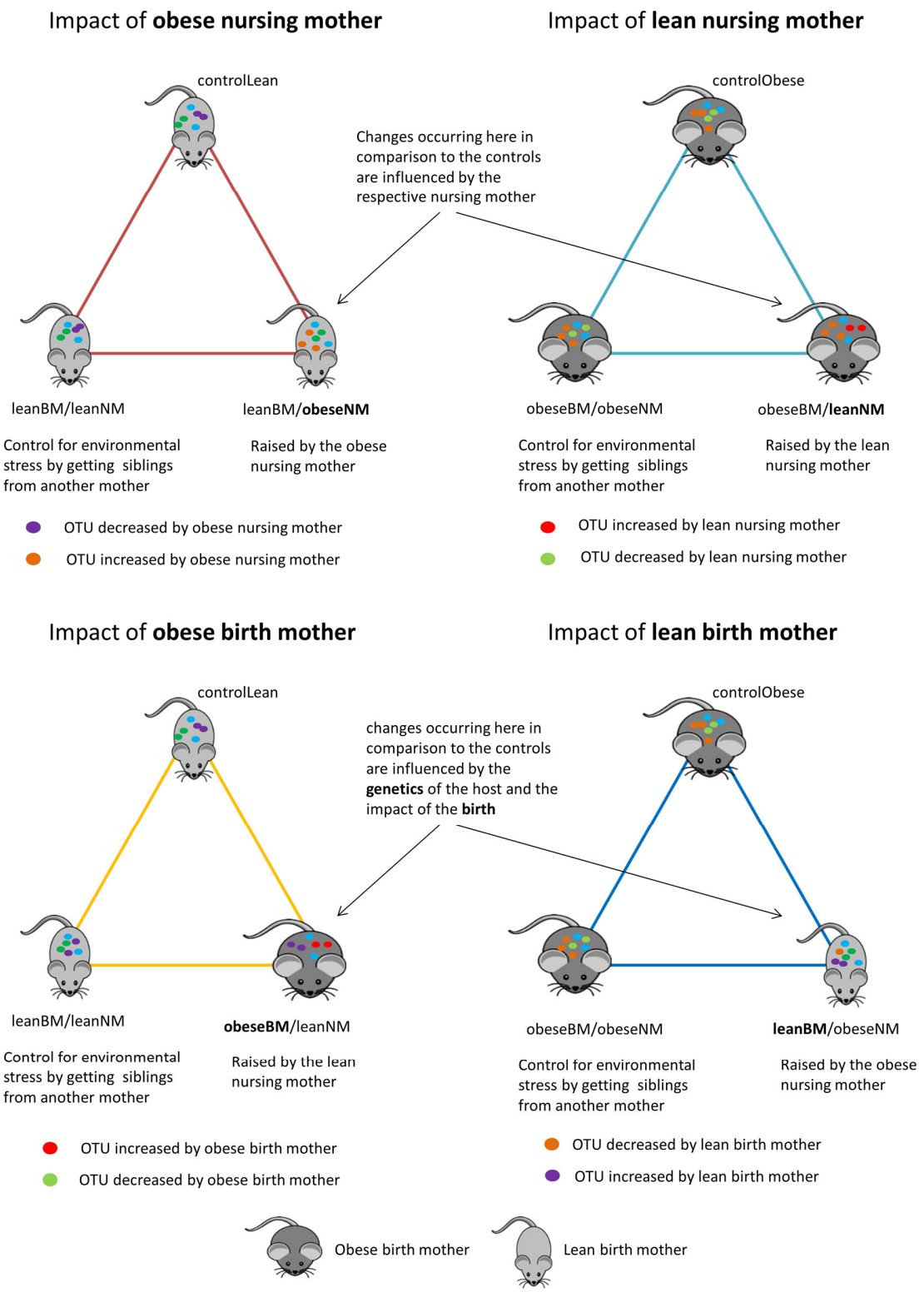


Figure 2: Experimental design of the cross-foster study [#]

3.2.1.2 Setup of high fat diet experiment

Fourteen female C57BL/6 mice were randomly assigned to standard chow and high-fat diet, to create groups of seven animals each diet. The high-fat diet contained 34 % fat, 24 % proteins, 1 % starch and 9.4 % sugar. The control diet contained 5.1 % fat, 18 % proteins, 37 % starch and 11 % sugar. Both diets contained the same amount of crude fibers and ash. Energetic value of high fat diet was 1.43 times higher than of control diet (21.6 MJ/kg vs. 15.1 MJ/kg). Concentrations of vitamins, macro and microelements was the same in both diets.

It has been demonstrated that there are gender specific differences in the synthesis of bile acids and the pool size of bile acids tends to be higher in females [67]. Therefore it was decided to use female mice only.

The mice were housed in polycarbonate cages containing wood chip bedding. The climatic conditions were set to a temperature of 21 ± 2 °C, 40–70 % of humidity and a light dark cycle of twelve hours each during the whole experiment. Acidified water and chow (high-fat and control) were accessible ad libitum.

Mice were sacrificed at six weeks of age and intestinal samples with content were divided into four parts (colon, cecum and lower and upper small intestine) immediately snap frozen and stored at -80 °C. For analysis the cecal samples were chosen, as the production of secondary bile acids in mice takes place there. Weight and 14 blood parameters (VetScan Comprehensive Diagnostic Profile, Abaxis Inc.) of the mice were measured weekly from age of three weeks on (Supplementary Table 2).

At time point of six weeks the mice fed a high-fat diet didn´t differ significantly in body weight from the one fed with control diet. Therefore, the effect on the cecal microbiome is not induced by a change in body weight but the high-fat diet.

3.2.2 DNA extraction

Extraction of the DNA from the colon and cecum samples of the cross-fostering experiment for 16S amplicon sequencing was conducted by transferring the frozen samples to a bead beating tube

included in the PowerSoil DNA Isolation Kit. After thawing the samples were treated using a tissue homogenizer at the speed of 5500 rpm for 30 seconds. Further on the DNA was extracted by applying the PowerSoil DNA Isolation Kit according to the manufacturer's protocol. To identify contaminating OTUs derived from the extraction kit a Blank sample only containing buffer was treated identical to the samples.

The frozen cecum samples of the feeding trial first off were ground on liquid nitrogen to obtain homogenized samples for DNA metagenomics and metabolomics measurement. Afterwards the DNA was extracted again applying the PowerSoil DNA Isolation Kit according to the manufacturer's protocol but, due to the pre grinding, using a different setting for the tissue homogenizer: speed of 5000 rpm for 15 seconds. Here also a Blank extraction was included to serve as control during the whole workflow.

In both cases concentration of the DNA was measured using the Quant-iT PicoGreen dsDNA Assay Kit according to the manufacturer's instructions.

The extracted DNA was stored at -20 °C.

3.2.3 High throughput sequencing

3.2.3.1 16S RNA gene sequencing

For the amplification of the 16S rRNA genes 50 ng of template DNA each sample was used. The total volume of one PCR reaction was a 25 µL and included 12,5 µL NEBNext® High-Fidelity 2X PCR Master Mix, 0.4 µL of the primer pair S-D-Bact-0008-a-S-16 S-D-Bact-0343-a-A-15 (10 µM) [19], which amplifies the V1/V2 region of the 16S rRNA, and 1.25 µL DMSO. The forward and reverse primers contained overhangs, which were compatible with Nextera XT indices for multiplexing, at their 5′ ends. Amplification was performed in duplicates with the following conditions: a denaturation step of 98 °C for 5 min, followed by 30 cycles of 20 s denaturation at 98 °C, 30 s of primer annealing at 59 °C and a 30 s elongation step at 72 °C. The final elongation step of 72 °C for 5 min completed the PCR. After checking the specificity of the PCR products by gel electrophoresis the PCR products were purified using the NucleoSpin Gel and PCR Clean-up

kit with NTI buffer diluted 1:4. Success of the purification was checked using a Bioanalyzer DNA 7500 Assay and the DNA content was measured with the Picogreen dsDNA assay kit.

For the sequencing library preparation 10 ng of template DNA, primers of the Nextera XT Index Kit v2 Set A and Set C and the NEBNext High-Fidelity 2X PCR Master Mix were used. The indexing PCR was conducted with the following conditions: a denaturation step of 30 s at 98 °C followed by eight cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Quality and quantity of the 16s amplicons were again analyzed as described above.

The sequencing of the samples, PCR negative controls and blank extraction control was conducted on a MiSeq System using the MiSeq Reagent Kit v3 (600 cycles) for paired end sequencing according to the instructions in the "Preparing Libraries for Sequencing on the MiSeq" protocol. The final libraries were diluted to 4 nM final concentration. The sequencing run was conducted according to the MiSeq System User Guide using 13 pM of DNA and 3 % PhiX.

The obtained reads are available in the Sequence Read Archive (SRA) of the NCBI under accession number SRP107967.

3.2.3.2 Shotgun metagenomics sequencing

1 µg of the extracted DNA was sheared with a Covaris M220 ultrasonicator for 25 seconds using 50 W peak incident power, 20 % duty factor and 200 cycles per burst. The fragment length was checked with the Fragment Analyzer using the Standard Sensitivity NGS Fragment Analysis Kit. The NEBNext Ultra II DNA Library Prep Kit for Illumina was used according to the manufacturer's protocol to prepare 200 ng of DNA and 6 µl of the blank extraction for shotgun sequencing, with the adjustment of the size selection to obtain fragments with 400-500 bp length. The PCR was conducted with 8 cycles and the quality of the metagenomic libraries was checked using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit, before the sequencing was conducted on a MiSeq System using the MiSeq Reagent Kit v3 for 600 cycles.

The metagenomic datasets obtained from shotgun sequencing are available in the Sequence Read Archive (SRA) of the NCBI under project accession number PRJNA503842.

3.2.4 Bioinformatics and statistical analysis*

For analysis of the 16S RNA gene amplicon reads, the demultiplexed raw data was processed with the open source software package QIIME v. 1.9. (Boulder, CO, USA) (Python v. 2.7.6) [68]. Sequencing primers were identified and removed by the MiSeq® System software. Merging of the obtained reads was done using FLASH v. 1.2.11 [69]. Reads identified as phiX or mouse genome by DeconSeq were removed as contamination [70]. The quality filtering (Phred score of 30) and length selection (320 bp and 400 bp) were conducted using QIIME and Biopieces [71], respectively. The filtered sequences were clustered at 97 % identity by UCLUST (v. 1.2.22q). For subsequent taxonomic affiliation the RDP classifier (release 2.11) [72] retrained with the Greengenes database (v. 13_5) was used. Steps were parallelized using GNU Parallel [73].

Selected sequences were further analyzed with the Standard Nucleotide BLAST tool using the MegaBLAST program and the 16S ribosomal RNA sequences database [74, 75].

Significant differences among taxa were identified by Bonferroni corrected T-test. Log2 fold changes were calculated for the abundance of the significantly changing taxa as follows:

$$\log2 ((a+0.0001)/(b+0.0001)) \quad (1)$$

where a and b are the average relative abundances of the taxa within the treatments compared. Pseudo-counts of 0.0001 were added to both abundance values to avoid a division by zero.

A serial group comparison, with pairwise Fisher's exact test and p-value correction by Benjamini-Hochberg method using Rhea [76], was conducted to analyze the impact of the nurturing mother and the birth mother on OTU level.

The core microbiome was defined as OTUs that were shared among all replicates of one sample group and Venn diagrams were created by an online tool provided by the University of Ghent [77].

The shotgun metagenomic dataset was analyzed using a bioinformatics pipeline developed in-house for Illumina paired-end data, with the following modifications. The Illumina adapters were identified and removed using AdapterRemoval v2.1.0 [78]. The same program was used for merging of paired end reads, trimming them based on quality (> 15), and length filtering (> 50 bp). For the removal of phiX and mouse genome reads again DeconSeq was used [70]. The quality-

controlled reads were assigned to taxa by Kaiju v1.4.4 [79], using the NCBI taxonomy and reference database of protein sequences (January 2017). Analysis steps were parallelized using GNU Parallel [73].

For analysis of functional genes involved in the bile acid 7α-dehydroxylation pathway alignment of the metagenomic reads to the respective protein database by DIAMOND (V 0.5.2.32) was conducted. The 827 representative protein sequences currently available for the functional genes were obtained from the UniProt Knowledgebase (UniProtKB). The gene numbers found were normalized by the reads per sample. The taxonomy of the respective *baiE* reads was analyzed using Kaiju v1.4.4 [79] as indicated above.

For analysis of the *bsh* gene the already validated HMM model PF02275 [80] was used to create the protein reference database, as the catalytic region of the *bsh* gene is conserved but the surrounding sequence is highly variable. Therefore the HMM model was search against the NCBI non-redundant protein database (June 2017) using the software HMMER 3.1b2 [81].
The metagenomic libraries then were analysed using this reference database using DIAMOND v0.5.2.32.

Statistical analysis and data visualization was conducted using the statistical program R (v3.1.1 and v3.2.2) [82].

*The pipelines for annotation of the shotgun and amplicon metagenomic reads were implemented by Dr. Gisle Vestergaard (Department of Biology, University of Copenhagen, Denmark).

3.2.5 Metabolomics

The bile acid quantification was conducted on 3 mg of the homogenized frozen wet cecal sample by ultra-high pressure liquid chromatography coupled to ion trap mass spectrometry (UHPLC-MS) according to Sillner et al 2018 [83] by Dr. Alesia Walker*.

*(Research Unit Analytical Biogeochemistry, Helmholtz Zentrum München).

4 Results

4.1 Impact of birth and nursing mother on the offspring's gut microbiome at early age

4.1.1 Quality of 16S RNA gene sequencing data

Sequencing of the 116 - 58 colon and 58 cecum - samples resulted in a total of 19,897,784 raw reads (for details see Supplementary table 3). The data of the colon samples has been used for **publication I** [84]. The average number of reads per sample after sequencing was 85,766, of which on average 79.02 % paired-end reads could be combined. Two samples from the obeseBM/leanNM treatment were excluded from the data set, because of a low number of combined reads. After quality filtering and removal of contaminating sequences (phiX and mouse genomic DNA) an average number of 66,068 reads per sample remained. For the colon samples the OTU calling at 97 % sequence identity revealed 3,146,700 total reads and for the cecum samples 3,239,874 total reads; the number of observed OTUs after an abundance cut off (0.005 % of the total reads) was for the colon samples was 864 and for the cecum samples 849. To remove possible bias from the variation in sequencing depth the samples were rarefied to 27,282 reads per sample for the colon samples and to 28,589 reads for the cecum samples.

To exclude a potential bias introduced by contamination from the extraction kits, the presence of the two OTUs with the highest abundance in the blank extraction control was analyzed in the samples. As they were only found in at maximum nine of the 56 colon samples and at maximum in eleven of the 58 cecum samples and had a relatively low abundance, I concluded that the contamination effects in this study as a result of the presence of microbial residues in the DNA extraction kit were minimal.

Sufficient sequencing depth was confirmed by rarefaction curves (Figure 3), which reached saturation at the respective subsampling level for observed OTUs for both, cecum and colon samples.

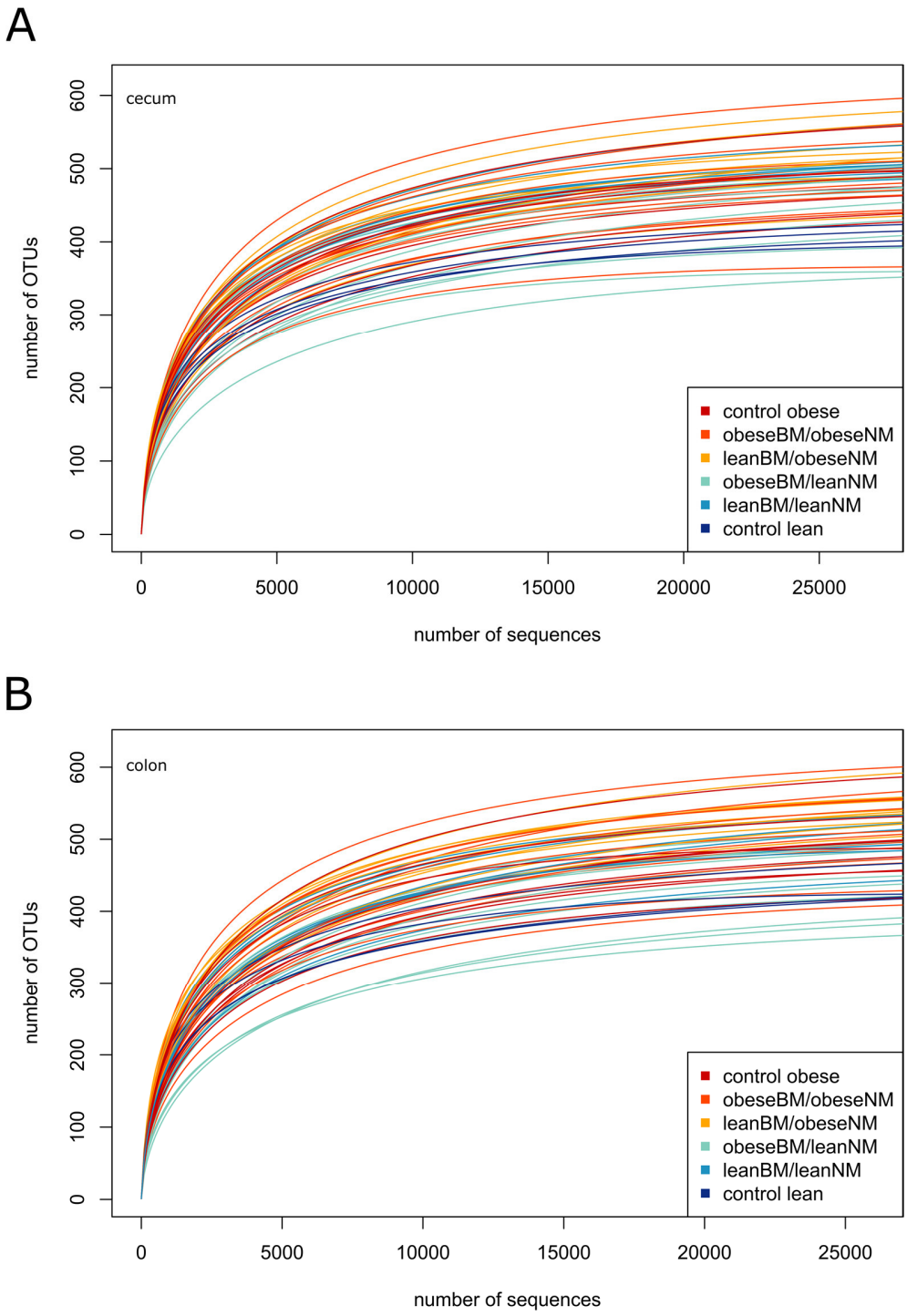


Figure 3: Rarefaction curves for the sequenced bacterial 16S rRNA gene

The rarefaction curves of observed OTUs reached saturation for the level of subsampling for (A) cecum (28,589 reads per sample) and (B) colon (27,282 reads per sample) samples. The sample groups are indicated as controlObese (red), obeseBM/obeseNM (orange), leanBM/obeseNM (green), obeseBM/leanNM (purple), leanBM/leanNM (yellow) and controlLean (blue).

4.1.2 Comparison of bacterial community in cecum and colon

To evaluate if there is a difference in the bacterial community between cecum and colon significant changes in the relative abundancies at family level were checked for by an ANOVA (significant = $p < 0.05$). The only family showing such a change in abundance was *Ruminococcaceae* in the lean control samples ($p = 0.047$) (Figure 4). But this could not be verified by comparing the colon and cecum samples of the leanBM/leanNM samples.

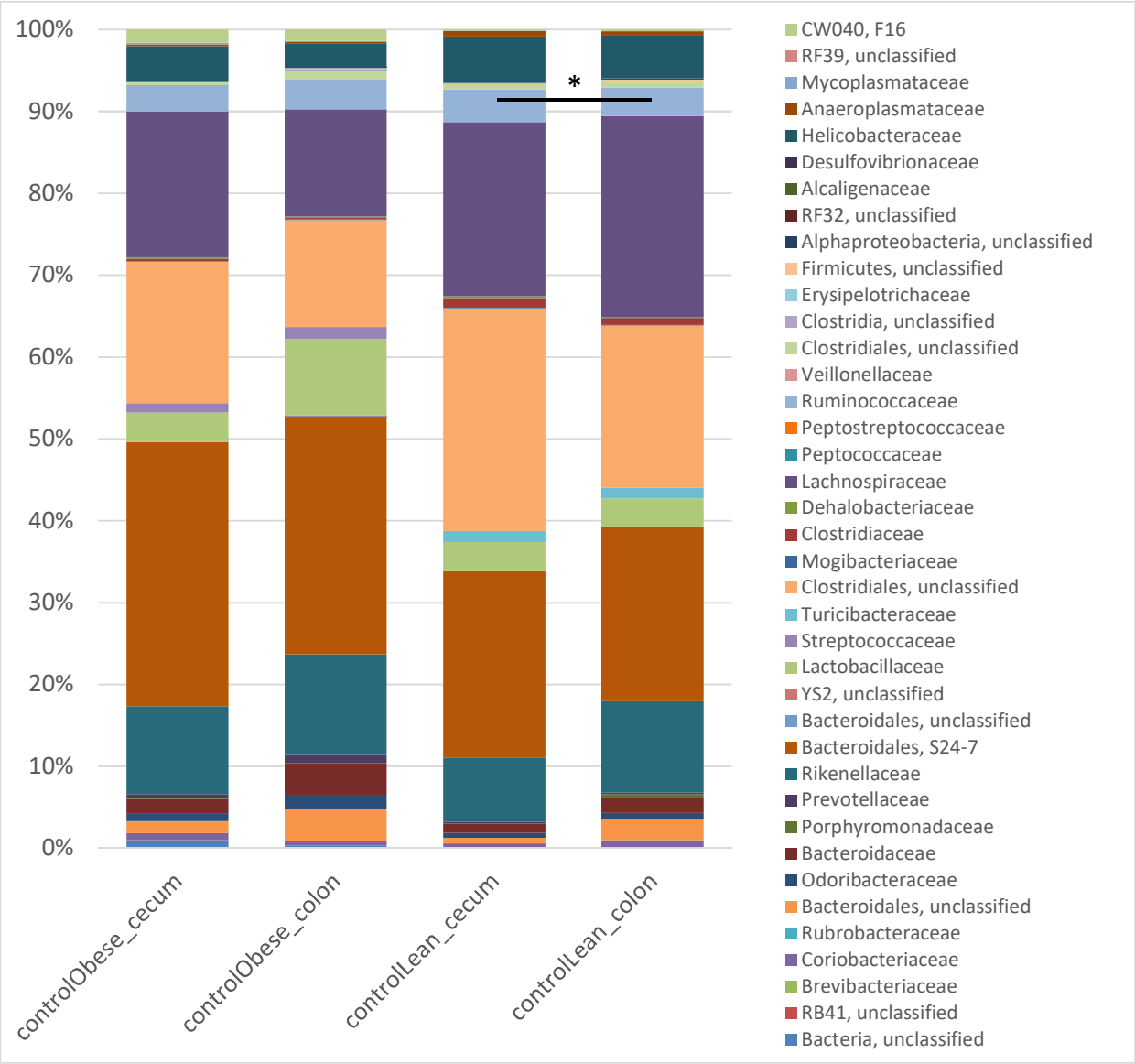


Figure 4: Comparison of bacterial community of cecum and colon samples at family level
Relative abundance of bacterial families in lean and obese control samples of cecum and colon. * refers to statistically significant differences ($p < 0.05$). Significances were calculated by ANOVA.

4.1.3 Impact of maternal factors on the diversity of the gut microbiome in colon and cecum at early age

The effect of the type of birth mother was analyzed by comparison of the samples of offspring exchanged between mothers (leanBM/obeseNM, obeseBM/leanNM) to the respective controls with the same kind of nursing mother (Figure 2). For analysis of the impact of the nursing mother the exchanged offspring were compared to the respective controls with the same kind of birth mother. Therefore, the changes that occurred when the obeseBM/leanNM samples were compared to the leanControl and leanBM/leanNM samples were indicating an impact of the obese birth mother, as the microbiomes of the offspring were all derived from the same kind of nursing mothers. Respectively the changes detected when the leanBM/obeseNM samples were compared to the obeseControl and obeseBM/obeseNM samples indicated an impact of the lean birth mother. The same scheme was used for the analysis of the impact of the nursing mother. The differences between the leanBM/obeseNM samples and the lean controls (leanControl, leanBM/leanNM) were representing an impact of the obese nurturing mother, as the offspring all had the same birth mother and genetic background. To detect the impact of the lean nurturing mother, the same was done for the obeseBM/leanNM samples and the obeseControl and obeseBM/obeseNM samples.

The impact of the birth mother on the OTU richness and evenness of the offspring's microbiome was more pronounced in the cecum than in the colon (Figure 5). Compared to the controls (controlObese, obeseBM/obeseNM) the lean birth mother (leanBM/obeseNM) showed a tendency to increase the OTU richness (average $S_{cecum} = 533.4$; $S_{colon} = 564.6$), but no impact on the evenness for both, cecum and colon samples. The obese birth mother did not have an effect on OTU richness in cecum and colon, as the obeseBM/leanNM samples is located in between the controls (leanBM/leanNM, LeanControl). However the obese birth mother did significantly decrease the evenness (on average $J_{cecum} = 0,69$; $J_{colon} = 0,68$) compared to the controls in cecum and colon samples.

The nursing mother had a significant impact on the OTU richness and evenness of the offspring's microbiome, which was also more distinct in the cecum. The lean nursing mother showed the tendency to decrease the OTU richness in the cecum as well as in the colon samples, as the number of observed OTUs was lower in obeseBM/leanNM samples (on average $S_{cecum} = 462.9$; $S_{colon} =$

464.1) compared to the obeseControl and obeseBM/obeseNM samples. The evenness of the gut microbiome community was decreased by the lean nursing mother. This was more pronounced in the cecum samples, were the difference in evenness was significant for the comparison of the obeseBM/leanNM samples (on average J_{cecum} = 0.69; J_{colon} = 0.68) in comparison with both controls (controlObese, obeseBM/obeseNM).

The effect of the obese nursing mother showed the contrary effect, as the OTU richness was increased in the leanBM/obeseNM samples (on average S_{cecum} = 533.4; S_{colon} = 564.6) in comparison to the leanBM/leanNM samples and this increase was significant for both colon and cecum samples when compared to the leanControl samples. The Evenness of the offspring's gut microbial community in cecum and colon was not affected by the obese nurturing mother, as the leanBM/obeseNM samples were in the same range as the controls (on average J_{cecum} = 0.73; J_{colon} = 0.75).

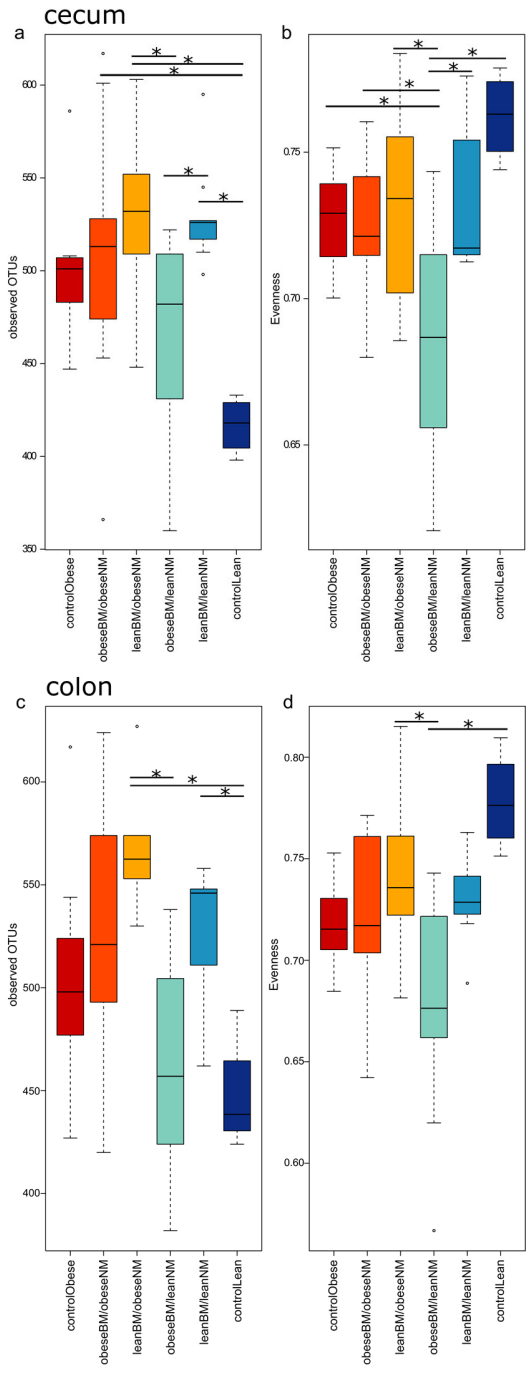


Figure 5: α-diversity measures of the microbiota in colon and cecum

The boxplots are based on the subsampled OTU tables (cecum: 28,589 reads per sample, colon: 27,282 reads per sample; at 97% identity level). Depicted are the number of observed OTUs (a, c) and the Evenness (b, d) of the six sample groups controlObese, obeseBM/obeseNM, leanBM/obeseNM, obeseBM/leanNM, leanBM/leanNM and controlLean for the cecum and colon samples. * refers to statistically significant differences ($p < 0.05$). Significances were calculated by nonparametric t-tests and were Bonferroni corrected. [#]

Analysis of the β-diversity showed a clear separation of samples by type of nursing mother for cecal and colon microbial communities (Figure 6). For both sites there was a clear separation of the leanControl and the obeseControl samples on the x-Axis of the PCoA-Plot, explaining 18.4 % of difference in diversity for the cecum samples and 20.8 % of the difference in diversity for colon samples. In the cecum samples both the leanBM/leanNM samples and the obeseBM/obeseNM samples clustered with the respective controls. While in the colon samples the obeseBM/obeseNM samples clustered with the obeseControl samples and the leanBM/leanNM samples clustered marginally separate from the leanControl samples.

The cecum and colon microbiome samples of the offspring exchanged between mothers (obeseBM/leanNM, leanNM/obeseBM) clustered with the ones of their respective nursing mother.

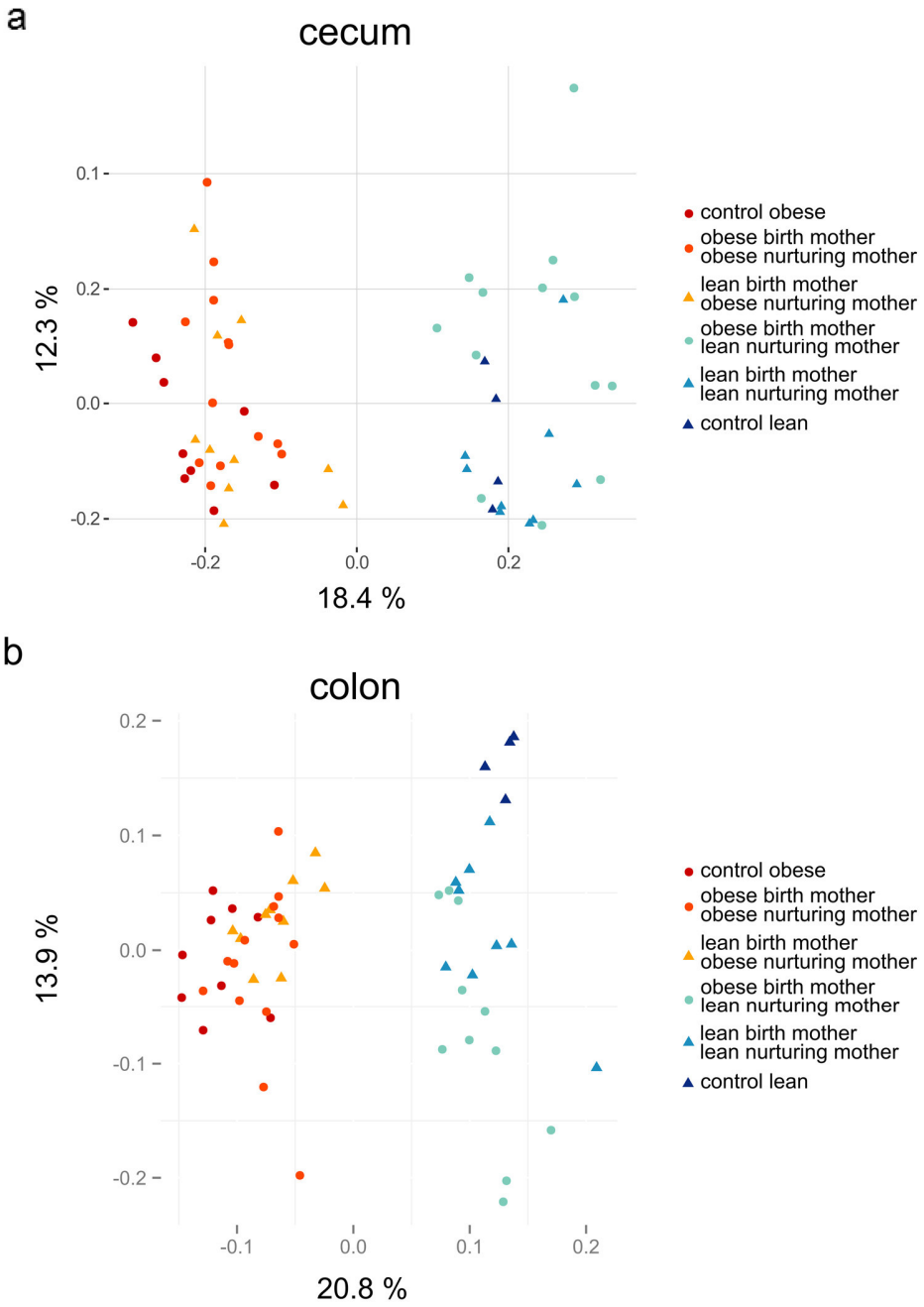


Figure 6: Clustering of the gut microbiome (β-diversity) in colon and cecum with respect to the type of nurturing and birth mother.

The PCoA plots for cecum (A) and colon (B) samples are based on Unweighted UniFrac distances of the microbial communities of the colon. The six sample groups controlObese, obeseBM/obeseNM, obeseBM/leanNM, leanNM/obeseBM, leanBM/leanNM and controlLean are distinguished by color (red/orange shade: obese nurturing mother; blue shade: lean nurturing mother) and shape (dot: obese birth mother; triangle: lean birth mother). [#]

4.1.4 Impact of maternal factors on the gut microbial community composition in colon and cecum at early age

To identify significant differences in the abundance of bacterial families between sample groups Bonferroni corrected pairwise t-tests (significant = $p < 0.05$) were applied to the different sample groups. The log2 fold changes of the abundance of significantly changing families were plotted as a heat map (Figure 7, Figure 8). To analyze the impact of the particular type of nursing and birth mother the differences in bacterial community composition in the exchanged offspring was again compared to the respective controls as described above.

To prove differences in bacterial community composition between the two genetically different types of mothers, the controls of cecum and colon samples were compared to each other on family level. The difference between the controls, based on the number of significantly changing families, was more pronounced in the colon. For the colon samples there was a significant difference in the abundance of fifteen taxa between leanControl and obeseControl samples, which was the most changes occurring between two sample groups. In contrast in the cecum only eight families changed significantly between the obeseControl and the leanControl samples. This confirms the difference in bacterial community within the controls also found by β-diversity analysis.

In the colon samples *Peptococcaceae*, *Veillonellaceae*, *Mycoplasmataceae*, *CW040 F16*, *Odoribacteraceae*, *Lactobacillaceae* and OTUs which could not be further assigned than to the class *Clostridia* were higher abundant in the obeseControl samples, while the abundance of the families *Peptostreptococcaceae*, *Desulfovibrionaceae*, *Porphyromonadaceae*, *Anaeroplasmataceae*, *Turicibacteraceae*, *Clostridiaceae*, *Lachnospiraceae* and OTUs which could not be further assigned than to the order *Clostridiales* was increased in the leanControl samples.

In the cecum samples the abundance of the families *Peptococcaceae* and *Mycoplasmataceae* was increased in the obeseControl samples, whereas the families *Mogibacteriaceae*, *Clostridiaceae*, *Anaeroplasmataceae*, *Turicibacteraceae*, and two OTUs which could not be further assigned than to the class *Clostridia* were increased in the leanControl samples.

To evaluate the robustness of the gut offspring´s microbiome and to control for the impact of stress, induced by the exchange of siblings to a foreign mother, cecum and colon samples from the leanBM/leanBM and obeseBM/obeseNM samples were compared to the respective controls.

The comparison of the obeseControl samples with the obeseBM/obeseNM samples showed one significant increase in the family *Peptococcaceae* in the cecum and three significant changes in the families *Prevotellaceae*, *Anaeroplasmataceae* and an OTU which could not be further assigned than unclassified bacteria. This low amount of changes indicates a sturdiness of the gut microbiome originated from the obese mouse line to stress.

The gut microbiome originated from the lean mouse line was less robust to the stress induced by the exchange of siblings, as in the cecum samples the six families *Mogibacteriaceae*, *Peptococcaceae*, *Desulfovibrionaceae*, *Lactobacillaceae*, an OTU which could not be further assigned than *Bacteroidales* and one OTU which could not be further assigned than *Clostridiales*, changed significantly in their abundance when the leanControl was compared to the leanBM/leanNM samples. In the colon a significant change in abundance was detected for the eight families *Peptococcaceae*, *Desulfovibrionaceae*, *Coriobacteriaceae*, *Lachnospiraceae* and four OTUs which could not be further assigned than *Clostridia*, *Clostridiales*, *Bacteroidales* and *Firmicutes* respectively.

To analyze the impact of the type of mother on the gut microbiota of the offspring the exchanged offspring were compared to the respective controls. In general, the obese birth mother and the lean nursing mother influenced the abundance of the most families (Table 2). If the abundance of a family was impacted by a certain type of mother in colon and cecum, the change was consistent, either increasing or decreasing in both parts.

The obese birth mother had a decreasing effect on *Erysipelotrichaceae* in the cecum and on *Peptostreptococcaceae*, *Porphyromonadaceae* in the colon. The *Turicibacteraceae* were decreased by the obese birth mother in both parts of the gut, while *Rikenellaceae* were increased.

The lean birth mother was just impacting the family *Streptococcaceae* in the colon, increasing its abundance.

The impact of the obese nursing mother on the abundance of the bacterial families also was limited to a family of unclassified *Firmicutes* being decreased in the colon. The lean nursing mother was correlating with the most families, significantly changing in abundance. Two unclassified families, belonging to the order *Clostridiales* and the phylum *Firmicutes* were linked to the impact of the lean nursing mother in the cecum. The families *Alcaligenaceae*, *Dehalobacteriacae*, *Bacteroidales S24-7* and an unclassified family of the order *Bacteroidales* were increased by the impact of the lean nursing mother in the cecum. In the colon the lean nursing mother seemed to have an

increasing effect on the family *Coriobacteriaceae* and a second unclassified family belonging to the order *Bacteroidales*.

Families showing an especially strong association with the lean nursing mother, as their abundance was significantly influenced in both parts of the gut sampled, were *Mycoplasmataceae*, *Streptococcaceae* and *CW040 F16*, which were increased and *Rikenellaceae*, which were decreased.

Table 2: Impact of the type of birth and nurturing mother on the abundance of bacterial families

	Bacterial family	**Change in abundance**	**cecum**	**colon**
Obese birth mother	*Erysipelotrichaceae*	decrease	**X**	-
	Turicibacteraceae	decrease	**X**	**X**
	Rikenellaceae	increase	**X**	**X**
	Peptostreptococcaceae	decrease	-	**X**
	Porphyromonadaceae	decrease	-	**X**
Lean birth mother	*Streptococcaceae*	increase	-	**X**
Obese nursing mother	*Firmicutes_unclassified*	decrease	-	**X**
Lean nursing mother	*Firmicutes_unclassified*	decrease	**X**	-
	Clostridiales, unclassified_2	decrease	**X**	-
	Alcaligenaceae	increase	**X**	-
	Dehalobacteriacae	increase	**X**	-
	Bacteroidales, unclassified_1	increase	**X**	-
	Bacteroidales, S24-7	increase	**X**	-
	Bacteroidales, unclassified_2	increase	-	**X**
	Coriobacteriaceae	increase	-	**X**
	Mycoplasmataceae	increase	**X**	**X**
	Streptococcaceae	increase	**X**	**X**
	CW040, F16	increase	**X**	**X**
	Rikenellaceae	decrease	**X**	**X**

X: significant change in abundance in this part of the gut
-: no significant change in abundance in this part of the gut

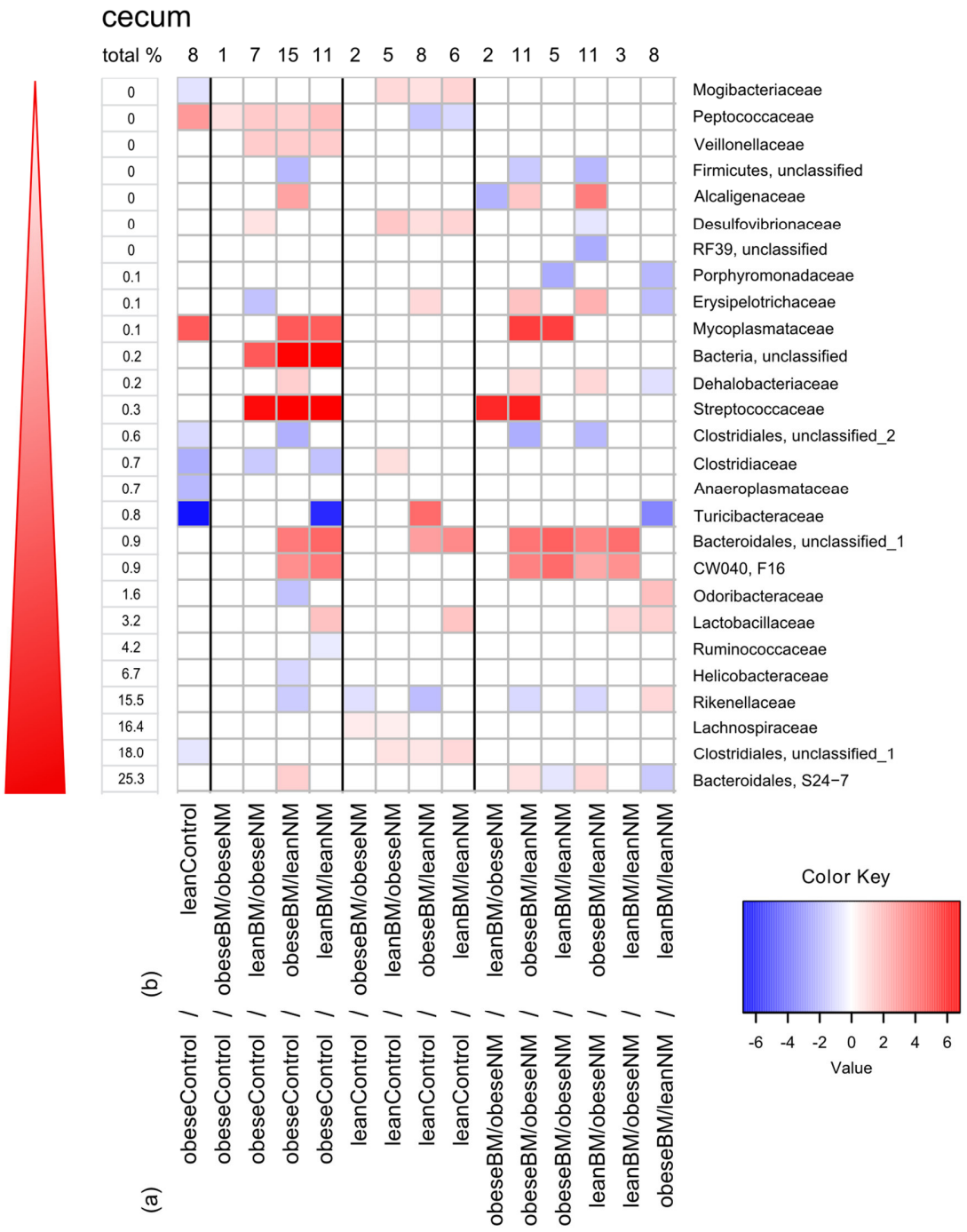


Figure 7: Heat map of the log2 fold change of the relative abundance of the bacterial families significantly changing among treatments in the cecum. White color indicates no significant change was detected. The treatments compared are stated at the bottom. On top the sum of observed significant changes for the treatments compared is depicted. The column to the left shows the total relative abundance of the bacterial families described to the right.

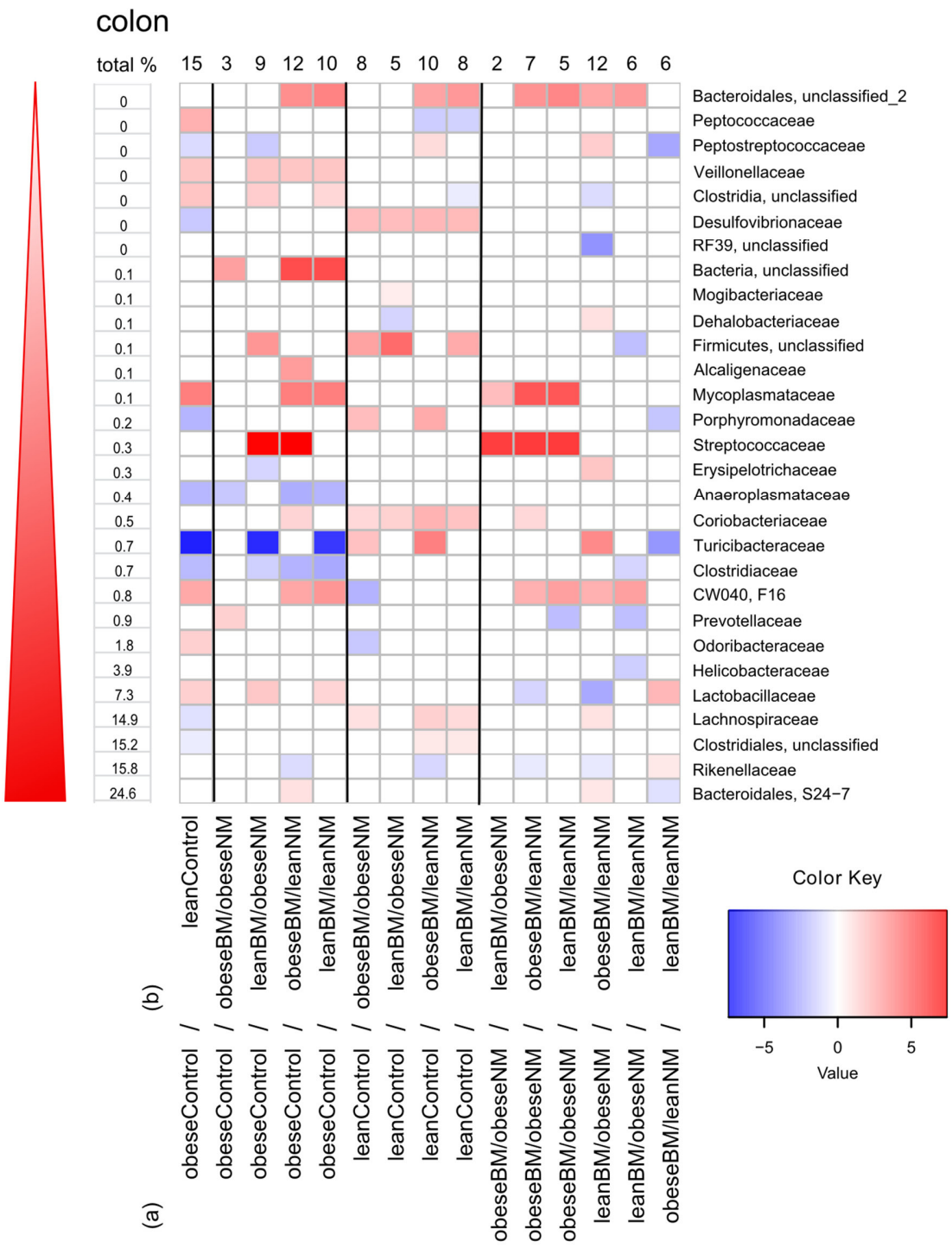


Figure 8: Heat map of the log2 fold change of the relative abundance of the bacterial families significantly changing among treatments in the colon. White color indicates no significant change was detected. The treatments compared are stated at the bottom. On top the sum of observed significant changes for the treatments compared is depicted. The column to the left shows the total relative abundance of the bacterial families described to the right. [#]

For a deeper analysis of the impact of the type of birth and nursing mother on OTU level a serial group comparison with pairwise Fisher's exact test and p-value correction by Benjamini-Hochberg method was applied. To analyze the impact of the particular type of nursing and birth mother the differences in bacterial community composition in the exchanged offspring (leanBM/obeseNM, obeseBM/leanNM) was again compared to the respective controls (controlLean, leanBM/leanNM, obeseBM/obeseNM, controlObese) as describes above (Figure 2). Representative sequences of the identified OTUs were annotated using the 16S ribosomal RNA sequences database of BLASTn with the MegaBLAST setting.

Overall the abundance of nine OTUs changed significantly in the obeseBM/leanNM samples compared to both, the controlLean and the leanBM/leanNM samples, indicating an impact of the obese birth mother on these OTUs (Table 3). Four of these OTUs changed only in the colon samples (289918, 336214, 354911, 348821) and their representative sequences were assigned to *Lactobacillus taiwanensis*, *Alistipes senegalensis* and *Lactobacillus reuteri*, which increased in the obeseBM/leanNM samples, and *Bacteroides acidifaciens*, which decreased in abundance in compared to the controls. One OTU (N2CUR OTU271), which was also assigned to *Alistipes senegalensis*, was significantly increased in by the obese birth mother in the cecum only. Another four OTUs changed in colon and cecum samples (214919, 276172, 276629, 351623). The representative sequences of these four OTUs, which were significantly affected by the obese birth mother in the colon were assigned to *Turicibacter sanguinis*, *Muribaculum intestinale* and *Cuneatibacter caecimuris*, which were negatively related to the obese birth mother and *Alistipes putredinis*, which was increased in abundance in the obeseBM/leanNM samples.

In contrast no significant changes related to the lean birth mother were found.

The obese nursing mother had an influence on seven different OTUs, four of them changing significantly in the colon (343630, NCUR OTU885, NCUR OTU90, NCUR OTU999), two in the cecum (339905, N0CUR OTU1555), and one in both parts of the gut (276629) (Table 3). The OTUs decreasing related to the obese nurturing mother in the colon were assigned to *Roseburia intestinalis*, *Alistipes senegalensis* and *Clostridium bolteae*. The OTUs which was increased in the colon was assigned to *Butyricimonas faecihominis*. Both OTUs affected by the obese nurturing mother in the cecum increased. They were assigned to *Alistipes shahii* and *Muribaculum intestinale*. In contrast to this the OTU changing significantly in both, cecum and colon, which

was also assigned to *Muribaculum intestinale,* was decreased in the leanBM/obeseNM samples in comparison to the controls (controlLean, leanBM/leanNM).

Shifting the offspring from an obese to a lean nursing mother caused a significant change in abundance of nineteen OTUs, of which six changes took place in the cecum only (268734, 272273, 342105, N0CUR OTU1555, N2CUR OTU1594, N2CUR OTU271), twelve in the colon only (275580, 276172, 276509, 277293, 463794, NCUR OTU119, NCUR OTU1812, NCUR OTU287, NCUR OTU445, NCUR OTU785, NCUR OTU885, NCUR OTU999) and one in both parts (336214) (Table 3). The OTUs assigned to *Muribaculum intestinale*, *Blautia producta*, *Alistipes senegalensis*, *Anaeroplasma abactoclasticum* and *Alistipes senegalensis* were increased related to the lean nursing mother in the cecum, while the OTUs assigned to *Eubacterium fissicatena* and *Alistipes shahii* were decreased. The OTU increased in both parts, was assigned to *Alistipes senegalensis*.

The majority of the OTUs affected by a certain kind of mother belong to one of the three families *Lachnospiraceae*, *Porphyromonadaceae* or *Rikenellaceae*. The latter two were already found to respond to effects of the mother during the analysis at family level. Particularly the obese birth mother showed a consistency in impact on family and OTU level.

Table 3: OTUs significantly changing in relation to an effect of a certain kind of mother

OTU ID	BLASTn assignment	type of mother correlating with	Change in abundance	part
214919	*Turicibacter sanguinis*	obeseBM	decrease	cecum
214919	*Turicibacter sanguinis*	obeseBM	decrease	colon
268734	*Muribaculum intestinale*	leanNM	increase	cecum
272273	*Blautia producta*	leanNM	increase	cecum
275580	*Roseburia intestinalis*	leanNM	decrease	colon
276172	*Alistipes putredinis*	obeseBM	increase	cecum
276172	*Alistipes putredinis*	leanNM	increase	colon
276172	*Alistipes putredinis*	obeseBM	increase	colon
276509	*Muribaculum intestinale*	leanNM	decrease	colon
276629	*Muribaculum intestinale*	obeseBM	decrease	cecum
276629	*Muribaculum intestinale*	obeseNM	decrease	cecum
276629	*Muribaculum intestinale*	obeseNM	decrease	colon
276629	*Muribaculum intestinale*	obeseBM	decrease	colon

277293	*Eisenbergiella massiliensis*	leanNM	decrease	colon
289918	*Lactobacillus taiwanensis*	obeseBM	increase	colon
336214	*Alistipes senegalensis*	leanNM	increase	cecum
336214	*Alistipes senegalensis*	leanNM	increase	colon
336214	*Alistipes senegalensis*	obeseBM	increase	colon
339905	*Muribaculum intestinale*	obeseNM	increase	cecum
342105	*[Eubacterium] fissicatena*	leanNM	decrease	cecum
343630	*Roseburia intestinalis*	obeseNM	decrease	colon
348821	*Bacteroides acidifaciens*	obeseBM	decrease	colon
351623	*Cuneatibacter caecimuris*	obeseBM	decrease	cecum
351623	*Cuneatibacter caecimuris*	obeseBM	decrease	colon
354911	*Lactobacillus reuteri*	obeseBM	increase	colon
463794	*Lactobacillus murinus*	leanNM	increase	colon
N0CUR OTU1555	*Alistipes shahii*	leanNM	decrease	cecum
N0CUR OTU1555	*Alistipes shahii*	obeseNM	increase	cecum
N2CUR OTU1594	*Anaeroplasma abactoclasticum*	leanNM	increase	cecum
N2CUR OTU271	*Alistipes senegalensis*	obeseBM	increase	cecum
N2CUR OTU271	*Alistipes senegalensis*	leanNM	increase	cecum
NCUR OTU119	*Odoribacter splanchnicus*	leanNM	increase	colon
NCUR OTU1812	*Anaeromassilibacillus senegalensis*	leanNM	increase	colon
NCUR OTU287	*Alistipes senegalensis*	leanNM	decrease	colon
NCUR OTU445	*Gabonia massiliensis*	leanNM	decrease	colon
NCUR OTU785	*Prevotella shahii*	leanNM	increase	colon
NCUR OTU885	*Alistipes senegalensis*	leanNM	increase	colon
NCUR OTU885	*Alistipes senegalensis*	obeseNM	decrease	colon
NCUR OTU90	*Clostridium bolteae*	obeseNM	decrease	colon
NCUR OTU999	*Butyricimonas faecihominis*	leanNM	decrease	colon
NCUR OTU999	*Butyricimonas faecihominis*	obeseNM	increase	colon

Balstn search (MegaBlast) (17.09.18) against 16S ribosomal RNA sequences

X: present in core microbiome; - not present in core microbiome

Subsequent to the analysis of the impact of the birth and nursing mother effect on the OTU level, the impact of the kind of mother on the OTUs belonging to the core microbiome was investigated. The core microbiome of a sample group was defined as OTUs present in all replicates of this group. The core microbiomes of the controls (controlLean, leanBM/leanNM and controlObese, obeseBM/obeseNM) were calculated as one combined core microbiome each (Figure 9). The number of OTUs was put in relation to the total amount of OTUs found in the data set of cecum (849 OTUs) and colon (864 OTUs) samples. The number of OTUs included in the core

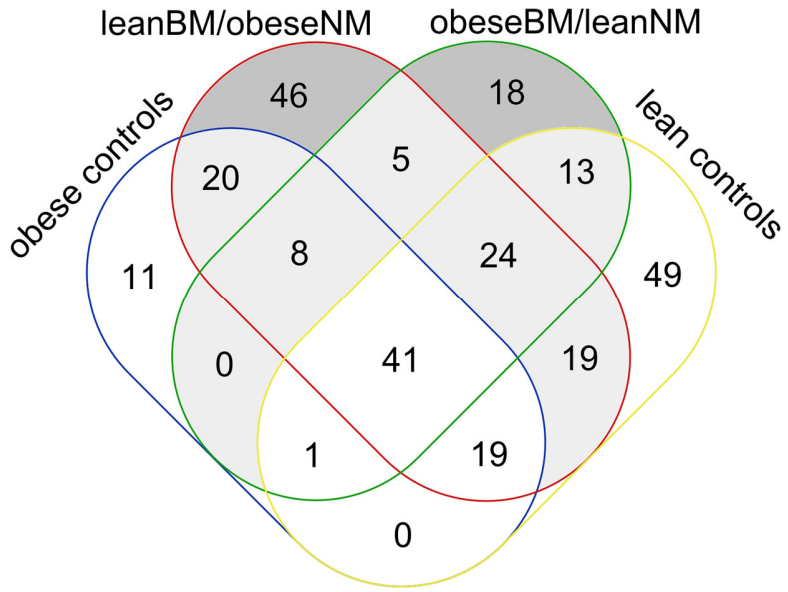
microbiome of the obese control samples was similar in cecum (100 OTUs, 11.48 % of total OTUs) and colon samples (99 OTUs, 11.46 %). Whereas the number of OTUs in the core microbiome of the lean control samples was higher in the cecal samples (cecum: 166 OTUs, 19.55 %; colon: 139 OTUs, 16.09 %). The core microbiome of the leanBM/obeseNM samples contained 184 OTUs (21.44%) in the cecal samples and 220 OTUs (25.46 %) in the colon samples. The core microbiome of the obeseBM/leanNM samples consisted of 110 OTUs (12.96 %) in the cecum and 131 OTUs (15.16%) in the colon.

The impact of the type of mother was analyzed by plotting the core microbiomes of all groups as Venn diagram. The overlap of the core microbiome of the swapped offspring and the controls following the introduced scheme was used as measure for the core OTUs influenced by a certain type of mother.

The OTUs which are part of the core microbiome of the leanBM/obeseNM samples (red circle) and are not part of the lean control core microbiome (yellow circle) are defined as affected by the obese nursing mother. As this regards only 9.31 % (79 OTUs) of core OTUs in the cecum, but 16.09% (139 OTUs) of core OTUs in the colon, the impact of the obese nursing mother seems higher in the colon. The impact of the lean nursing mother affected a similar amount of OTUs in the cecum (7.07 %, 60 OTUs) and the colon (8.10%, 10 OTUs). The core OTUs affected by the obese birth mother made up 3.65 % of the total OTUs (31 OTUs) in the cecum and 5.09 % (44 OTUs) in the colon samples. This is a low amount in comparison the core OTUs affected by the lean birth mother in cecum (15.39 %, 133 OTUs) and colon (11.07 %, 94 OTUs).

Of the twenty-eight OTUs significantly changing in relation to a certain kind of mother, eighteen are included in the core microbiome (Table 4). The most OTUs included in the core microbiome of a certain treatment group belong to the genera *Alistipes*, *Muribaculum* and *Lactobacillus.* This again emphasizes the role of the families *Rikenellaceae* and *Porphyromonadaceae,* and brings up the relevance of the family *Lactobacillaceae.*

A) cecum



B) colon

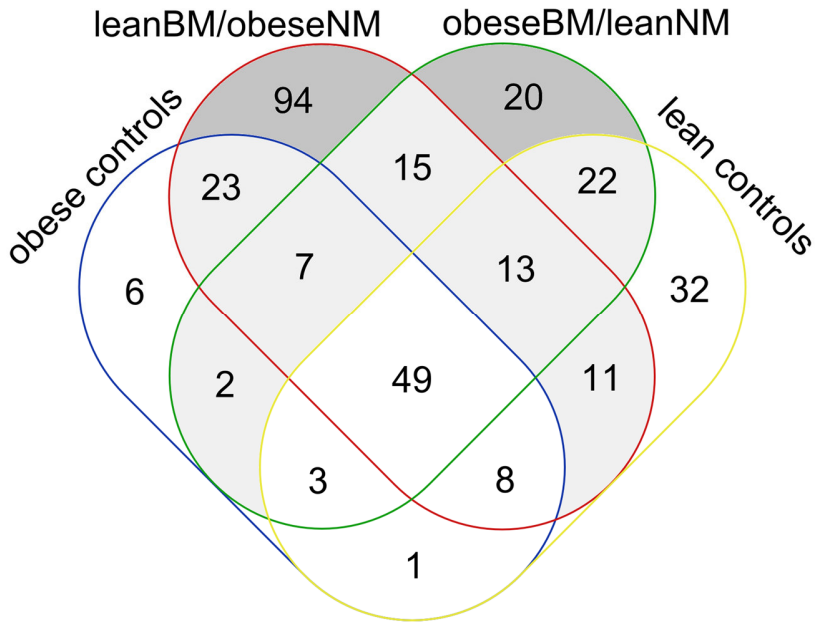


Figure 9: Venn diagram of the core microbiomes of exchanged offspring and controls

The core microbiome was defined as OTUs present in all replicates of the treatment groups. The core microbiomes of the controls (controlLean, leanBM/leanNM and controlObese, obeseBM/obeseNM) were calculated as one combined core microbiome each. A) shows the core microbiome of the cecum samples and B) the ones of the colon samples. The dark grey areas are the OTUs which are specific for the core microbiome of the sample group. Together with the light grey areas, these are forming the core microbiome OTUs, which were affected by a certain kind of mother.

Table 4: OTUs which are affected by a certain kind of mother and are part of the core microbiome

OTU ID	BLASTn assignment	In the cecum included in the core microbiome of	In the colon included in the core microbiome of
214919	*Turicibacter sanguinis*	lean controls	lean controls, leanBM/obeseNM
342105	*[Eubacterium] fissicatena*	lean controls, leanBM/obeseNM, obeseBM/leanNM	all
276172	*Alistipes putredinis*	lean controls, leanBM/obeseNM, obeseBM/leanNM	lean controls, obeseBM/leanNM
336214	*Alistipes senegalensis*	lean controls, leanBM/obeseNM, obeseBM/leanNM	leanBM/obeseNM, obeseBM/leanNM
N2CUR OTU271	*Alistipes senegalensis*	lean controls, obeseBM/leanNM	non
N0CUR OTU1555	*Alistipes shahii*	leanBM/obeseNM, obese controls	non
348821	*Bacteroides acidifaciens*	lean controls, leanBM/obeseNM, obese controls	all
NCUR OTU999	*Butyricimonas faecihominis*	non	leanBM/obeseNM, obese controls
NCUR OTU90	*Clostridium bolteae*	non	leanBM/obeseNM
351623	*Cuneatibacter caecimuris*	all	all
463794	*Lactobacillus murinus*	all	all
354911	*Lactobacillus reuteri*	all	all
289918	*Lactobacillus taiwanensis*	all	all
268734	*Muribaculum intestinale*	leanBM/obeseNM, obese controls	leanBM/obeseNM, obese controls
339905	*Muribaculum intestinale*	all	all
276509	*Muribaculum intestinale*	non	obese controls
276629	*Muribaculum intestinale*	lean controls, leanBM/obeseNM, obese controls	lean controls, leanBM/obeseNM, obese controls
NCUR OTU119	*Odoribacter splanchnicus*	non	all

4.2 Impact of high fat diet on the gut microbiome at early age

Based on the previous results, which emphasized the impact of the nursing mother and therefore the quality of food intake on the gut microbiome of the offspring at early age, I decided to further investigate this factor. As the fat content of breast milk was shown to influence the gut microbial composition in infants [85, 86], I concentrated on this aspect. As high-fat diet was shown to affect the gut microbiome composition independently of obesity [36], six weeks old mice (line C57BL/6) were used, as there was no difference in body weight that could interfere with the impact of the high fat diet. Consequently, the time period of high-fat feeding was three weeks, which was similar to the time span mice were nursed and therefore influenced by breast milk. The significant changes, which were prominent in the first experiment, were the same for cecum and colon, therefore the cecum samples were used, as the (metabolic) activity of bacteria in the mouse gut is highest there.

4.2.1 Quality of whole genome shotgun metagenomic sequencing data

The gut microbial community in the cecum of female C57BL/6 mice, which either were fed a high-fat (n = 7) or standard diet (n = 7), were investigated by metagenomics. This data is used for **publication II**. The quality of the data was checked by plotting the taxonomy per sample, generated by the analysis of the data with Kaiju, as rarefaction curves (Figure 10). The rarefaction curves reached saturation on the family level. Therefore, the taxonomic analysis took place at that level.

As the blank sample, which was included from the extraction step on, contained only 23 reads after removal of mouse reads no noteworthy contamination was caused by the extraction kit.

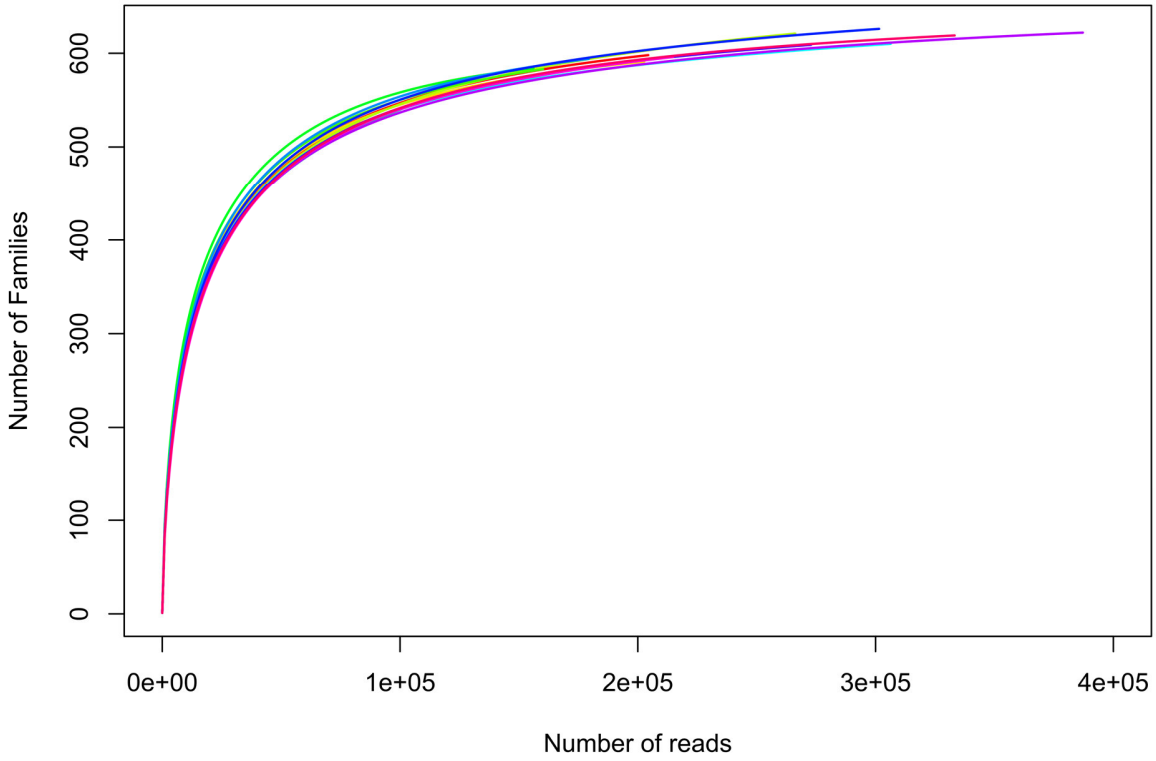


Figure 10: Rarefaction curves of metagenome data per sample based on Kaiju output.
The number of families in the metagenomes is presented as a function of the number of sequenced reads per sample.

4.2.2 Changes in the gut microbial diversity induced by high-fat diet

The high-fat diet significantly decreased the richness of the bacterial composition in the cecum from on average 546 to 521 bacterial OTUs at family level ($p = 0.041$), while simultaneously significantly increasing the evenness of the community from on average $J = 0.52$ to $J = 0.54$ ($p = 0.023$) (Figure 11).

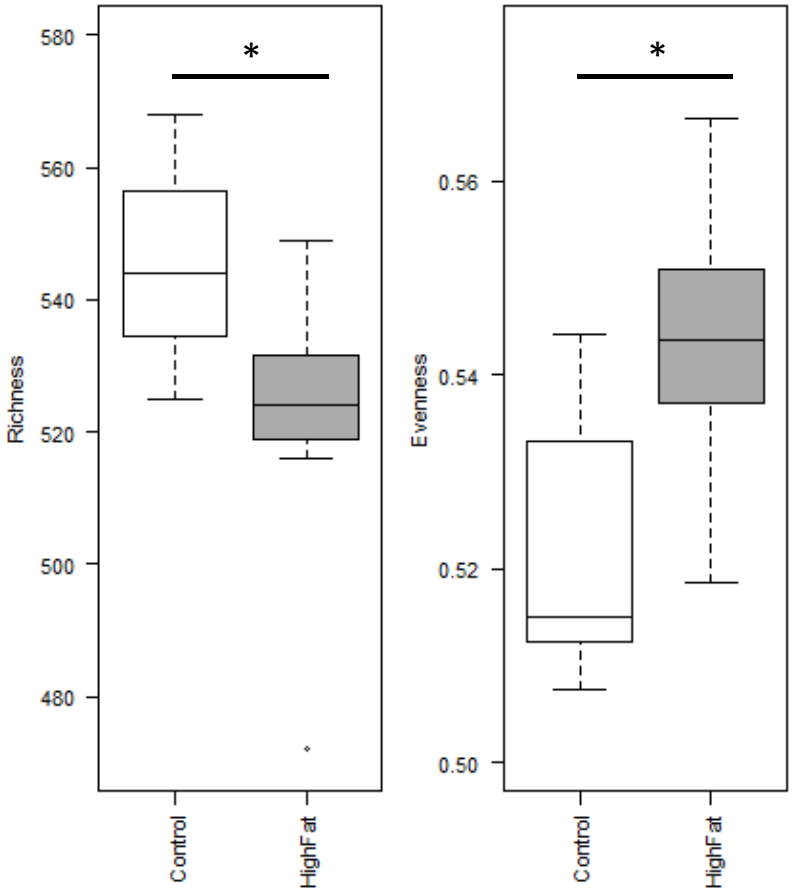


Figure 11: α-Diversity measurements of bacteria within the microbiota of the cecum
The boxplots are based on normalized reads assigned at family level by Kaiju using the NCBI taxonomy. Depicted are the Richness and the Evenness of the cecum samples of mice fed a control diet (n=7; white) and mice fed a high fat diet (n=7; grey). * refers to statistically significant as $p < 0.05$. Significances were calculated by nonparametric t-tests and were Bonferroni corrected.

The reads of the metagenomics datasets were annotated at family level by Kaiju using the NCBI taxonomy. The sequences of all samples were normalized by the total number of reads per sample. Based on the relative abundances of bacterial families per sample a NMDS analysis was performed. The ordination plot showed a separation of the composition of bacterial families in the cecum samples by type of diet relative to each other (Figure 12).

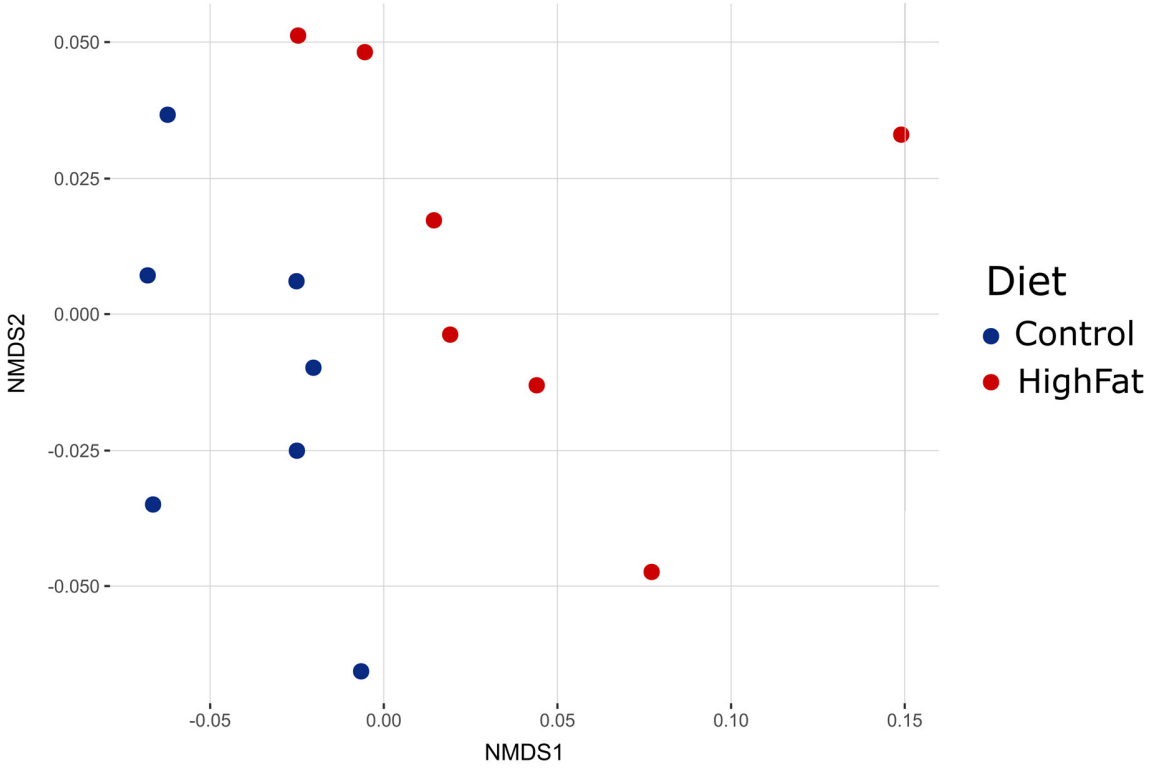


Figure 12: NMDS analysis of taxonomy in metagenomics samples on family level
The similarity of the bacterial composition on family level of the samples relative to each other is shown as NMDS plot. Samples are color coded according to diet. Stress value: 0.106.

4.2.3 Changes in the taxonomic composition of the gut microbiome induced by high-fat diet

As previous studies showed an impact of high-fat diet especially on the phyla Firmicutes and Bacteroidetes we investigated the abundance of these (Figure 13). The Firmicutes were increased by the high-fat diet, but the change was not significant. In contrast the Bacteroidetes were significantly decreased in the cecum of the mice fed a high-fat diet from on average 27.5 % to 21.4 % ($p = 0.013$).

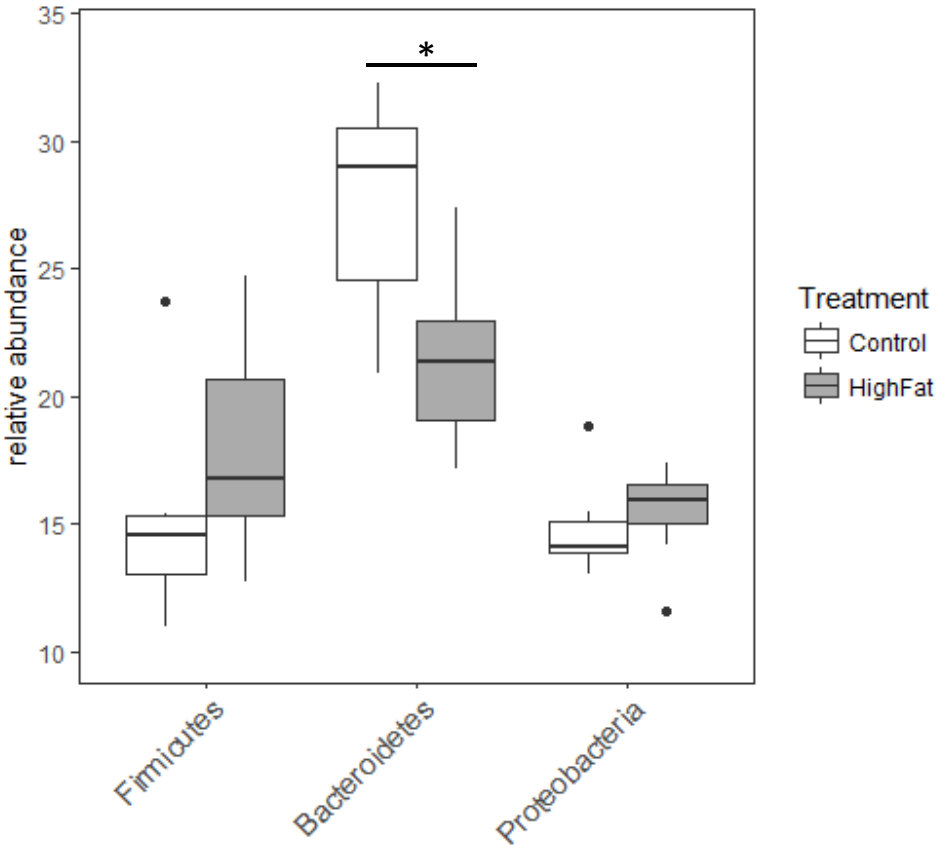


Figure 13: Relative abundance of the phyla known to be influenced by high fat diet
Abundance of the three phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* in % of total reads shown by treatment (each n=7). * refers to statistically significant as $p < 0.05$. Significances were calculated by nonparametric t-tests and were Benjamini-Yekutieli corrected.

When analyzed on family level fourteen bacterial families with an average abundance above 0.05 % showed significant changes in abundance by high-fat diet (Figure 14, Table 5). The relative abundance of the seven families *Oscillospiraceae*, *Chlamydiaceae*, *Streptomycetaceae*, *Enterobacteriaceae*, *Mycobacteriaceae*, *Acidaminococcaceae* and *Hymenobacteraceae* was significantly increased, while the relative abundance of *Porphyromonadaceae*, *Deferribacteraceae*, *Desulfovibrionaceae*, *Desulfohalobiaceae*, *Desulfomicrobiaceae*, *Desulfobacteraceae* and *Desulfobulbaceae* was significantly decreased by the high-fat diet. It is noteworthy that the latter five families all belong to the sulfate reducing bacteria.

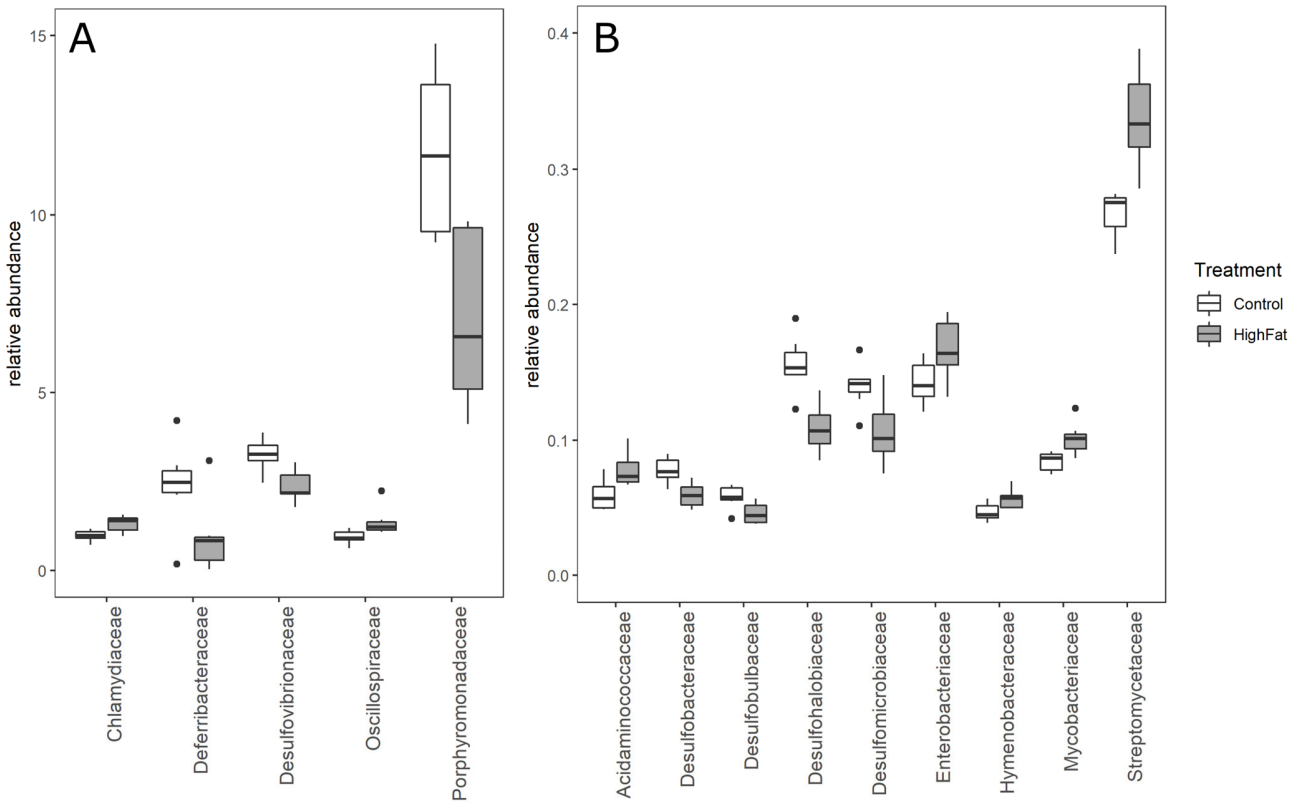


Figure 14: Relative abundance of significantly changing families

The average relative abundance of bacterial families, which differ significantly between control and high-fat diet ($p > 0.05$) is shown for families with an average abundance above 0.05%. A) shows the families with a higher abundance (up to 15%) and B) the families with a lower abundance (up to 0.4%).

Table 5: Average of relative abundance and p-value of significantly changing bacterial families (> 0.05%)

	Control [%]	High-fat [%]	p-value
Porphyromonadaceae	12.47	7.14	0.0034
Desulfovibrionaceae	3.27	2.39	0.0037
Oscillospiraceae	0.96	1.38	0.0270
Chlamydiaceae	1.00	1.32	0.0104
Deferribacteraceae	2.42	0.92	0.0285
Streptomycetaceae	0.27	0.34	0.0005
Enterobacteriaceae	0.14	0.17	0.0351
Desulfohalobiaceae	0.16	0.11	0.0007
Desulfomicrobiaceae	0.14	0.11	0.0146
Mycobacteriaceae	0.08	0.10	0.0070
Acidaminococcaceae	0.06	0.08	0.0133
Desulfobacteraceae	0.08	0.06	0.0024
Hymenobacteraceae	0.05	0.06	0.0252
Desulfobulbaceae	0.06	0.05	0.0156

4.3 Impact of high fat diet on secondary bile acid producing by bacteria

One reason for the impact of high-fat diet on the gut microbiome is argued to be the consequent increase of bile acid concentration [38, 39]. Bile acids are amphipathic molecules, which are toxic to bacteria mainly by membrane damage. Certain bacteria can degrade bile acids, which yields so called secondary bile acids. To investigate how bacteria involved in the formation of potential harmful secondary bile acids are affected by high-fat diet during early age, I analyzed the bile acid profile and bacteria carrying the genes *bsh* and *baiE.*

4.3.1 Alteration of the bile acid composition by high-fat diet

The targeted metabolomics analysis showed a significant increase in total bile acids by high-fat diet in the mouse cecum, in comparison with standard chow (rank sum test $p < 0.05$) (Figure 15). Overall 17 different bile acids were detected (Supplementary Table 4), of in particular the concentration of the primary bile acid α-Muricholic acid (αMCA) and the three secondary bile acids deoxycholic acid (DCA), hyodeoxycholic acid (HDCA) and ω-Muricholic acid (ωMCA) was significantly increased in samples of mice fed a high fat diet (rank sum test $p < 0.05$) (Figure 15, Supplementary Table 4).

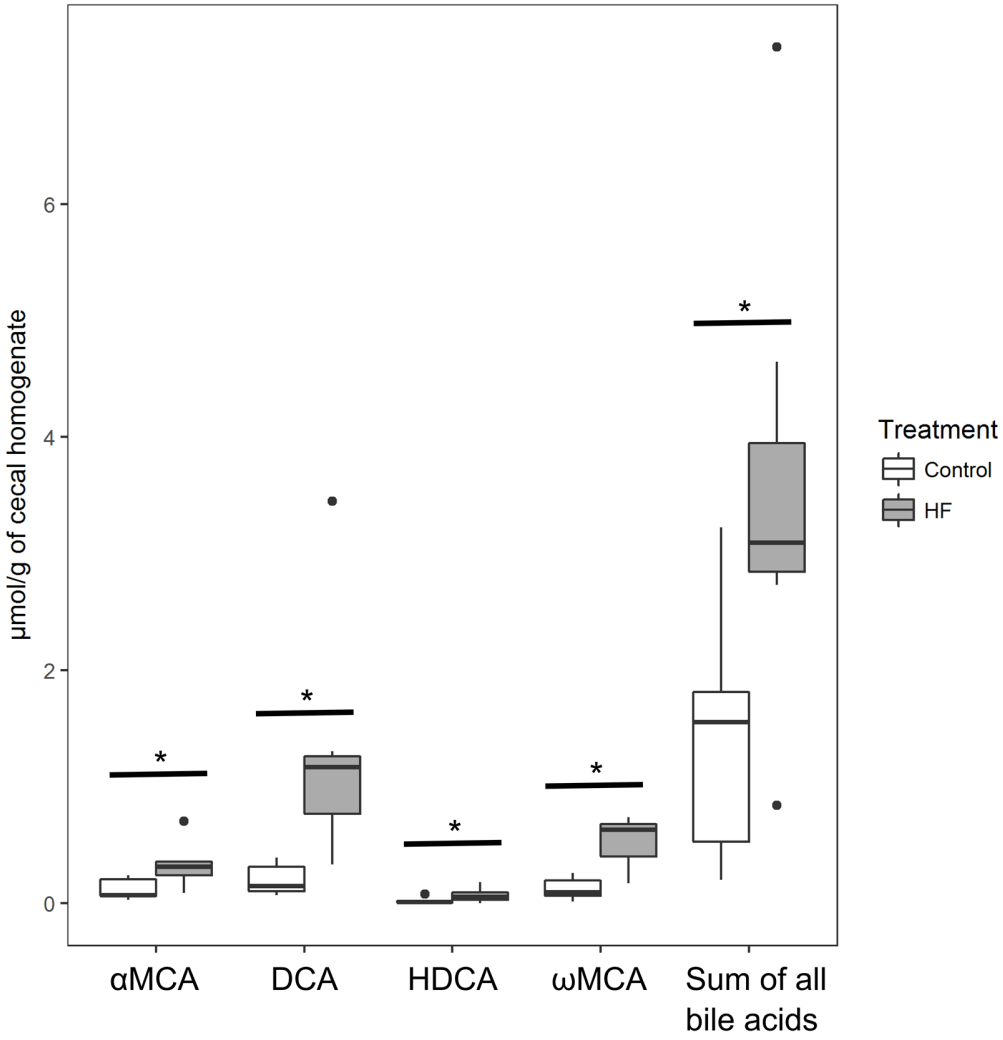


Figure 15: Concentrations of bile acids significantly changed by high-fat diet in the mouse cecum
The amount of significantly (rank sum test * = $p < 0.05$) changing bile acids is shown as µmol/g of cecal homogenate for the samples of mice fed a standard chow (Control, white) (n=7) and mice fed a high-fat diet (high-fat, grey) (n=7) as measured by UHPLC-MS. (αMCA: α-Muricholic acid, DCA: Deoxycholic acid, HDCA: Hyodeoxycholic acid and ωMCA: ω-Muricholic acid)

4.3.2 Impact of high-fat diet on bacteria with bile salt hydrolase function

The *bsh* gene encodes the enzyme bile salt hydrolase, which is responsible for the splitting of the side chain of primary bile acids. As this step is necessary for all following modifications of bile salts by bacteria, I searched the metagenomics libraries for *bsh* genes by alignment to a protein

database containing representative protein sequences. The obtained reads were normalized by the total read number of the respective sample. In the control samples the relative amount of *bsh* genes was on average 0.027 % (± 0.0057 %), while in the high-fat treatment samples showed a decrease of *bsh* genes to 0.024 % (± 0.0051 %) (Figure 16 A). There was no significant difference in *bsh* gene abundance between control and high-fat diet treatment (Benjamini-Yekutieli corrected t-test: $p = 0.28$).

The α-diversity of the cecal bacterial community containing *bsh* reads at family level decreased in richness and increased significantly in evenness (Benjamini-Yekutieli corrected t-test: $p= 0.014$) under high-fat diet (Figure 16 B).

The reads for *bsh* genes were taxonomically analyzed at phylum and family level. Of the *bsh* reads found in the cecum of mice fed the standard diet 59.33 % were assigned to the phylum *Bacteroidetes* and 30.83 % to the phylum *Firmicutes* (other phyla: 3.22 %; unassigned: 6.63 %). The high-fat diet treatment decreased the abundance of *bsh* reads assigned to *Bacteroidetes* to 51.81 % while the ones assigned to *Firmicutes* were increased to 33.83 % (other phyla: 3.78 %; unassigned: 10.58 %). On family level most *bsh* reads were assigned to six families, which are in decreasing order: *Porphyromonadaceae*, *Lachnospiraceae*, *Odoribacteraceae*, *Rikenellaceae*, *Prevotellaceae* and *Bacteroidaceae* (Figure 16 C). 25.10 % of the *bsh* reads from the cecum of mice fed the control diet were assigned to *Porphyromonadaceae*, while this number was decreased to 17.73 % by the high-fat diet. The second most *bsh* genes were assigned to was *Lachnospiraceae* with 13.62 % for the control and 13.08 % for the high-fat diet, respectively. While high-fat diet increased the *bsh* reads that were assigned to the three families *Odoribacteraceae*, *Rikenellaceae* and *Prevotellaceae*, fewer reads in high-fat samples were assigned to the *Bacteroidaceae.*

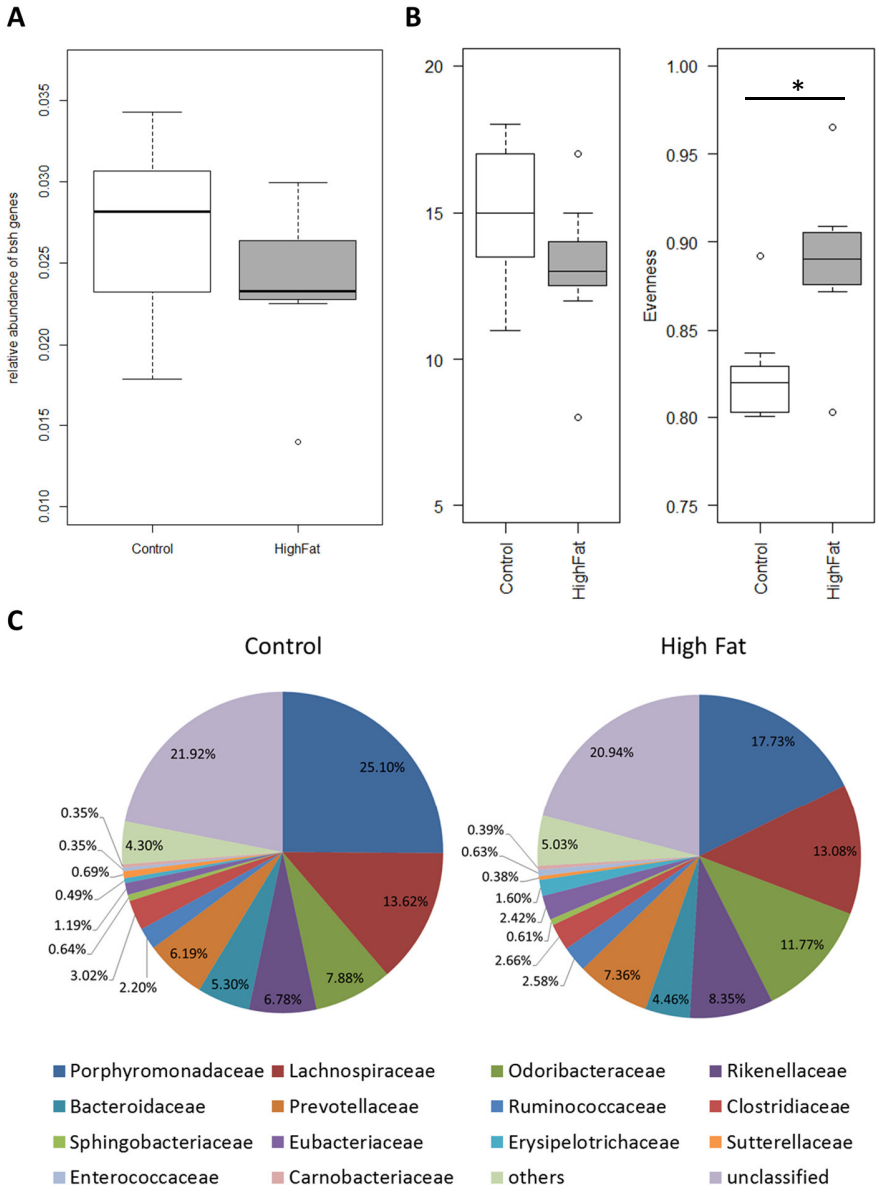


Figure 16: Relative abundance, α-diversity and taxonomy of bile salt hydrolase genes (*bsh*) in the cecal microbiome per treatment.

Reads for *bsh* genes were identified by alignment of metagenomic reads to a protein database consisting of representative protein sequences by DIAMOND. **A)** shows the abundance of genes found for the control and high-fat diet samples normalizes by the reads per sample. The *bsh* reads were taxonomically assigned at family level by Kaiju using the NCBI taxonomy. **B)** shows the richness and evenness of the assigned *bsh* reads and **C)** shows the relative abundance of the families the reads were assigned to. All significant differences between the controls (n=7, white) and high-fat diet treatment (n=7, grey) were calculated by Benjamini-Yekutieli corrected t-test (*= $p < 0.05$)

4.3.3 Impact of high-fat diet on bacteria capable of 7-α-dehydroxylation

After removal of the side chain from the primary bile acids by BSH, the deconjugated bile acid can be further processed by the intestinal bacteria. I analyzed the bacteria capable of conducting following multi-step 7α-dehydroxylation pathway by measuring the abundance of the *baiE* gene. This gene encodes a bile acid 7α-dehydratase, which catalyzes the rate-limiting and irreversible step in the multi-step 7α-dehydroxylation pathway. The average amount of *baiE* reads in the control samples was 0.006 % (± 0.00123 %) of total reads, while in high-fat diet decreased the abundance significantly (Benjamini-Yekutieli corrected t-test: $p = 0.047$) to 0.005 % (± 0.00118 %) of total reads (Figure 17 A).

The α-diversity of the cecal bacterial community containing *baiE* reads decreased in richness and increased in evenness under high-fat diet at family level (Figure 17 B).

The taxonomic assignment of the *baiE* reads on phylum level showed 77.57 % of *baiE* reads being assigned to the phylum *Bacteroidetes* in the control samples, while high-fat diet decreased the amount of *baiE* reads assigned to *Bacteroidetes* to 72.99 %. The high-fat diet increased the *baiE* reads assigned to the phylum *Firmicutes* from 9.51 % in control samples to 10.87 % in high-fat diet samples, and decreased the *baiE* reads assigned to *Proteobacteria* in control samples from 6.15 % to 3.12 % in the high-fat diet samples. On family level, the majority of the *baiE* reads were assigned to *Porphyromonadaceae* (control: 55.51 %; high fat: 49.82 %) and *Bacteroidaceae* (control: 9.26 %; high fat: 11.24 %) (Figure 17 C).

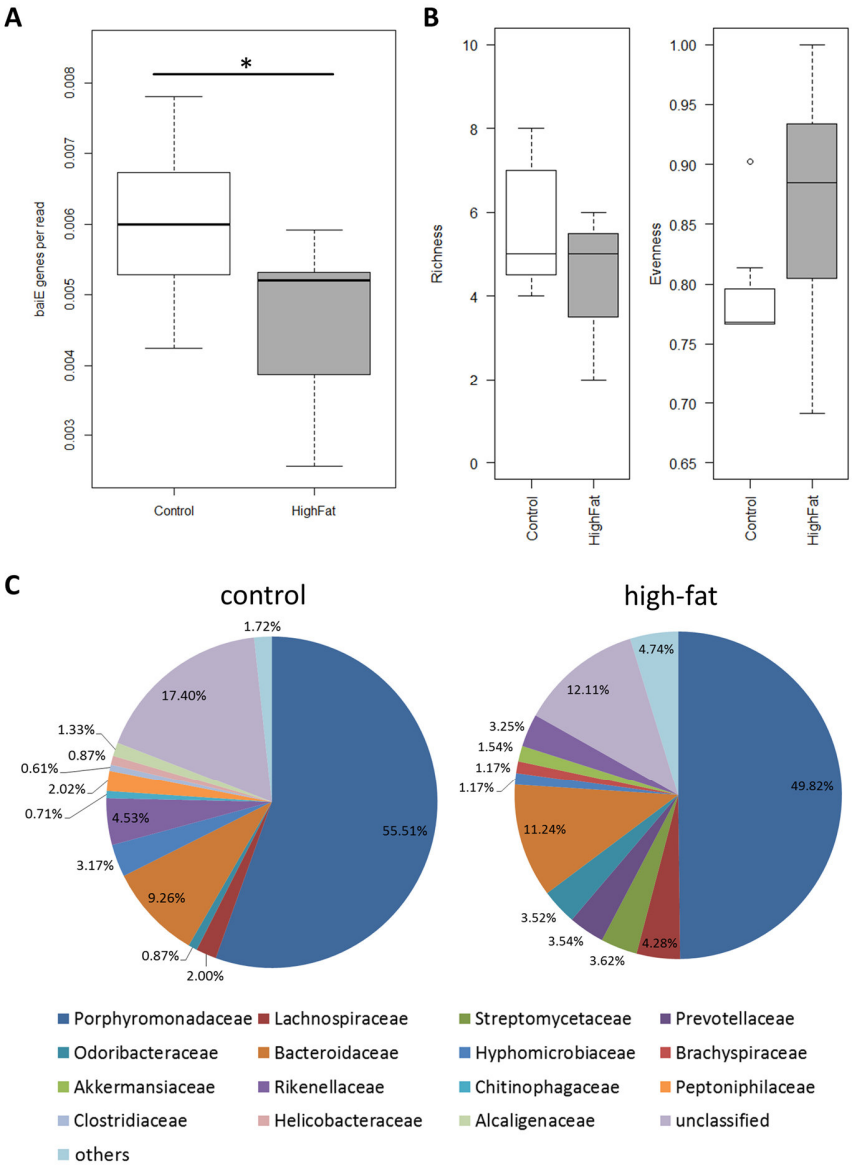


Figure 17: Relative abundance, α-diversity and taxonomy of bile acid inducible (bai) gene E in the cecal microbiome per treatment.

Reads for *baiE* genes were identified by alignment of metagenomic reads to a protein database consisting of representative protein sequences by DIAMOND. **A)** shows the abundance of genes found for the control and high-fat diet samples normalizes by the reads per sample. The *baiE* reads were taxonomically assigned at family level by Kaiju using the NCBI taxonomy. **B)** shows the richness and evenness of the assigned *baiE* reads and **C)** shows the relative abundance of the families the reads were assigned to. All significant differences between the controls (n=7, white) and high fat diet treatment (n=7, grey) were calculated by Benjamini-Yekutieli corrected t-test (*= $p < 0.05$).

5 Discussion

In the frame of the first part of the thesis I investigated the impact of maternal factors on the gut microbiome of the offspring at early age. I assumed that birth and nursing mother have distinct impacts on the gut microbiome of the offspring.

5.1 Validity of experimental set up

To investigate the impact of the birth and the nursing mother on the offspring's gut microbiome, a cross-fostering experiment with genetically predisposed lean and obese mice was performed. Colon and cecum samples from offspring exchanged between mothers (leanBM/obeseNM, obeseBM/leanNM) were compared to the respective controls by 16S rRNA gene sequencing as explained above to assess changes in their gut microbiome (4.1.2. and Figure 2).

I expected the gut microbial composition of the offspring serving as controls (controlObese, controlLean) to show a significant difference, due to selection of a specific microbiome within the certain mouse strain over several generations by its genetics and behavior (food amount and choice), enabling us to investigate the distinct impacts of birth and nursing mother. This was confirmed by a distinct clustering in the PCoA Plots pointing out the difference of the composition between the controls in colon and cecum (Figures 6), as well as by families changing significantly in abundance (Figure 7 and 8). It is of notice, that on family level the controls of the colon samples differed more than the one of the cecum samples, indicating a higher selective pressure on the community in the cecum. The low number of mothers influencing the control samples was take care of, by the addition of the second type of controls (leanBM/leanNM, obeseBM/obeseNM), with four different nursing mothers each, contributing to the robustness of the analysis.

Both, the impact of the birth and of the nursing mother include several different factors. The impact of the birth mother includes host genetics, as well as microbiota transferred during birth and by first milk (colostrum). As the mice were housed in a controlled environment with sterile food and bedding material, the impact of the nursing mother includes the transfer of microbiota associated with the mother´s body and effects of breast feeding. Following the factors of each type of mother will be discussed in detail.

5.2 Impact of the birth mother on the gut microbiome in colon and cecum at early age

The birth mother had an impact on the α-diversity of the gut microbiome of the offspring (Figure 5). The samples showed the same tendency for colon and cecum samples, but the differences between the exchanged offspring and the controls were more pronounced in the in the cecum. The impact of the birth mother on the β-diversity was minor, but a contribution of the birth mother to the microbiome composition was indicated within the samples of lean nursing mothers (Figure 6). This might indicate that the impact of the birth mother on the β-diversity might be overwritten by effects of the obese nursing mother, and not be relevant in the cecum. But due to the low amount of birth mothers in the controlLean group this tendency should be interpreted with caution, as the leanBM/leanNM samples cluster with the obeseBM/leanNM.

It was supposed that the birth mother has an impact by microbiota being transferred during birth [9], but it has been shown that offspring go through a biphasic assembly of a diverse gut microbiome which already has established by day 21 [10]. As the bacteria transferred during birth are adapted to the ecological conditions in the vagina, which include a more acidic pH, they are replaced by bacteria well adapted to the intestinal environment.

Like mentioned before the impact of the birth mother could be caused by inoculation of the intestinal environment during first milk (colostrum) feeding. Colostrum is the first form of breast milk produced by mammals, and it contains a high amount of antibodies and lymphocytes, as well as a higher amount of proteins than the later mother milk. As the abundance of *Turicibacteraceae* is positively correlated with the intake of proteins [87], this could be a reason for the decrease of this family within the cecum and colon microbiome of offspring with an obese birth mother. The obese birth mothers having a different quality of the first milk, could have had an influence, priming the gut microbiota towards a low *Turicibacteraceae* abundance. Another hint for the colostrum playing a role in mediating the effect of the birth mother on the gut microbiome of the offspring is the increase of two OTUs assigned to the genus *Lactobacillus* in the colon microbiome of offspring of obese birth mothers. A study in humans showed a higher maternal BMI correlating to higher numbers of Lactobacillus in colostrum [88].

The probably most dominant impact on the gut microbiome determined by the birth mother is the genetics of the offspring. The genetics of the host has been shown to influence the gut microbiome

in previous studies [12, 14]. Different taxa have been linked to host genetics and one mechanism of this selection is the immune system of the host [89]. The family *Streptococcaceae* was positively associated with the microbiome of the colon in offspring of lean birth mothers in this study. The OTUs assigned to *Streptococcaceae* could be further annotated to the genus *Lactococcus* by BLASTn search, which was correlated with quantitative trait loci associated with body weight in former studies [90]. Additionally, also the family *Turicibacteraceae* was linked to host immunity by previous research [14, 90], and this could be another reason for the negative association of this family with the obese birth mother in this study. Moreover the family *Rikenellaceae* was found to negatively correlate with leukocytes in a study about weight loss intervention [91]. OTUs belonging to this family were assigned to the genus *Alistipes*. They significantly increased in correlation to obese birth mothers, which is in accordance with a study in which *Alistipes* correlate with QTL for body fat content [90].

5.3 Impact of the nursing mother on the gut microbiome of the offspring

The nursing mother influenced the α-diversity of the gut microbial composition of the offspring. The impact showed the same trend in colon and cecum samples (Figure 5). Despite previous studies showing obesity to decrease the microbial diversity of the gut [32, 33], in this study the OTU richness was positively correlated with the obese nursing mother and negatively with the lean nursing mother. The reason of this difference in comparison to other studies might be that the obesity was not induced by a high fat diet, but by genetic predisposition. Also, the offspring did not differ in weight irrespectively of the genotype or the type of nursing mother. Another common observation of other obesity studies I could not confirm was an increase of the *Firmicutes* to *Bacteroidetes* ratio [92], as this finding probably correlates with high-fat diet instead of obesity [36]. Nevertheless, in compliance with previous studies, the two phyla *Firmicutes* and *Bacteroidetes* also were the most abundant in the murine gut microbiome analyzed by us [92, 93]. From the twenty main genera described for the murine gut microbiome of the colon [94] thirteen genera were also found in our study. The lack of *Faecalibacterium, Anaerotruncus, Enterococcus, Pseudoflavonifractor, Butyrivibrio, Alistipes* and *Blautia* in this study might be a result of difference between 16S rRNA gene analysis compared to metagenome sequencing.

The impact of the nursing mother was also emphasized by β-diversity analysis (Figure 6), which is in accordance with a previous cross-fostering study [95]. But as the mouse strains and sample types are not identical, the genera significantly changing are different from ours. In contrast to this study, in which colon and cecum parts including tissue were used, to also cover bacteria attached to the gut wall, Daft et *al*. used fecal pellets. Furthermore, in their cross-fostering study was a diabetic mouse line (NOD) and a non-diabetic mouse line (NOR) was used, while this mouse model was designed to investigate characteristics of obesity. Daft et *al*. found *Parabacteroides*, *Odoribacter*, *Bacteroides*, *Prevotella*, *Clostridium*, *Sutterella*, *Lysobacter*, *Anaeroplasma*, *Stenotrophomonas* and *Akkermansia* to be major responders to the impacts of the nursing mother. Remarkably, this study confirms the impact of the nursing mother on the three genera *Odoribacter*, *Prevotella*, and *Clostridium,* despite the differences in the experimental set ups. Interestingly several studies have indicated health-beneficial properties of these genera [96-98]. Below the individual factors by which the nursing mother potentially shapes the gut microbiome of the offspring during early age will be examined.

The first post-natal maternal factor considered to impact the microbiome of the offspring is the transfer of bacteria associated with the mother's body. This includes skin microbiota, oral microbiota and, especially concerning our study took place in mice, fecal microbiota. A difference in the gut microbiome of the nursing mother would cause a variance in the gut microbiome of the offspring. Unfortunately, this study did not include microbiome samples of the nursing mothers themselves, but the β-diversity analysis showed a distinct microbiome composition of the offspring from different types of nursing mothers. The microbiome of the nursing mothers could have been shaped by their genotype, either by forming their behavior and food preferences or by shaping their immune system. An impact of genetics on the host´s gut microbiome has been shown in previous studies [12, 99]. One example for this in our study is the impact of the obese nursing mother on OTUs assigned to *Clostridium bolteae,* as the family *Clostridiaceae* has been linked to genetic traits of the host previously [100]. And as already mentioned, the family *Streptococcaceae,* which is positively correlated with a lean nursing mother in colon and cecum in this study, was associated with body weight in a QTL study [90], indicating a mechanism determined by host genetics impacting members of this family.

Another essential factor influencing the gut microbial development at early age is breast milk. A difference in quality of breast milk of lean and obese nursing mothers could be a reason for the differences in gut microbial composition of the offspring. Breast milk mainly contains *Staphylococci*, *Streptococci*, lactic acid bacteria and *Bifidobacteria,* and has an important impact on the development of the gut microbiome [24, 26, 101]. To cover the longest possible period of exposure to breast milk the offspring were sampled at an age of three weeks. This overlaps with weaning and the start of solid diet intake, nevertheless I found significant changes in breast milk associated taxa. Predominantly the genera *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Bifidobacterium* are reported to be transferred by breast-feeding [24, 26]. Therefore, the impact of the nursing mother on of the family *Streptococcaceae* and the OTU belonging to *Lactobacillus murinus* might be explained by this. To verify maternal milk as medium these bacteria are transferred by, it would be necessary to sample the microbiome of the breast milk and the skin of the mothers to compare it to the offspring´s microbiota in future studies. Additionally, lactic acid producing strains cross-feeding butyrate producers like *B. faecihominis* and *Roseburia intestinalis,* might impact these genera influenced by the nursing mother indirectly [45-47].

Furthermore, the nutritional composition of the breast milk could influence the gut microbiome development of the offspring, as different substrates select for different bacteria. The milk oligosaccharides of humans for example were shown to promote the growth of bifidobacteria and two species of the *Bacteroides* [102] and a high amount of fat has been linked to increase of *Clostridiaceae* and a decrease of *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae* [36]. In compliance the family *Rikenellaceae* was negatively associated with the lean nursing mother in our experiment, which means a relative decrease in the controls with obese nursing mothers and implicates an in relation higher amount of fat in the maternal milk of obese nursing mothers.

Another property of the maternal milk of mammals is that it comprises bioactive molecules, like immunocompetent cells, immunoglobulins and antimicrobial peptides, which could select for different microbiota. A previous study showed the family *F16* of the order *CW040,* from the candidate phylum *Saccharibacteria* correlating with low IgA levels [103]. Therefore, a reason for the decrease of this family within the colon and cecum of mice raised by a lean nursing mother in this study, could be a higher amount of these in the milk provided by the lean nursing mothers.

5.4 Comparison of the maternal effects on colon and cecum samples

Despite if partial included in the previous discussion, this section will summarize the comparison of colon and cecum samples in detail. The bacterial community composition at family level did not differ between the controls, except for a change in abundance of *Ruminococcaceae* between the lean controls of colon and cecum (Figure 4). As previously mentioned, the number of replicates of the lean controls was lower than for the other sample types, therefore the result was double checked with the leanBM/leanNM samples, which did not verify the change. Thus, I used the cecum and colon samples equivalently for showing maternal impacts earlier.

As already mentioned throughout the previous discussion the cecum and colon samples showed the same tendency for the impact of the birth and the nursing mothers on α- and β-diversity (Figure 5 and 6). This is in accordance with a study of Gu et al., who mapped the bacterial community of the mouse intestinal tract [104]. They found a similar gut community profile at family level for cecum and colon samples, which was also the case in this work. A reason for the similarity in these anatomical regions could be the similarity of physiochemical conditions, which exert a selective pressure on the bacterial community. Furthermore, bacteria are associated with the epithelial surface in the cecum and proximal colon [105], as the inner mucus layer is less dense and more penetrable than in the distal colon [106]. I used the proximal and distal colon as one sample type, which could have contributed to the similarity as well.

The families responding to impacts of the birth and nursing mother showed differences between colon and cecum samples. Of the families, that could be distinctly assigned at this level, the four families *Erysipelotrichaceae, Alcaligenaceae, Dehalobacteriacae* and *S24-7* were only influenced by maternal effects in the cecum, while the families *Peptostreptococcaceae, Porphyromonadaceae, Streptococcaceae and Coriobacteriaceae* were influenced in the cecum samples. But the impact was not visible on OUT level, except for the impact of the obese nursing mother on the family *Porphyromonadaceae.* As the OTUs assigned to *Porphyromonadaceae* showed a response in both colon and cecum, I assumed the pre- and post-natal maternal factors to have a similar effect on the microbiota in colon and cecum at the early age of three weeks.

5.5 High-fat diet following weaning alters the cecal gut microbiome

Based on the previous results I choose to investigate the impact of high-fat diet on the bacterial community in the cecum at an early age. High-fat diet has been associated with changes in the gut microbial community, such as an reduction in diversity [35, 36], which I also found to be true for the impact of high-fat diet at early age (Figure 11 and 12). It was also reported to alter the community composition by decreasing *Bacteroidetes* and increasing *Firmicutes* and *Proteobacteria* [35, 36]. Both trends were already visible in our mice at the age of six weeks (Figure 13).

A striking change induced by high-fat diet in this study was the decrease of five families belonging to the sulfate reducing bacteria (Figure 14). These bacteria are able to utilize sulfate (SO_4^{2-}) as terminal electron acceptor for anaerobic respiration, producing hydrogen sulfide (H_2S) and they are commonly found in gastrointestinal samples. Moreover sulfate-reducing bacteria are hydrogenotrophic, therefore contributing to the disposal of hydrogen via cross-feeding and enabling efficient microbial fermentation processes in the intestinal tract [107]. As the occurrence of hydrogen sulfide has been linked to GIT diseases such as irritable bowel syndrome and colorectal cancer [108, 109], but also to health promoting effects such as maintaining the integrity of the mucus layer [110], it is important to understand how high-fat diet impacts the abundance of sulfate-reducing bacteria.

Interestingly, all five families of sulfate reducing bacteria decreased by high-fat diet (*Desulfovibrionaceae*, *Desulfohalobiaceae*, *Desulfomicrobiaceae*, *Desulfobacteraceae* and *Desulfobulbaceae)* belong to the phylum *Proteobacteria*, whose overall abundance was increased in the high-fat diet samples. Hildebrandt et al. also found an increase of the phylum *Proteobacteria* in their study about the impact of high-fat diet, but in contrast to my data, theirs showed the sulfate-reducing bacteria to be increased [36]. The reason for this might be the use of a different mouse strain and sample type (fecal pellet instead of cecum), but most likely is caused by their application of antibiotics with the drinking water, which could have induced a selective pressure favoring the growth of sulfate-reducing bacteria.

A reason for the impact of high-fat diet on the abundance of sulfate-reducing bacteria could be a change in availability of sulfate. Thus the effect could be caused directly by the composition of

the diet, as dietary proteins are a substrate for sulfide generating bacteria in the large intestine [111]. But as the amount of protein was higher in the high-fat diet than in the control diet used in this study, this does not explain the decrease of the sulfate reducing bacteria. Another reason for the negative impact of the high-fat diet on the abundance of the sulfate-reducing bacteria might be the disturbance of a cross-feeding interaction. In culture the release of sulfate from sulfated mucins was shown to promote the growth of sulfate-reducing bacteria [112]. Rey et al. found an increase of sulfatase expression in Bacteroides species to correlate with an increase in *Desulfovibrio piger* abundance and metabolic activity [113]. As the phylum Bacteroidetes was significantly decreased by high-fat diet in this study, a cross-feeding relation between the sulfate-reducing bacteria and *Bacteroides* species might be interrupted. It is also possible that high-fat diet induces a physiological response of the host tissue in the intestinal tract that is to the detriment of sulfate-reducing bacteria, as high-fat diet was shown to induce alteration of mucins in the colon of mice [114].

Another taxa affected by the high-fat diet, which is of high interest is the family *Porphyromonadaceae,* which was significantly decreased (Figure 14). It stands out by its high abundance and the fact that it already responded to the obese nursing mother in the previous experiment. This family is especially of interest as *Porphyromonadaceae* have been associated with protective effects against colitis and colorectal cancer in mice [115, 116]. They have been demonstrated to be sensitive to bile acids, as a decrease in bile acids favored an overgrowth [117]. Additionally, Daniel et al. showed that high-fat diet affected cecal metabolic pathways including bile acid and bilirubin metabolism [37].

It is therefore likely that the effect of high-fat diet in the gut microbial community is mediated by elevated levels of bile acids. To investigate this in more detail I choose to analyze the bile acid profile and the bacteria capable of bile acid degradation of the same samples.

5.6 High-fat diet alters the bile acid profile in the gut at early age

Similar to previous studies, high-fat diet increased the amount of primary and secondary bile acids in the cecum of the mice (Figure 15) [118]. Despite the challenge of comparing actual numbers of the total bile acid concentrations, due to different mouse strains and sample types, the control

group of a study with the same measuring technique supported that my finding (1.38 µmol/g) is in an adequate range (1.95 µmol/g). It has been shown that the changes induced by high-fat diet in the gut microbial composition are mediated to a high extent by alterations in the bile acid profile, especially by an increase of CA [39]. The most prominent increase in abundance in this study was detected for the secondary bile acid DCA, which derives from modifications of CA. DCA is reabsorbed and recycled back to the liver and again secreted as taurine conjugated form (TDCA) [119], therefore the increase in abundance could result from enrichment. This is especially of interest as DCA is associated with the formation of cancer [120].

5.7 Impact of high-fat diet on bacteria capable of bile acid degradation

As the modifications on primary bile acids are performed by bacterial enzymes arising questions are which bacteria are involved in the formation of secondary bile acids and how the increase in bile acids is affecting the abundance of these bacteria capable of bile acid modification.

The first step in the conversion of a primary bile acid to a secondary bile acid is the removal of the side chain by the bile salt hydrolase. Bile salt hydrolase activity is widely distributed among bacteria colonizing the intestinal tract. Within the Gram-positive bacteria, it has been detected in *Clostridium*, *Enterococcus*, *Bifidobacterium* and *Lactobacillus*, while *Bacteroides* and *Parabacteroides* are the only members of Gram-negatives so far known to exhibit BSH activity [121-125].

This study shows more than half of the *bsh* reads were assigned to the phyla *Bacteroidetes*, with a further annotation to *Porphyromonadaceae*, *Odoribacteraceae*, *Rikenellaceae*, *Prevotellaceae*, *Bacteroidaceae* and *Sphingobacteriaceae* (Figure 16 C). Of these six families, BSH activity has been described for members of the *Bacteroidaceae (Bacteroides*) and the *Porphyromonadaceae* (*Parabacteroides*) before [125, 126]. As most studies were based on cultivation essays the BSH activity of the other four families might have been missed so far. To confirm the BSH activity it would be necessary to screen members of these families by cultivation and enzyme activity assays in future studies. In addition, a single cell genomics approach would enable the assembly of draft genomes from the whole-genome sequencing data and hence reconstruction of molecular and regulatory networks [127].

Concerning the abundance of *bsh* reads an increase of bacteria capable of metabolizing bile acids by high-fat diet would have been expected. First, a selective advantage by access to additional nutritional resources could favor the growth of bacteria with BSH activity. The deconjugation of primary bile acids by BSH yields glycine and taurine. Bacteria encoding this enzyme could catabolize glycine to ammonia and carbon dioxide and taurine to ammonia and carbon dioxide with the additional release of sulfite, to promote their growth and reduce their need for competing for nutrients. Second, despite still being controversially discussed, BSH mediates higher resistance to conjugated bile acids and thus a selective advantage [128, 129].

However, contrary to my expectations, no increase in abundance of *bsh* genes by high-fat diet and total bile acids increase respectively, was detected (Figure 16 A). This could be due to the fact that most of the *bsh* reads were assigned to *Bacteroidetes*, which were significantly decreased and eventually replaced by bacteria without *bsh* gene in the high-fat samples. This assumption is supported by the α-diversity analysis, which indicated a decrease in family richness and a significant increase in evenness for bacterial families harboring the *bsh* gene (Figure 16 B). Another probable explanation would be a change at the transcriptional level. The *bsh* gene is generally not regulated by bile salts, but an exception to this has been demonstrated for *L. johnsonni.* Here an upregulation of *bsh* expression by bile acid concentration has been demonstrated, with the same study showing the proliferation rate being impaired at certain bile acid concentrations (2%) [130].

Although it is difficult to determine, if the bile acid concentration has an inhibiting effect on the gut bacteria in the cecum in this study, the bacterial community shows a clear compositional change. The phylum *Bacteroidetes* shows a significant decrease, which has been detected in studies of high-fat diet previously [36]. On family level, this decrease was continued by the significant decrease of *Porphyromonadaceae.* This higher susceptibility to damage by bile acids could be due to a characteristic of the bacterial cell, like the outer membrane composition, the missing of an efficient bile acid efflux pump or the lack of a sufficient DNA repair mechanism. However, the decrease of *Porphyromonadaceae* is also likely to be caused by ecological factors like substrate availability and competition and therefore the expected increase in bacteria with *bsh* gene might be prevented by environmental factors.

Further modification of the primary unconjugated bile acids takes place by a multistep 7α-dehydroxylation pathway, yielding secondary bile acids. The enzyme bile acid 7α-dehydratase, encoded by the gene *baiE*, catalyzes the rate-limiting an irreversible step in this pathway [56]. As the formation of DCA, which takes place by 7α-dehydroxylation of CA, is significantly increased by high-fat diet in this study, it was expected to find a higher amount of bacteria with *baiE* genes in the high-fat diet samples. Moreover, it has been suggested that this pathway provides bacteria with an additional electron acceptor [51, 55], which could favor their survival and proliferation when more bile acids are available. Also intuitively on would expect that bacteria which metabolize bile acids, developed strategies to cope with their toxicity. Interestingly the results show a significant decrease of *baiE* reads in the high-fat samples compared to the controls (Figure 17 A).

This decrease could be caused by accumulating DCA reaching a concentration, which is critical for the bacteria with the potential to produce it [131]. This is affirmed by α-diversity measures for baiE reads, where a decrease in richness and an increase in evenness on family level were observed (Figure 17 B).

Another reason could be selective pressure by the high-fat diet. As mentioned before the phylum *Bacteroidetes* – which has been shown to be negatively affected by high-fat diet – and within it, the family *Porphyromonadaceae,* was significantly decreased by the high-fat diet. In accordance with this, the amount of *baiE* reads assigned to the phylum *Bacteroidetes* is decreased by 4.58 % by high-fat diet, and by 5.69 % concerning the family *Porphyromonadaceae*. As the overall amount of *Porphyromonadaceae* was decreased from 12.47 % to 7.14 % the decrease in reads assigned to this family could have been expected to be even more pronounced. As this was not the case this supports the hypothesis, that the intestine environment selects for bacteria able to modify bile acids [132, 133].

Furthermore, the 7α-dehydroxylation of bile acids has been demonstrated for the genera *Eubacterium* and *Clostridium* so far [56, 134]. Interestingly the amount of reads assigned to the phylum *Firmicutes* was low in comparison to the amount of reads assigned to the phylum *Bacteroidetes* in both, controls and high fat treatment samples. But the increase of *Firmicutes* and decrease of *Bacteroidetes* by the high-fat diet, which has been detected analyzing the taxonomy of all reads, was detected among the assigned *baiE* reads, too (Figure 17 C).

Another reason for the decrease of bacteria capable of 7a-dehydroxylation could be the dependence on the deconjugation of the bile acids secreted by the host by BSH. As I haven't detected an increase in BSH activity, this tendency might be amplified further downstream of the microbial inter-species co-metabolism.

Finally, also host factors could influence the selection of distinct bacterial families. Bile acids have endocrine functions in the body through their binding to the Farnesoid X Receptor (FXR) and the G-protein-coupled bile acid receptor TGR5. Alterations of the bile acid profile influence the gut microbial composition by inducing genes encoding anti-microbial peptides via FXR [49]. The TGR5 is i.a. a negative regulator of gastric inflammation [135]. As DCA has a higher potency to activate TGR5 than CA [136] the inflammatory potential might be decreased and favor the growth of bacteria replacing bacteria with *baiE* gene.

6 Conclusions and Outlook

As more and more studies emphasize the importance of the gut microbial bacteria for health, also its role during early development comes into focus. The composition of the gut bacterial community is critical for the development of the immune system and its disturbance can lead to disease later in life. It is therefore important to investigate the different factors shaping the bacterial composition at early age.

With this study I contributed to the knowledge about these factors influencing the gut microbial composition at early age. I confirmed my first hypothesis, according to which there is a distinct influence of the birth and nursing mother on the gut microbiome of the offspring at early age.
The results showed that the influence of the various pre- and post-natal maternal factors impact the bacterial community in cecum and colon in a similar way.
The effect of the birth mother on the microbiome in colon and cecum of their offspring indicated a contribution of the quality of colostrum, but the impact of the host genetics seemed to play a major role in mediating the impact of the birth mother on certain bacterial families. The genetics seemed to exert its impact largely by determining the hosts immune system, as the bacterial families responding to factors of the birth mother – *Turicibacteraceae*, *Streptococcaceae* and *Rikenellaceae* – had been shown to correlate with genetic traits before. However, it is difficult to distinguish which factor of the birth mother exactly determines the impact on the offspring´s gut microbiome, due to the variety of physical parameters involved. To gain insight in these complex interactions in future studies the parameters should be investigated separately and it should be aimed for a higher taxonomic resolution.

Furthermore, the study demonstrated the importance of the nursing mother in shaping the gut microbial composition of the offspring during early age. This impact included the general transfer of microbiota from the mother, which might be selected by her genetics and behavior, via close contact. However, the data indicated that the predominant nursing mother factor shaping the offspring´s gut microbiome was the maternal milk, but it is not clear if the bacteria are transferred by the maternal milk or selected for by its composition. To refine our insight in how the maternal milk shapes the gut microbial community and to determine the mechanism behind it, future analyses have to include bacterial metabolism and function and also the sampling has to be extended to include samples of the mothers and the maternal milk. Moreover, it would be of high

interest if the effects described for the maternal impacts just reflect the moment of sampling or determine the health of the offspring also later on in life. This remains to be investigated in future long-term studies.

To isolate the potential contribution of the fat content of the maternal milk I further on investigated the impact of high-fat diet on the gut microbial composition at early age. As assumed high-fat diet had a major impact on the cecal gut microbiome composition already at early age. It largely resembled the effect observed in adults and was visible independent of obesity. Especially interesting was the reduction of the sulfate-reducing bacteria and the family *Porphyromonadaceae*. Here it would be interesting to further analyze the cross-feeding mechanisms sulfate-reducing bacteria are involved in, as well as the influence the alterations the high-fat diet could cause in mucus molecule composition.

I choose to further concentrate on the impact of high-fat diet on the bile acid profile and the intestinal bacteria capable of bile acid degradation. The data showed that a high-fat diet increased the amount of secondary bile acids and affected the diversity of bacteria capable of secondary bile acid formation. Especially the concentration of the secondary bile acid DCA was elevated, leading to a higher risk of cancer formation. I found indication for participation of novel families of the *Bacteroidetes* on DCA production. Analysis of the bacteria responsible for DCA formation detected potential bile acid 7α-dehydroxylation activity for some novel families, of which especially the abundance of the family *Porphyromonadaceae* stood out. Despite the negative effect of bile acids on the bacteria metabolizing them, there was still a significant enrichment of the host's bile acid pool with secondary bile acids. The decrease of certain families involved in bile acid metabolism, especially *Porphyromonadaceae*, might be compensated on functional level by an increase in gene expression. Therefore high-fat diet and consequential bile acid increase harm both, microbiome and host, by enrichment of secondary bile acids, hence affecting the intestinal environmental conditions that affect other bacterial taxa and steer their metabolic activities. Further studies should investigate the potential of members of the *Porphyromonadaceae* within the formation of secondary bile acids in more detail.

In conclusion my study contributed to the knowledge on which factors are involved in shaping the gut microbial community at early age and emphasized the importance to further investigate these factors on a mechanistic level. The prospective elucidation of these relations and mechanisms will

contribute to understand and cure diseases associated with the disturbance of the gut microbiota at this stage of development.

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List of abbreviations

baiE	Bile acid 7α-dehydratase
BLAST	Basic Local Alignment Search Tool
BM	birth mother
bp	base pairs
bsh	bile salt hydrolase
DEPC-H_2O	distilled water treated with DEPC
DCA	deoxycholic acid
CA	cholic acid
MCA	muricholic acid
HPLC	High Performance Liquid Chromatography
NCBI	National Center for Biotechnology Information
NM	nursing mother
NMDS	non-metric multidimensional scaling
NTC	non template control
OTU	operational taxonomic unit
PCoA	Principal Coordinates Analysis
PCR	polymerase chain reaction
SRA	Sequence Read Archive
UHPLC-MS	ultra-high pressure liquid chromatography coupled to ion trap mass spectrometry

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Consent for publication

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This applies to Figure 2 (upper half), Figure 5 (c, d), Figure 6 (B) and Figure 8 of this thesis.

Appendix

Supplementary Table 1: Body weight of exemplary mice from cross-fostering study

Type of control	Body weight [g]	Average [g]
obese	11,22	
obese	9,79	
obese	11,57	10,86
lean	12,32	
lean	11,64	
lean	13,56	
lean	12,46	12,495

Supplementary Table 2: Average blood test results and body weight measures of the six weeks old mice of the high-fat diet feeding trial

	high-fat (n=7)	high-fat STD	Control (n=7)	control STD	unit
ALB	41.71	2.56	41.14	2.91	g/L
ALP	213.57	14.35	310.43	37.32	U/L
ALT	77.00	30.82	79.14	47.95	U/L
AMY	923.29	61.87	913.86	129.97	U/L
BUN	5.91	0.77	8.61	0.73	mmol/L
CA	2.40	0.03	2.34	0.07	mmol/L
CRE	19.43	0.00	19.57	13.52	µmol/L
GLOB	9.00	2.37	7.43	1.29	g/L
GLU	10.83	1.12	9.17	0.53	mmol/L
K+	8.44	0.20	8.43	0.07	mmol/L
NA+	143.29	0.98	146.14	1.98	mmol/L
PHOS	3.31	0.41	3.20	0.47	mmol/L
TBIL	5.00	0.49	4.14	0.45	µmol/L
TP	50.86	2.19	48.29	3.73	g/L
body weight	17.96	1.06	16.78	1.40	g

Supplementary Table 3: Number of reads per sample of 16S RNA gene sequencing

Sample ID	Number of raw reads	Number of filtered reads used for analysis
B009	129292	34515
B013	79829	35194
B017	78166	49617
B021	87061	56005
B025	93542	54057
B029	100641	41350
B033	101420	44023
B037	97908	51404
B041	116398	35961
B045	77046	61455
B049	68646	44854
B053	54671	28792
B057	121561	71749
B061	88182	55143
B065	97983	62436
B069	84428	57572
B073	96590	73378
B077	80461	57364
B081	88603	65436
B085	106267	74184
B089	86480	46968
B093	38424	27282
B097	94955	64088
B101	87134	66001
B105	87662	60588
B109	103075	58657
B113	68622	42806
B117	90843	67358
B121	86775	67285
B125	81816	61745
B129	71788	53528
B133	87822	67374
B137	80240	60314
B141	85176	67266

B145	81165	64084
B149	67145	52760
B153	85551	68338
B157	68541	50920
B161	72073	57081
B165	95336	69509
B169	78884	61973
B173	94262	71438
B177	60934	45937
B181	75440	60636
B185	60502	49070
B189	77093	54327
B193	87184	54669
B197	65261	41294
B201	97388	52426
B205	84345	57127
B209	78347	59764
B213	122129	87946
B217	70268	53903
B221	63722	51054
B225	79324	53188
B229	87615	63507
Blank	85596	X
NTC110	108606	X
NTC2010	19563	X
NTC210	102786	X

Supplementary Table 4: Average bile acid concentration in mouse cecum (µmol/g) measured by UHPLC-MS

	Full Name	**high-fat (n=7)**	high-fat STD	**control (n=7)**	control STD
12-oxoLCA	12-oxolithocholic acid	0.034	± 0.021	0.019	± 0.017
3-dehydroCDCA	3-dehydrochenodeoxycholic acid	0.049	± 0.026	0.029	± 0.028
7-oxoDCA	7-oxodeoxycholic acid	0.050	± 0.073	0.037	± 0.070
AlloCA	Allocholic acid	0.038	± 0.041	0.026	± 0.019
βMCA	α-Muricholic acid	0.326	± 0.189	0.124	± 0.087
CA	Cholic acid	0.262	± 0.427	0.068	± 0.068
CA-7S	Cholic acid 7-sulfate	0.318	± 0.540	0.090	± 0.105
DCA	Deoxycholic acid	1.283	± 1.034	0.204	± 0.132
HDCA	Hyodeoxycholic acid	0.077	± 0.059	0.018	± 0.0271
LCA	Lithocholic acid	0.034	± 0.027	0.009	*
TCA	Taurocholic acid	0.008	± 0.005	0.190	± 0.405
TDCA	Taurodeoxycholic acid	0.014	± 0.005	0.013	± 0.013
βTMCA	β-Tauromuricholic acid	0.011	± 0.005	0.222	± 0.502
UDCA	Ursodeoxycholic acid	0.072	± 0.071	0.014	± 0.013
ωMCA	ω-Muricholic acid	0.525	± 0.210	0.125	± 0.095
ωTMCA	ω-Tauromuricholic acid	0.026	± 0.016	0.068	± 0.154
βMCA	β-Muricholic acid	0.559	± 0.394	0.266	± 0.191
Sum of all bile acids		3.551	± 2.013	1.377	± 1.054

Bold: significantly different between treatments

* only detected in one replicate, no STD applicable