Diploma Thesis

The role of the gut microbiota in tumor-induced cachexia

submitted by

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to obtain the academic degree of

Doctor of Medicine

(Dr. med. univ.)

at the

Medical University of Graz

performed at the

Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine

under supervision of

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Graz, 27.11.2020

AFFIDAVIT

I hereby declare that I have written this thesis independently, without outside help other than the stated sources and that I have marked the textual or content-related passages taken from the sources as such.

Graz, 27.11.2020 *Daniela Fulterer eh*

ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor Univ.-Prof.[in] Dr.[in] habil.rer.nat. Christine Moissl-Eichinger for her endless support, guidance through this demanding assignment and inspiring conversations, as well as her team, especially Lisa Wink, for their patience and help with the lab work.

Furthermore, I am deeply grateful to my family and friends for always believing in me, enabling me to pursue my goals in life and getting me back on track when I lose direction.

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ABBREVATIONS

DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
NGS	next generation sequencing
SCFAs	short-chain fatty acids
ILCs	innate lymphoid cells
IgA	immunoglobulin A
TH cells	T-helper cells
IL-22	interleukin-22
EHEC	enterohaemorrhagic Escherichia coli
LDL	low-density lipoprotein
ROS	reactive oxygen species
POS	pectic oligosaccharides
MCA	fibrosarcoma cell line, non-cachexigenic
CHX	cachexigenic tumor cell line
C26	colon cancer cell line, cachexigenic
C26nc	colon cancer cell line, non-cachexigenic
DMEM	Dulbecco's modified eagle's medium
FCS	fetal calf serum
PBS	phosphate-buffered saline
CD2/F1	murine hybrid model (Charles River)
C57Bl6J	murine model (Charles River)
ABX	antibiotic treatment
16S rRNA	small subunit (16S) ribosomal ribonucleic acid
OTU	operational taxonomic unit
PICRUSt	phylogenetic investigation of communities by reconstruction of unobserved states
RSVs	ribosomal sequence variants
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis effect size

PCoA	principal coordinates analysis
RDA	redundancy analysis
CHXABX	cachexigenic tumor cell line and antibiotic treatment
iWAT	inguinal white adipose tissue
gWAT	gonadal white adipose tissue
DMH	1,2-dimethylhydrazine
FMT	fecal microbiota transplant

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ZUSAMMENFASSUNG

Hintergrund

Veränderungen des Darmmikrobioms stehen in Verbindung mit der Entwicklung von zahlreichen (chronischen) Krankheiten, darunter Krebs (1–3) und Tumorkachexie (4–9). Die Analyse des Mikrobioms könnte wichtige Informationen liefern und möglicherweise als neue therapeutische Strategie dienen.

Material und Methoden

Das Ziel dieser Arbeit ist es, die Veränderungen des Darmmikrobioms in Verbindung mit Tumorkachexie von drei unabhängigen Mäusekohorten, die mit Kachexie-erzeugenden oder nicht-Kachexie-erzeugenden Tumorzellen injiziert wurden, zu vergleichen. Das Tumorwachstum sowie das Körpergewicht wurden während der Studie überwacht und Fäzesproben wurden zu verschiedenen Zeitpunkten im Verlauf der Kachexie entnommen. Die extrahierte DNA von den Stuhlproben der Mäuse wurde anschließend zur PCR und NGS-basierenden Amplikon-Sequenzierung übermittelt. Um die Zusammensetzung des Mikrobioms und die taxonomische Vielfalt zu analysieren wurden die entnommenen Rohdaten, unter Verwendung von QIIME2 (10), durch eine Galaxy (11) Leitung verarbeitet. Calypso (12) wurde zur Berechnung der alpha- und beta- Diversität, Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaft und zur Visualisierung der Resultate verwendet.

Ergebnisse und Diskussion

Eine Änderung in der Zusammensetzung des Mikrobioms und eine Verminderung der bakteriellen Vielfalt und Reichtums war in allen drei Mausstudien mit dem Voranschreiten der Kachexie nachweisbar. Des Weiteren zeigten kachektische Mäuse Veränderungen in der Abundanz spezifischer Mikroben, insbesondere wurde ein signifikanter Anstieg von *Lactobacillus spp.* beobachtet. In Bezug auf spezifische mikrobielle Profile assoziiert mit Tumorkachexie, zeigen unsere Ergebnisse teils andere Resultate als andere Forschungsarbeiten (5,7). Mehr Studien, vor allem mit menschlichen Probanden, werden in diesem jungen Forschungsfeld gebraucht, um die Rolle des Mikrobioms in der Tumorkachexie bestimmen zu können und gegebenenfalls einen neuen Therapieansatz zu etablieren.

ABSTRACT

Background

Changes of the gut microbiome are associated with the development of multiple (chronic) diseases, including cancer (1–3) and tumor-induced cachexia (4–9). Analysis of the microbiome could provide important information and possibly serve as a new therapeutic strategy.

Material & Methods

This thesis aims to compare changes in the gut microbiome in relation to tumor-induced cachexia in three independent mouse cohorts inoculated with cachexigenic or non-cachexigenic cancer cells. Tumor growth and body weight was monitored throughout the study and fecal samples were taken at different time points with the progression of cachexia. Extracted DNA from murine fecal samples was subjected to PCR and NGS-based amplicon sequencing. To analyze the microbial community composition and taxonomic diversity the obtained raw reads were processed through a Galaxy (11) pipeline, using QIIME2 (10). Calypso was used to calculate alpha and beta diversities, differences in community composition, and visualize the results (12).

Results and Discussion

A shift in microbiota composition and a decrease of bacterial diversity and richness was detectable with progression of cachexia in all three murine studies. Moreover, cachectic mice showed changes in abundance of specific microbes, notably a significant increase of *Lactobacillus* spp. was observed. Regarding specific microbial profiles associated with tumor-induced cachexia, our findings provide partly different results when compared to other research (5,7). More studies, above all with human subjects, will be needed in this young field of research to determine the role of the microbiome in tumor-induced cachexia and to potentially establish a new therapeutic approach.

1 INTRODUCTION

1.1 The human microbiome

Trillions of microbes inhabiting the human body form a complex ecosystem with their host (13). The microbes, their genetic information, and the milieu in which they thrive is called the microbiome (14), whereas the term microbiota describes the microorganisms that compose the microbiome (14). The human microbiota consists of eukaryotes, archaea, bacteria and viruses/phages, noting that the bacteria alone outnumber the amount of human cells of its host (15,16).

1.1.1 Defining a healthy microbiome

Early research sought to define a healthy microbiome as a core set of taxa, being universally present in healthy individuals (17). Nevertheless, significant variation in microbial abundance was detected in large-cohort studies even among the healthy adults (17,18). Regardless of the changes in microbial composition, the metagenomic carriage and metabolic pathways seemed stable (17,18). Therefore, an alternative hypothesis of a healthy microbiome as a functional foundation, consisting of common gene families and pathways, was proposed (19). Furthermore, high diversity, in terms of richness (number of taxa present) (17), evenness (homogeneity of microbial constituents) (17) and temporal stability (20) of the microbiome has been associated with good health.

1.1.2 The microbes across the human body

The most intensively studied area of the human body is the colon with the lower gastrointestinal tract containing almost 100 trillion microorganisms and approximately 160 different species (21). Most of the detected microbes are bacteria, from which 93.5% are represented by the phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (22,23).

The oral cavity also harbors a highly diverse microbiome (24), similar to the gut (18). The predominant species at the beginning of the digestive tract are *Streptococcus spp*. (18).

Skin regions differ in their microbial composition mainly because of the local conditions of the skin (e.g. moist versus dry) (25). They are primarily inhabited by *Corynebacterium, Propionibacterium and Staphylococcus* (25).

The microbial ecosystem of the vagina is dominated by *Lactobacillus spp*., consisting of five community types. Those types are characterized by the predominant prevalence of a single species of *Lactobacillus* or a combination with other microbes, for example *Gardnerella* (26,27).

Other areas of the human body are more difficult to classify, concerning their microbial composition, because they demonstrate a lower microbial biomass and thus prove technical challenges for the detection of microbes. For example, the lung (28–30), breast milk (31), the placenta (32), the circulating blood (33) and the tissue microbiome (34,35) are regions of interest for future studies.

1.1.3 Archaea, viruses, eukaryotes and protozoa

Microbiome research in the past has been largely limited on bacteria (18,36), but now more and more studies characterize and highlight the importance of other microbial domains inhabiting the human body. Research findings remain limited though, as detection and classification techniques are still being developed (37,38).

Archaea have been described as a component of the healthy human gut microbiome. The dominant archaeal genera found in the human gut is the genus of *Methanobrevibacter* (37) and one member - *Methanobrevibacter smithii* has been reported to the improve efficiency of bacterial digestion of polysaccharides (39) and overrepresentation might promote adiposity (40).

Viruses are also arousing interest in the scientific world and it is estimated that every human is host to a distinctive virome (41,42) as part of their healthy microbial ecosystem (43). The virome has been specified to consist mostly of

bacteriophages (43), which are indicated to provide vehicles of horizontal gene transfer for remotely related bacteria (44).

Eukaryotes, namely *Candida, Malassezia* and *Saccharomyces,* are also being regularly detected in samples of healthy individuals and their importance for the host is being investigated (45–48). Moreover, multicellular eukaryotes, for example helminths, have also been a constituent of the human gut microbiome in previous generations (49). With their eradication from the human microbial ecosystem, modern societies might lack an important educator of the immune system and scientists hypothesized that this aspect might contribute to the increasing incidence of metabolic and inflammatory diseases (49).

Particular protozoa are commonly found as part a healthy microbial ecosystem as well (48,50), albeit they show a larger variability between individuals than bacteria (48). The presence of one member, namely *Blastocystis*, has been brought into correlation with a decreased risk of gastrointestinal disease (51).

While research on the other microbial domains is still in the beginning, studies indicate the importance of trans-kingdom interactions for the balance of a healthy microbiome. For instance, analysis of skin samples exhibited a rivalry between bacteria and fungi depending on the physiological attributes of collection sites (52). Furthermore, the genus of *Lactobacillus* is well-known for keeping the colonization of fungi, both in the gut (45) and vagina (53), under control.

1.2 Functions of the healthy gut microbiome

The microbiome has coevolved with humans to have multiple functions that benefit the health of their host. These functions include harvesting elsewise inaccessible nutrients from the diet, maintaining integrity of mucosal barriers, obtaining protection against pathogens and contributing to immune system development and homoeostasis (14,18).

1.2.1 Digestion and Metabolism

Microorganisms in the gastrointestinal tract are substantial regulators of digestion. They are important for the extraction, synthesis and absorption of a wide range of nutrients and metabolites, including bile acids, lipids, amino acids, vitamins and short-chain fatty acids (54). In addition, they aid in the regulation of lipid homeostasis (54,55). Besides the production of diet-dependent metabolites and nutrients, the colonic microbiome is able to generate various diet-independent microbial products, for example the cell wall components lipopolysaccharide and peptidoglycan (54,56–60).

A core function of the human microbiome in the gut is carbohydrate fermentation. The microbes are essential for the degradation of plant-derived polysaccharides (61) and support oligosaccharide fermentation to release SCFAs and gases, which serve microbial cross-feeding communities and moreover about 10% of human daily energy demands are attributed to carbohydrate fermentation (62). The SCFAs butyrate and propionate can regulate intestinal physiology and immune function, while acetate acts as a substrate for lipogenesis and gluconeogenesis (63). When carbohydrates are depleted, the gut microbiota switches to other substrates, mostly proteins or simply amino acids. The fermentation of amino acids releases SCFAs, but also a some potentially harmful substrates. Studies have demonstrated that substrates like ammonia, phenols, p-cresol, certain amines and hydrogen sulfide, are associated with the initiation or advancement of a leaky gut, inflammation, DNA damage and even cancer progression (64). Conversely, carbohydrate fermentation through dietary fiber or consumption of

plant-based foods seems to inhibit the production of potentially harmful substrates (65).

1.2.2 Immune system

The microbiome is essential for the development and maturation process of the immune system. Firstly, an important energy source of the intestinal epithelial cells are microbial-derived SCFAs, which influence both oxygen usage and the reinforcement of the epithelial barrier (66). Secondly, microbes affect the development and function of myeloid cells at multiple times during cellular maturation. The lack of commensal microorganisms decreases myeloid-cell development in the bone marrow, which facilitates potential bacterial infections (67,68). The microbiome can also affect myeloid cells after hematopoiesis, namely by controlling the ageing of neutrophils (69) and the number of circulating basophils (70). In addition, the microbiota influences tissue-resident macrophages. For example, SCFAs serve as a signaling vehicle to modify the gene-expression profile of macrophages in the intestine (71,72). Finally, also innate lymphoid cells (ILCs) are reliant on the microbiome as microbes affect the maturation and proper functioning of ILCs (73–75).

Simultaneously, the intestinal immune system oversees the exposure of bacteria to host tissue to prevent a possible succeeding rise of pathologies, which could lead to an affection of key metabolic functions. This process happens at two levels: firstly, by reducing direct contact between gut bacteria and the intestinal epithelium (stratification) and secondly, by restricting pervasive microbes to intestinal sites and confining their exposure to the systemic immune system (compartmentalization) (76). One example is the colon, which demonstrates two mucus layers. Albeit the outer layer is able to curb a large amount of microbes, the inner layer is resistant to bacterial intrusion (77). The small intestine on the other hand, is lacking a structurally defined inner and outer mucus layer. Compartmentalization here is partly reached through secretion of antibacterial proteins by the intestinal epithelial cells (76). Secretion of immunoglobulin A (IgA) is essential for the stratification of bacteria on the luminal side of the intestinal

epithelium. Dendritic cells in the gastrointestinal tract take small numbers of bacteria which were able to pass through the epithelial border as specimens and then produce IgA directed specifically at these microbes. Furthermore, the intestinal dendritic cells are interacting with B cells to initiate them to produce IgA (76,78). Even though bacteria are largely contained on the luminal side of the intestinal epithelium, some microorganisms eventually penetrate the barrier and are disposed of by lamina propria macrophages (79). Also, innate lymphoid cells in the lamina propria support containment of intestinal bacteria as they exhibit effector cytokine profiles similar to those of T helper (TH) cells (80) and are able to produce interleukin (IL)–22, which is essential for preventing the spread of lymphoid-resident bacteria to systemic sites (81).

1.2.3 Protection against pathogens

The microbial community is also indispensable for the protection against a colonization by pathogenic bacteria that can be the result from the disturbance of the healthy microbiota. Mechanisms that control the capacity of the microbiota to limit the growth of pathogenic bacteria are complex and consist of competitive metabolic interplay, detection of intestinal niches and initiation of host immune responses (82). Commensal bacteria are able to produce bacteriocins, toxins that can impede the colonization of specific bacteria of the same or related species (83). For instance, *Escherichia coli* can produce a bacteriocin against a closely related pathogen, enterohaemorrhagic *E. coli* (EHEC), leading to an inhibition of its growth (84). The microbiota can also avert an infection caused by pathogens by changing the environmental conditions of the host and thereby preventing an colonization by pathogenic bacteria (83). A healthy microbiota of the vagina, for example, averts the colonization of urinary tract with pathogenic bacteria by reducing the local pH-value (85). Furthermore, commensal bacteria can cause starvation of competing and pathogenic microbes by favoring the consumption of nutrients required for them to grow (86–89). Another strategy of the microbiota in the prevention of pathogen colonization is enhancing host defense mechanisms indirectly, which includes enhancing the function of the epithelial barrier and promoting immune responses (82). Additionally, the production of IgA by the

intestinal epithelium can be stimulated by signaling of commensal microbes and it further boosts barrier function through binding bacterial antigens, alleviating pathogen activity and thereby averting infection (90–93).

Nevertheless, pathogenic bacteria have developed strategies to circumvent the mechanisms of commensal microbes (82). For instance, to avoid competition for nutrients with commensal bacteria, pathogens have adjusted to consuming more efficiently or switching to alternative nutrients (88,94). Moreover, to complicate survival of commensal microbes, some pathogenic bacteria are able to produce virulence factors that provoke inflammation of the intestine. The consequence of this triggered inflammatory reaction is diarrhea, which significantly reduces the number of commensal bacteria in the intestine and thereby benefits incoming pathogens and their chance of colonization due to lesser competition (95).

1.3 Influences on the microbiome

Population-studies conducted on a large scale have demonstrated, that the microbiome is highly influenced by ethnical, geographical and environmental factors (18,96–98). Moreover, underlying factors for the strong influence of geography and ethnicity on microbial communities, such as diet, cultural norms and genetical predispositions were also indicated (22,99,100).

Research suggests that genetics play a major role regarding the composition of the microbiome. This was shown in studies, which examined and compared the microbiome of related individuals. Especially twins were found to have a more similar microbial community patterns and species-level phylotypes compared to individuals who are not related (101,102).

Dietary habits greatly influence the microbial community of the gut (14,103,104). Notably, the macronutrient intake, primarily the type of carbohydrate consumed, seems to play a key role regarding the functional change of gut microbes (105,106). When intake patterns of the host change, microbial functions are highly likely to do so as well and with that also their strategies of nutrient-acquisition (107). The consumption of mostly fiber-rich and low- or non-processed foods, with fish and plants being the primary protein sources, is associated with a decreased risk of cardiovascular disease, cancer and mortality (108–111). Diets including highly processed foods, lots of snacking, whole milk and an overall higher calorie-intake, usually found in western cultures, were linked to a lower microbial diversity (104). Subjects with lower-diversity microbiomes were associated with a poor intake of vegetables, fruits and fish (112). They were found to have considerably higher levels of fasting serum triglyceride levels, an increased insulin resistance and were more likely to have elevated levels of LDL cholesterol, as well as inflammation, compared to subjects with high diversity microbiomes (113).

The microbiome composition can also be influenced by medication, especially antibiotics (114). However also proton-pump inhibitors (115) metformin (116), anti-depressants (114) and hormones (114) were indicated to possibly alter the gut microbiome. Even only being exposed to medication for a short period of time was found to significantly influence the microbial community (117).

1.4 Dysbiosis and Disease

Dysbiosis can be seen as a disruption of a microbial community in an otherwise stable ecology (118) and has been associated with multiple diseases, including metabolic syndrome (101,113,119–125) cardiovascular disease (65,126), inflammatory bowel disease (127–129), liver diseases (130,131), asthma/allergy (132,133), neurodevelopmental disorders (134) and cancer (1–3). Features of the microbiota that may cause diseases include functions such as the production of metabolites, extracellular enzymes or immunostimulatory surface structures. SCFAs are the most influential microbial metabolites in health and disease, as they affect a wide range of physiological functions, such as energy balance, endocrine function and shaping the immune regulation (135). Illness of the host is either a direct result of these functions or happens due to the initiation of damaging developments like immune system imbalances (136).

1.4.1 Cancer and the microbiome

As mentioned above, dysbiosis potentially contributes to the development of multiple diseases, including cancer (3). In colorectal cancer a diminished temporal stability and shift of diversity of gut microbiota was observed (137). Further studies in this field indicated, that microorganisms may facilitate susceptibility to cancers, either by being present within the tumor microenvironment or by interaction with far-off located microbiota through metabolic products (1).

Bacterial and viral infections have been suspected in tumor development for a long time, as they influence multiple metabolic and immunological processes. For instance, gastric cancer is associated with an infection caused by *Helicobacter pylori* (138). This bacterium can trigger chronic gastritis (139) and produces cytotoxins, which disturb cellular autophagy and apoptosis and thereby benefit carcinogenesis (138). Also, studies have associated prevalence of specific bacterial species with tumor sites in colorectal carcinoma (140). One of the most commonly reported bacterium is *Fusobacterium nucleatum*, which is able to influence carcinogenesis by causing inflammation of the gut, due to invasion of the colonic epithelium (141,142). As another example, some pathogenic strains of the

species Escherichia coli may cause a rise of DNA mutations in infected cells through encoding of colibactin, which is a genotoxin (143). Moreover, the presence of *Enterococcus faecalis* was associated with development of colorectal carcinoma as this bacterium can produce reactive oxygen species (ROS), which are able to damage the DNA of epithelial cells (144).

Besides the direct effects of microbiota on host tissues, studies suggest that commensal gut microbes might modify development and progression cancer through for instance the production of metabolites (145). Evidence suggests that the development of cancer comes along with an increase of protein-derived metabolites, whereas SCFA levels decline (2). Other than being an energy source and inhibiting the bacterial colonization with Gram-negative pathogens, SCFAs have been reported to curb inflammation and promote cellular apoptosis in cancer (71,146,147). Studies of colorectal cancer showed a lower abundance of SCFAs producing bacteria and murine models of cancer indicated a potential protection against colorectal tumorigenesis through dietary fiber (148,149). In addition, other metabolites, such as secondary bile acids are gaining interest as potential influences on susceptibility to cancers. Animal and experimental models indicate secondary bile acids are used by commensal gut microbes as an instrument to modify immune functions and affect antitumor immunosurveillance (150,151). Furthermore, the two substantial secondary bile acids, deoxycholic acid and lithocholic acid, were discovered to trigger mitosis and inflammation in the stellate cells of the liver, consequently promoting liver cancer development (152). Additionally, secondary bile acids are able to change microbiota composition, reduce SCFA production, facilitate oxidative stress, induce impairment of the DNA and increase resistance to apoptosis (153).

The intestinal microbiota is also important in the metabolism of xenobiotics and has been linked to treatment-related toxicity in cancer therapy (154–157). Gut microbes have been found to interfere with immune reactions, for instance associations between the microbiota and the response to immune checkpoint blockage and other anti-cancer therapies have been reported (158–165). Simultaneously, results from preclinical studies indicate that modulation of the gut microbiota could possibly improve therapeutic response (166).

1.5 Cachexia

Cachexia is defined as a multifactorial syndrome characterized by occurrence of involuntary weight loss and systemic inflammation (167). It occurs in patients with chronic diseases, such as cancer, chronic heart failure, chronic kidney disease or chronic obstructive pulmonary disease (168). The syndrome is marked by loss of skeletal muscle tissue and body fat due to different metabolic alterations caused by long-term illnesses (169). These alterations also affect several other organs, including the liver, brain and heart (170). Cachexia is reported in 50-80% of patients with advanced cancer (171). and estimated to be the indirect cause of death for 20% of all cancer patients, as muscle-wasting of the diaphragm and cardiac muscle possibly encourage respiratory and/or cardiac failure (172,173). Cancer cachexia also decreases the quality of life (169) and limits oncologic treatment options, thus worsening the prognosis of patients (174,175).

Currently a multimodal therapy, consisting of nutritional support, supportive drugs, and physical activity, is used to treat cancer cachexia (176). All of these options are regarded as palliative and ineffective for the long-term perspective (177). Considering the high impact of cachexia on life quality and the diminished life expectancy, a lot of research is now focused on detecting new therapeutic strategies. One field of research is the microbiome and the potential of treating cancer cachexia by modulation of the microbial community (178,179).

1.5.1 Tumor- induced cachexia and the microbiome

The starting point of microbiome research in cancer cachexia was, that in a mouse model of colon cancer the gut barrier was found to be disrupted along with tumor growth, resulting in systemic inflammation (4). This finding led to more experiments with other mouse models with cancer (acute leukemia and subcutaneous transplantation of colon cancer cells), which suggested that microbiota composition is altered by cancer and the development of cancer cachexia (5). Also, the study demonstrated that the gut barrier function is modified in tumor-induced cachexia and found several bacterial taxa, that correlated with

this change. On the one hand, *Enterobacteriaceae* and *Parabacteroides goldsteinii* levels were increased in the feces of cancer-bearing mice and on the other hand, *Lactobacillus* levels (*L. johnsonii/gasseri* and *L. reuteri*) were decreased (5). Moreover, the scientists illustrated that reestablishing the *Lactobacillus* levels by feeding a mixture of probiotics (*Lactobacillus reuteri* 100-23 and *Lactobacillus gasseri* 311476) to leukemic mice with cachexia, counteracted inflammation in the plasma and partially prevented the accession of muscle atrophy markers (5). In a following study administration of a prebiotic, namely pectic oligosaccharides (POS), to leukemic mice was able to mitigate tumor-induced cachexia, by postponing the weight loss (6). Non-digestible carbohydrates with prebiotic properties were therefore proposed by the authors as a potential new strategy in multimodal therapy of cancer cachexia (6). Building on the previous studies, scientists then administered a synbiotic (inulin-type fructans in combination with live *Lactobacillus reuteri* 100-23) to leukemic mice. They reported that this synbiotic treatment reinstated the *Lactobacillus* population, reduced the Enterobacteriaceae levels and moreover prolonged the survival of the mice due to reduced hepatic cancer cell proliferation, muscle wasting and morbidity (7). Studies later also sought to identify the reason for the modification in gut functions and a possible biomarker for cachexia- related gut dysbiosis in a mouse model and validated their findings in a patient cohort. It was discovered that the alterations were largely driven by interleukin 6 and found an increase of lipopolysaccharide-binding protein levels in both cachectic mice and human patients, making it a possible biomarker (8). Furthermore, to evaluate the increase of Enterobacteriaceae species *Klebsiella oxytoca* was identified as one of the main Enterobacteriaceae species increased in cancer cachexia. The study indicated *Klebsiella oxytoca* to act as a gut pathobiont by altering the normal gut barrier function in cachectic mice (9).

1.6 Hypotheses and aims of this thesis

1.6.1 Hypotheses

For this thesis, the hypotheses are as follows:

- Changes in the microbiota composition can be observed in tumor-bearing cachectic mice compared to control groups
- Tumor-induced cachexia correlates with the relative abundance of microbes, creating specific microbial profiles
- The microbiome mirrors the state of progression in tumor-induced cachexia

1.6.2 Aims

To explore the above-named hypotheses, three independent mouse studies were included in this thesis. The murine fecal samples were collected at several timepoints and then analyzed regarding their microbial content.

The following aims were formulated:

- To investigate changes in the microbiota composition between tumor-bearing cachectic mice compared to control groups
- To explore changes in bacterial diversity and richness
- To detect specific microbial profiles correlating with tumor-induced cachexia
- To investigate the gut microbiome over the course of time with progression of cachexia

2 MATERIAL AND METHODS

This study was part of an ongoing university cooperation-project to analyze the microbiome in tumor-induced cachexia. The fecal samples were provided by the collaboration partner of the Karl-Franzens University in Graz and further processed and analyzed at the Hygiene Department and the Core Facility Molecular Biology, Center for Medical Research at the Medical University of Graz, Austria

2.1 Mouse cohorts and sampling design

Mouse experiments were performed by Martina Schweiger and her team at the Karl-Franzens University in Graz.
Tumor cells (MCA207, CHX207, C26, and C26nc) were cultivated in DMEM + 10 % FCS + Penicillin/Streptomycin. On the day of injection, cells were harvested by trypsinization and washed 3 times in phosphate-buffered saline (PBS). Cells were counted using a hemocytometer and diluted to 1 x 10^7 cells/ml. 100 µl of the cell suspension in PBS were injected intramuscularly into mice.
For the C26/C26nc tumor-bearing mouse experiment, 11 weeks old male CD2/F1 mice were inoculated with 1 x 10^6 C26 (cachexigenic), C26nc (non cachexigenic) tumor cells or with PBS as control. Mice were sacrificed 16 days after inoculation. Feces was collected at nine timepoints: before (T -6, T -4) and after tumor inoculation (T1, T5, T7, T9, T12, T14, T16).
For the CHX/MCA tumor-bearing mouse experiment, 11 weeks old male C57Bl6J mice (Charles River) were inoculated intramuscularly with 1 x 10^6 cells CHX207 (cachexigenic), MCA207 (non cachexigenic) tumor cells or with PBS as control. Mice were sacrificed 12 days after inoculation. Feces was collected 8 days and 11 days after inoculation.
For antibiotics treatment experiment in CHX mice, 10 weeks old male C57Bl6J mice (Charles River) were treated with an antibiotics cocktail (following advice from Fredrik Bäckhed – Table 1) for one week, then (day 8) mice were intramuscularly inoculated with 1 x 10^6 cells CHX207 or PBS (100 µl), further

treated with antibiotics (in the drinking water, 150 ml, changed every 48 h) and sacrificed 17 days after tumor inoculation. Feces was collected before sacrifice. For analysis, the samples were sent to the work group Moissl-Eichinger at the Medical University of Graz and stored there until further processing at -80°C.

Abx Formulation	Concentrations		
	ug/mL	mg in 200 ml	
Neomycin	100	20	
Streptomycin	50	10	
Ampicillin	100	20	
Vancomycin	50	10	
Metronidazole	100	20	
Bacitracin	50 Units	10	
Ciprofloxacin	125	25	
Ceftazidime	100	20	

Table 1 - Antibiotic formulation after Frederick Bäckhed

2.2 DNA extraction and PCR amplification

DNA from all samples was extracted using the E.Z.N.A. stool DNA kit (Omega bio-tek), following the extraction protocol as given by the manufacturer. DNA concentration was determined via Qubit and a standardized amount (template volume 10-30ng or 1-2µl) was subjected to PCR. The 16S rRNA gene amplicons for the universal approach were amplified using Illumina-tagged primers F515 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GGACTACHVGGGTWTCTAAT-3′) (180). The cycling conditions for the universal approach were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 60°C for 60 s and elongation at 72°C for 90 s and a final elongation step at 72°C for 10 min.

2.3 Sequencing

Library preparation and sequencing were carried out at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University of Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was done using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600 cycles (2 × 251 cycles).

2.4 Bioinformatics, statistical analysis and visualization

To analyze the microbial community composition and taxonomic diversity, obtained raw reads were processed through an in-house Galaxy (181) pipeline, using QIIME2 (10), under the guidance of Alexander Mahnert from the center for microbiome research. To analyze the predicted functions of studied microbial communities, the sequence data was processed with QIIME (182) and closed-reference OTU picking based on GreenGenes taxonomy (13_8 database, (183)). A subsequent PICRUSt (version 1.0.0.) analysis was performed using the default settings (184). A biome table was constructed for downstream analyses, and RSVs represented by five or less sequences were removed. These data processing steps were performed in Galaxy, an open source web-based platform for data processing and analysis (181). This platform was made available by the Center for Medical Research (ZMF), Medical University of Graz.
To calculate alpha and beta diversities, differences in community composition, and visualize the results, we applied Calypso (Version 8.84), an online platform for mining, visualizing and comparing multiple microbial community composition data (cgenome.net/calypso; (12)). Data filtering with the following settings was used: Taxa with less than 0.01 percent relative abundance across all samples were removed. Top 20,000 taxa were included. Total-sum normalization was applied for 16S rRNA gene data. Statistical comparisons were performed using the linear discriminant analysis (LDA) effect size method (LEfSe) (185).

3 RESULTS

This study examined the fecal microbiota composition in three independent murine cohorts, intramuscularly inoculated with tumor-cells, which additionally caused cachexia or not, compared to control mice. Moreover, in the third murine study an antibiotic cocktail was administered to further analyze the influences on the microbiome. The mice were distributed to multiple cages to minimize cage effect and clinical parameters (e.g. weight loss) were noted for the time of the study.

3.1 Mouse study 1 – (C26/C26nc experiment)

In this experiment, mice were sorted into three groups: cachectic (C26), non-cachectic (C26nc) and control. Each group consisted of six mice and they were put into six cages to keep the bias of the cage effect as small as possible. Feces was collected at nine timepoints: before (T -6, T -4) and after tumor inoculation (T1, T5, T7, T9, T12, T14, T16). Additionally, body weight changes were measured over time. In total, 162 samples were obtained in this experiment. Two samples had to be excluded for further analysis due to the low number of received reads (<5000).

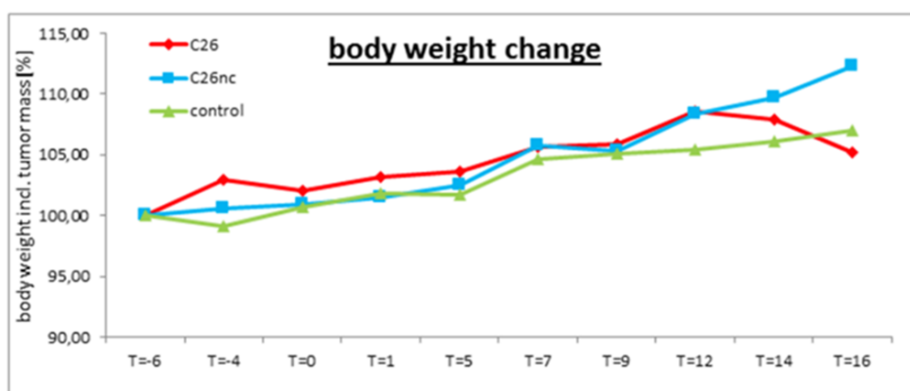


Figure 1 mouse group 1 - body weight change

Shows the C26nc-group increasing their weight around T9, probably due to the growing tumor mass and the C26-group decreasing their weight due to start of tumor-induced cachexia at T12.

3.1.1 The cage effect strongly influences the microbial community

To test for clusters of samples with similar community composition and identify environment- microbiota associations Principal Coordinates Analysis PCoA and Redundancy Analysis RDA were performed. Figure 2 shows that the variance in community composition is strongly influenced by the cage effect (p= 0.001). Comparing the early collection timepoints before injection (-6, -4) to the late timepoints after injection (12, 14, 16) there is no specific clustering of samples according to group even as the community composition shifts in the later timepoints. Despite these findings, general effects of tumor-induced cachexia should be very strong and independent from cage effect.

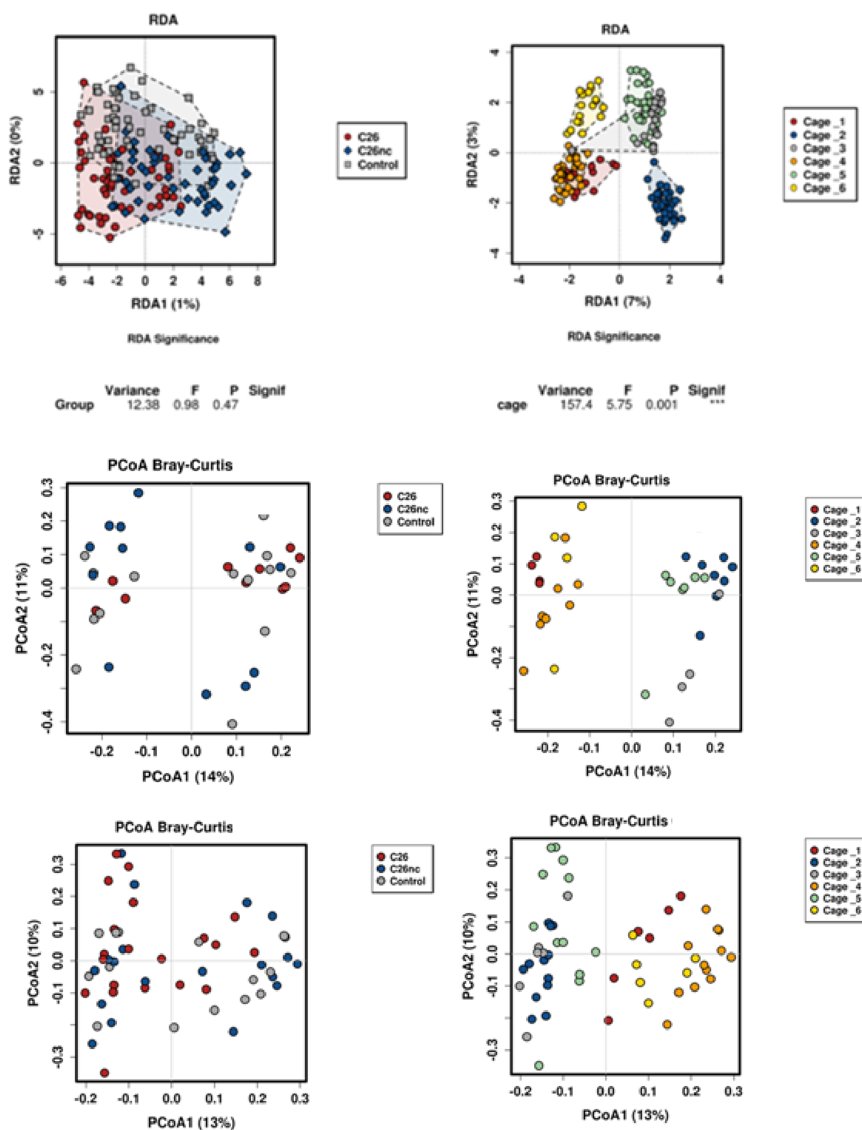


Figure 2 mouse group 1 - RDA Analysis of all timepoints. PCoA before injection and later timepoints (12, 14 & 16).

3.1.2 Diversity and richness are reduced with progression of cachexia

Microbial diversity was measured by Inverse Simpson index, a measure of overall present RSV and evenness. As illustrated in figure 3, diversity was reduced in the C26 group with progression of disease, but this was not significant (p=0.62). However, the most critical timepoint for a reduction in microbial diversity in the cachexia group was found to be timepoint 12, which marks the beginning of weight loss (see figure 1). There was also a small reduction in richness, a measure of present taxa/RSV observed in the C26-group comparing timepoints 9 and 12 with the other groups.

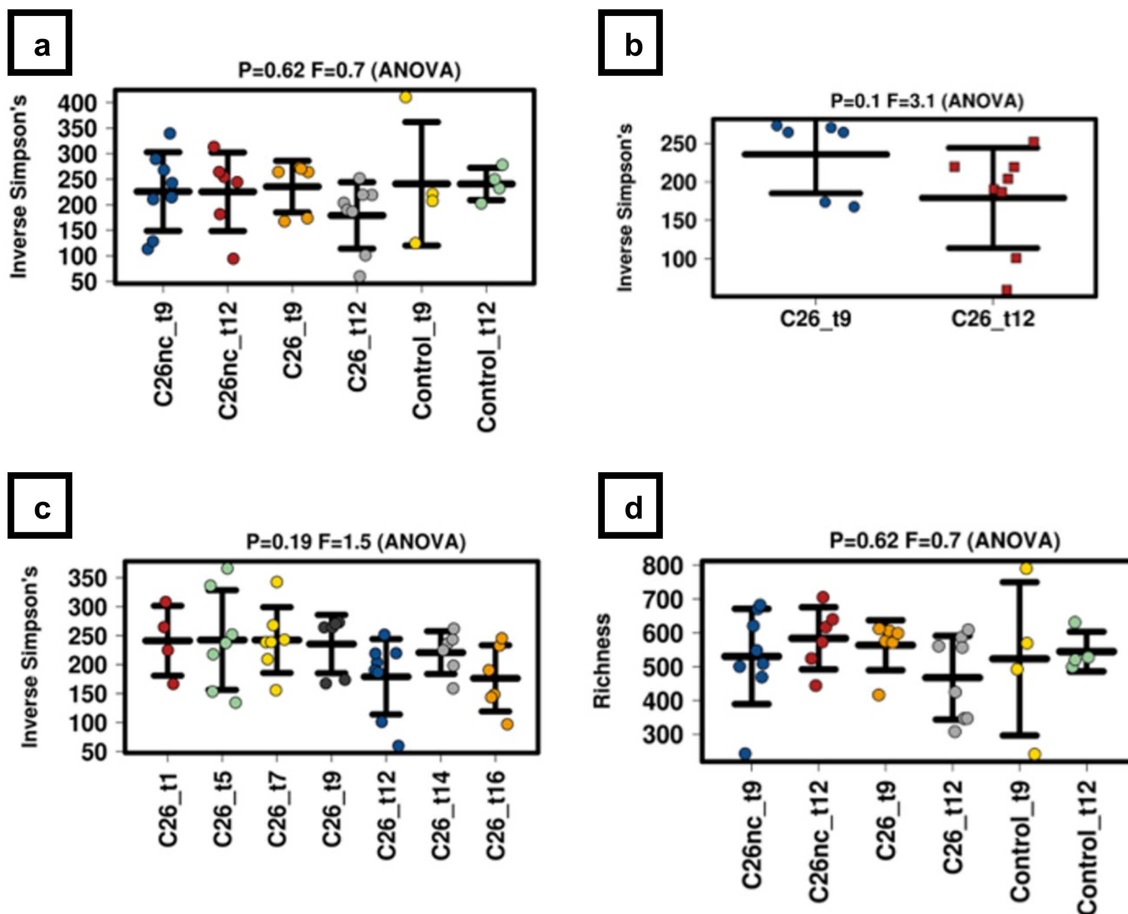


Figure 3 mouse group 1 - Inverse Simpson´s of timepoints 9 and 12 of all groups (a). Inverse Simpson´s of the C26 group timepoints 9 and 12 (b). Inverse Simpson of the C26 group after injection (c). Richness of timepoints 9 and 12 of all groups (d).

3.1.3 Tumor- induced cachexia correlates with the relative abundance of microbes creating specific signatures

Figure 4 visualizes the microbial composition on the early collection days (1, 5, 7) compared to late collection days (12, 14,16) in the C26 Group after tumor inoculation. A relatively constant microbial composition on the genus level was seen over the course of time but an apparent rise of *Lactobacillus* and decrease in abundance of *Ruminococcaceae_UCG014* was noticeable with progression of disease.

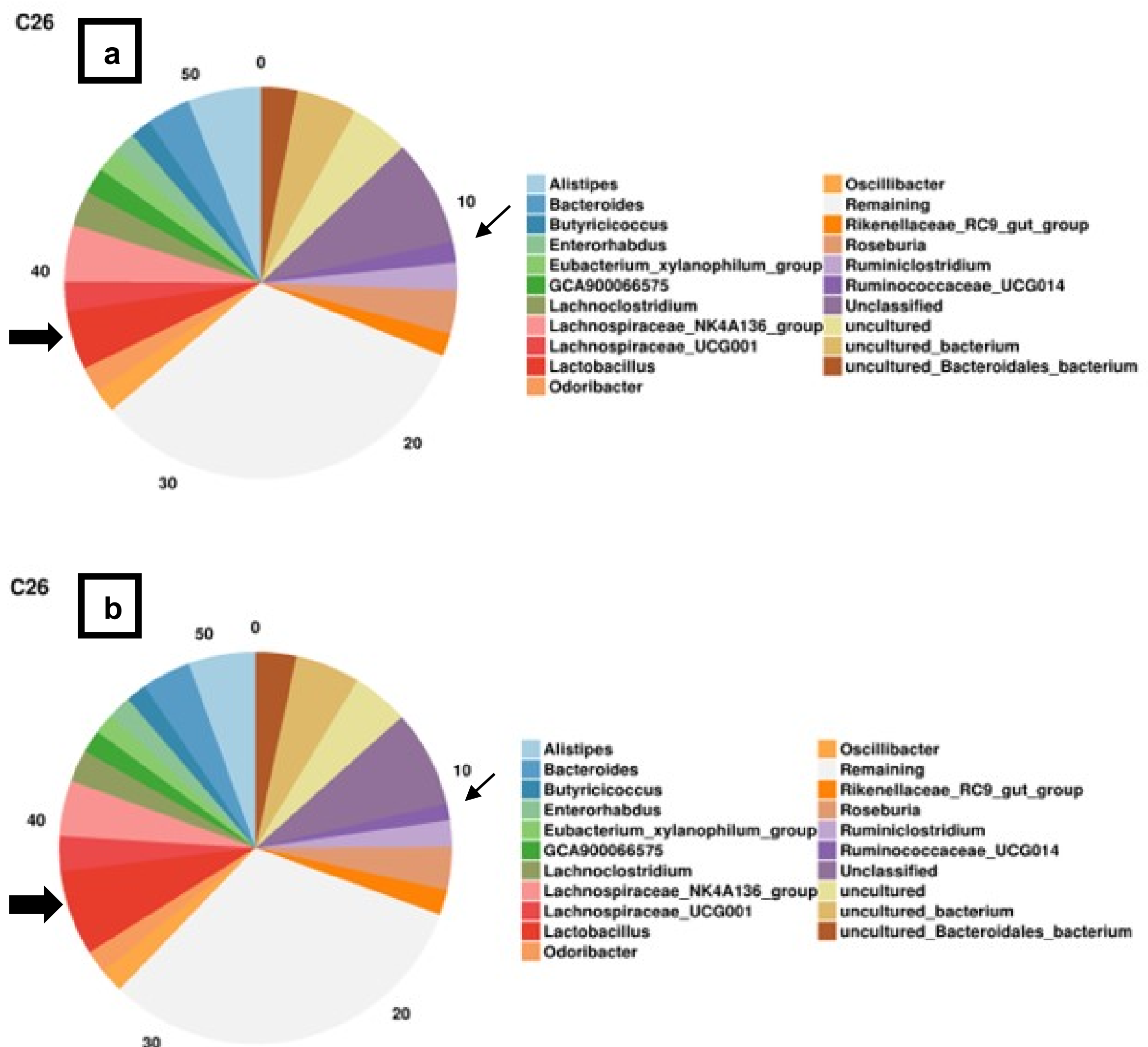


Figure 4 mouse group 1 - Pie chart C26 group genus level. Early collection timepoints (a - 1,5,7) vs. late timepoints (b - 12,14,16)

The LEfSe (linear discriminant analysis LDA effect size) analysis, shown in Figure 5, was used to identify bacterial taxa statistically associated with the changes around the most critical timepoints in the C26 group. On genus level *Lactobacillus* was linked to timepoint 12, while *Ruminococcaceae UGC014* was associated with timepoint 9. *Lactobacillus* increased significantly in abundance comparing the two timepoints (p= 0.018) and even more dramatically when compared to C26nc and control (p= 0.00039). *Ruminococcaceae UGC014* showed a significant decrease at timepoint 12 (p= 0.0092). The LEfSe Analysis on RSV level associated RSV 3046, closely related to *Lactobacillus salivarius,* with timepoint 12 and RSV 4611, corresponding to *Eubacterium xylanophilum*, with timepoint 9. *Lactobacillus salivarius* RSV 3046 showed a significant increase when comparing the two timepoints (p=0.044). There was no significant correlation between weight and microbiome composition at the later timepoints 12, 14 and 16 (not depictured).

Figure 6 shows the abundance of selected taxa. *Lactobacillus reuteri* and *L. gasseri* abundance in the C26 group showed a decrease at timepoints 5 and 14, but this was not significant (p= 0.27 and p= 0.34). *Enterobacteriaceae* and *Parabacteroides* also varied in abundance over the course of time, but no significant decrease/increase in the C26 group was detectable.

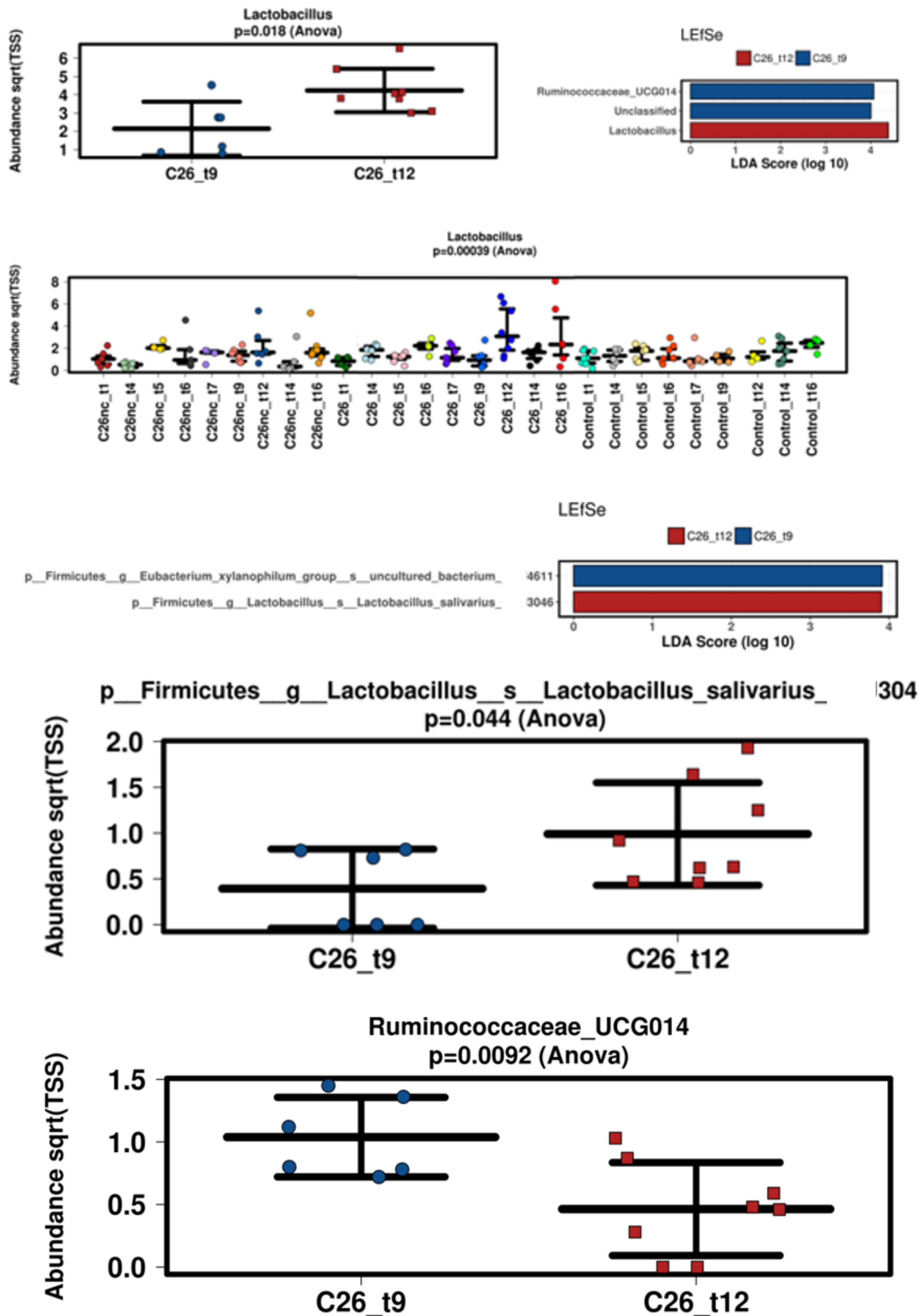


Figure 5 mouse group 1 - *Lactobacillus* abundance t9/12 C26 group. LEfSe Analysis on the genus level. *Lactobacillus* abundance in all groups after injection. LEfSe Analysis RSV level. *Lactobacillus salivarius* RSV 3046 abundance t9/12 C26 group. *Ruminococcaceae_UCG014* abundance t9/12 C26 group.

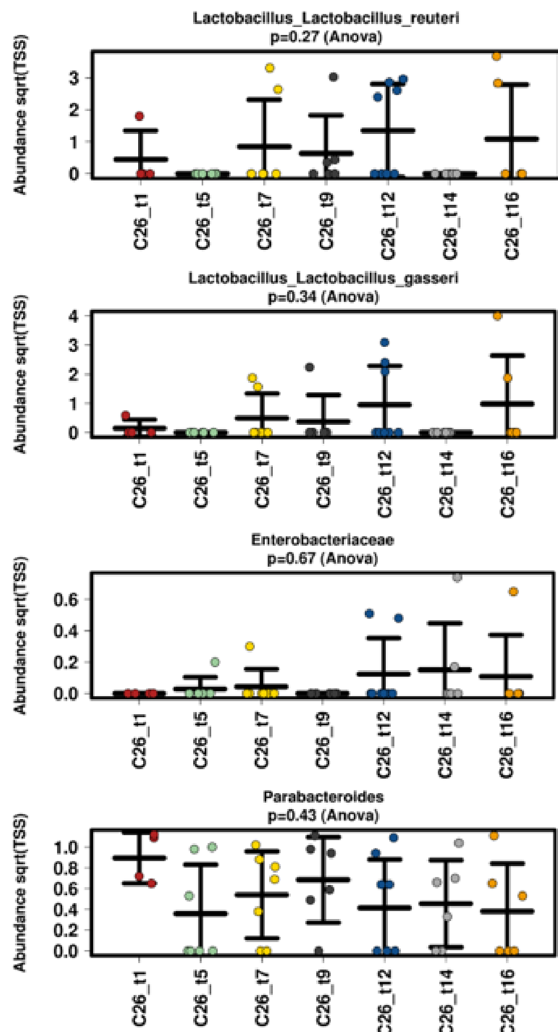


Figure 6 mouse group 1 - Abundance of selected taxa in the C26 group

3.1.4 Summary of mouse group 1

In the C26/C26nc experiment microbial composition was significantly influenced by the cage effect (p= 0.001), although a shift in community structure was detectable with progression of cachexia. A decrease in diversity and richness were also observed in the C26 group at later timepoints, but both were found to be insignificant. Nevertheless, the beginning of weight loss, timepoint 12, was identified as the most critical timepoint for a reduction in microbial diversity in the cachexia group and was statistically associated with *Lactobacillus* and RSV 3046 *Lactobacillus salivarius.* Comparing microbial abundances in the C26 group over time, *Lactobacillus* and *Lactobacillus salivarius* RSV 3046 showed a significant increase (p= 0.018 and p= 0.044) at timepoint 12, while *Ruminococcaceae UGC014* significantly decreased (p= 0.0092).

3.2 Mouse study 2 – (CHX/MCA experiment)

In this sub-study, mice were sorted into three groups: cachectic (CHX), non-cachectic (MCA) and controls. MCA group consisted of 4, CHX of 8 and control of 8 mice. They were sorted into 5 cages to keep the bias of the cage effect as small as possible. Feces was collected at two timepoints: 8 days (timepoint 1) and 11 days (timepoint 2) after injection. Additionally, body weight, tumor weight, fat mass and lean mass were measured over time. In total, there were 40 samples obtained from this study cohort. Two samples had to be excluded for further analysis due to a low number of sequence reads (<5000). Observational parameters, illustrated in figure 7, show the start of weight loss in the CHX group at day 9, as well as a rapid decrease of fat mass, a relatively consistent lean mass and increased tumor mass after sacrifice in CHX mice.

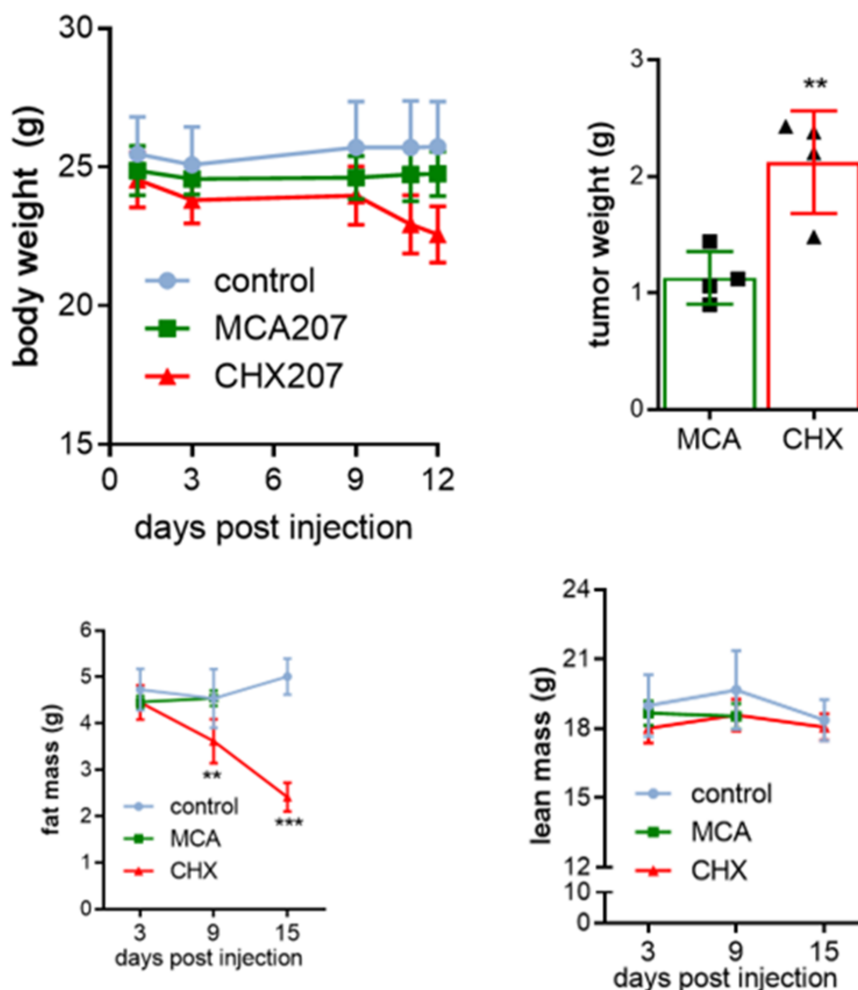
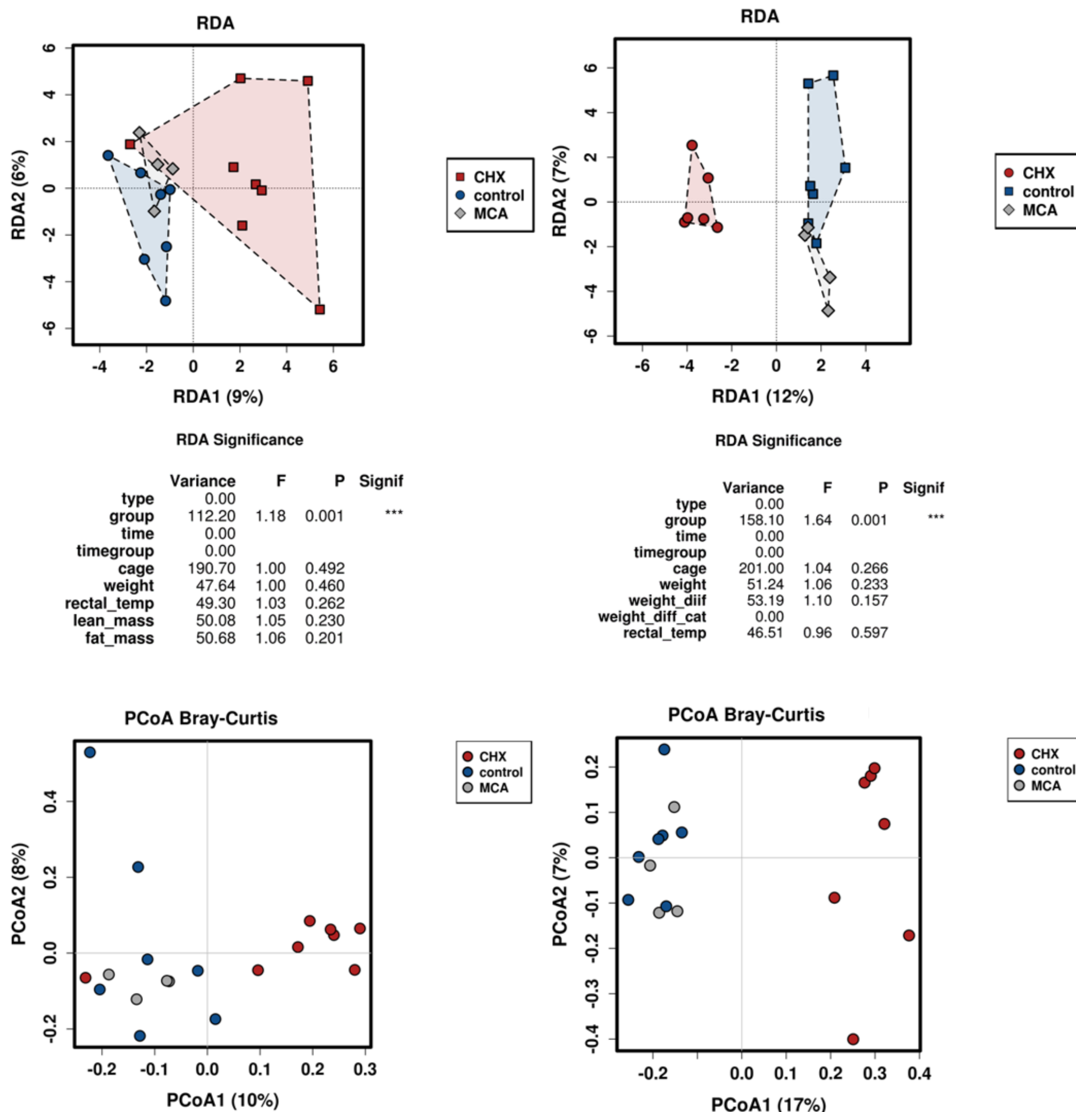


Figure 7 mouse group 2 - observational parameters

3.2.1 The microbial community showed a specific clustering according to group

To assess the influences on microbial community composition Redundancy Analysis RDA+ was performed, depictured in Figure 8. It showed a significant influence of the group selection on microbial community composition (p= 0.001) on both timepoints, whereas the cage effect was not significant (p= 0.492 and p= 0.266). To visualize clusters of samples with similar microbial composition Principal Coordinates Analysis PCoA was performed. Figure 8 shows PCoA of all groups at timepoints 1 and 2. A clear shift in community composition between the timepoints and a specific clustering at timepoint 2 becomes visible in the CHX group.



RDA Significance

	Variance	F	P	Signif
type	0.00			
group	112.20	1.18	0.001	***
time	0.00			
timegroup	0.00			
cage	190.70	1.00	0.492	
weight	47.64	1.00	0.460	
rectal_temp	49.30	1.03	0.262	
lean_mass	50.08	1.05	0.230	
fat_mass	50.68	1.06	0.201	

RDA Significance

	Variance	F	P	Signif
type	0.00			
group	158.10	1.64	0.001	***
time	0.00			
timegroup	0.00			
cage	201.00	1.04	0.266	
weight	51.24	1.06	0.233	
weight_diif	53.19	1.10	0.157	
weight_diff_cat	0.00			
rectal_temp	46.51	0.96	0.597	

Figure 8 mouse group 2 - RDA+ of all groups, timepoint 1 (left) and timepoint 2 (right). PCoA of all groups on RSV level, timepoint 1 (left) and timepoint 2 (right)

3.2.2 Diversity and richness are significantly reduced with progression of cachexia

Microbial diversity was measured by Inverse Simpson index, a measure of overall microbial richness and evenness. As illustrated in Figure 9, diversity at timepoint 1 was increased in the CHX group and then significantly (p= 0.00011) decreased with progression of disease. This significant reversal was also visible when assessing the microbial richness (p= 0.00017), a measure of the number of present taxa, and furthermore small changes in evenness were also observed between the timepoints (not depictured).

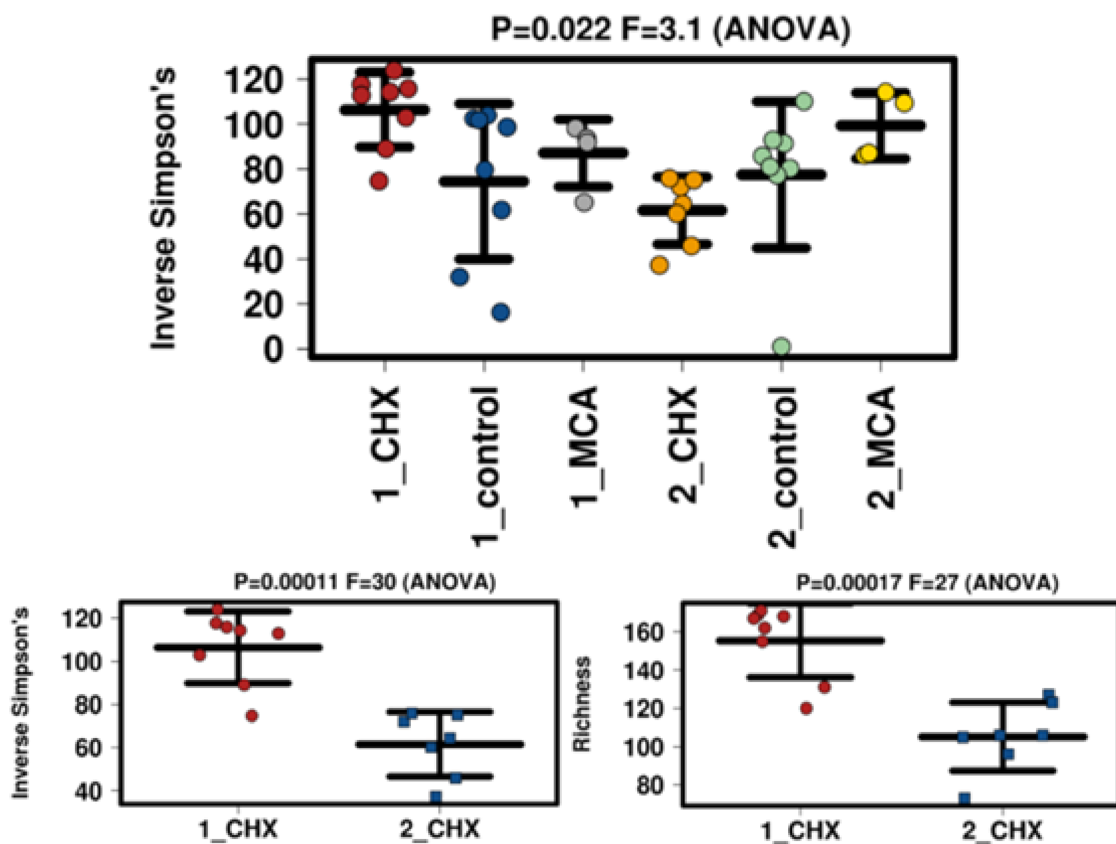


Figure 9 mouse group 2 - Inverse Simpson´s of all groups on timepoint 1 and 2 (above). Inverse Simpson`s and richness of the CHX group on both timepoints (below).

3.2.3 Tumor- induced cachexia correlates with the relative abundance of microbes creating specific signatures

Figure 10 visualizes the microbial composition at timepoint 1 compared to timepoint 2 in the CHX group. On genus level there was a visible change in microbial composition with progression of cachexia. Some of the major changes in microbial abundances were an increase of *Lactobacillus* and near depletion of *Ruminococcaceae UCG014*.

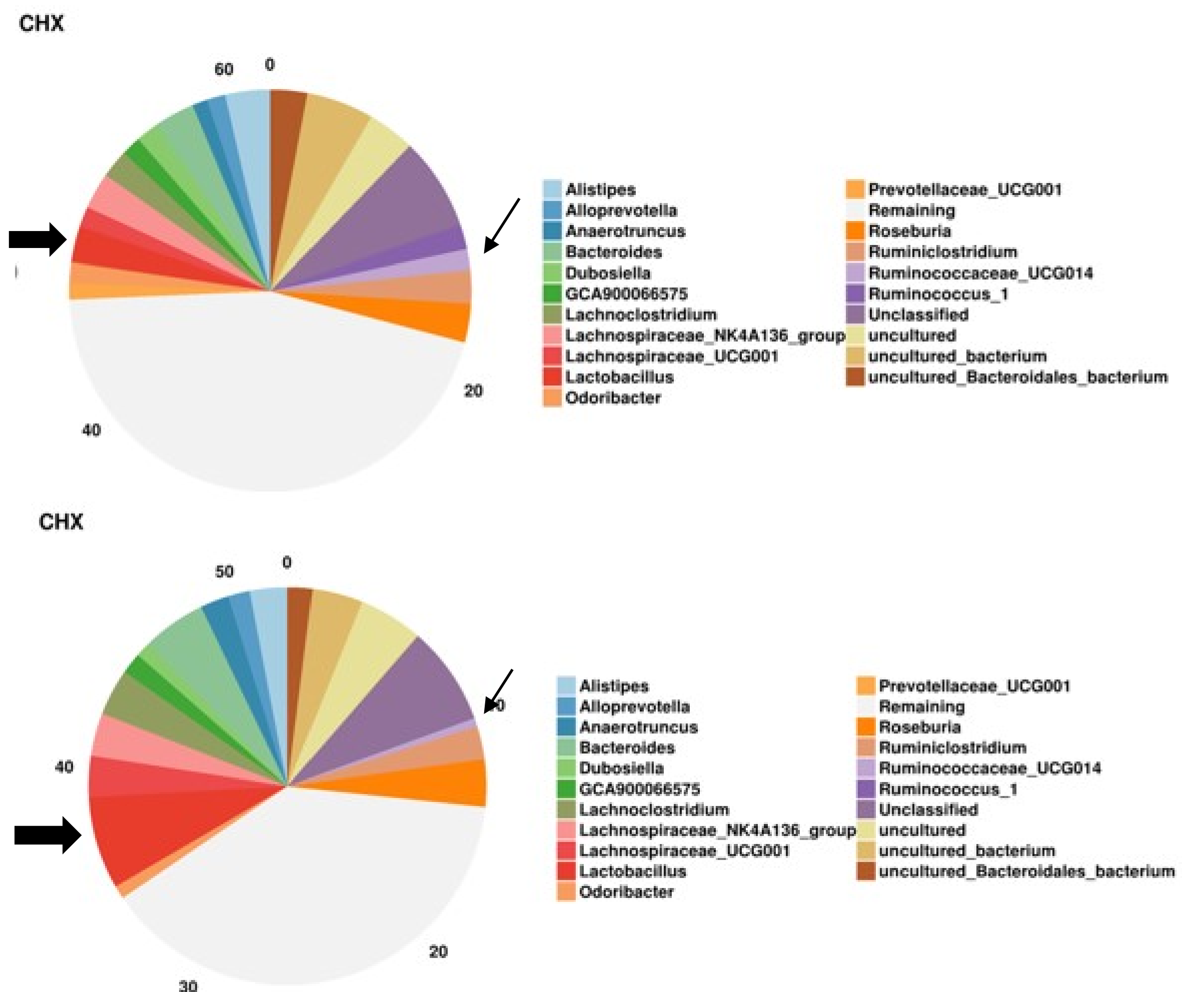


Figure 10 mouse group 2 - Pie Chart on the genus level of most abundant taxa in the CHX group on timepoint 1 (above) and 2 (below).

LefSE analysis on genus level as shown in Figure 11 was used to identify bacterial taxa, that differentiate the microbial composition at timepoint 2. A large number of genera were differentially represented between the three groups. The statistical associations of greatest magnitude with the sample groups were: *Prevotellaceae_UCG001* with MCA, *Rumicococcaceae_UCG014* with control,

Lactobacillus and *Bacteroides* with CHX. *Lactobacillus* increased significantly in abundance at timepoint 2 in the CHX group both, when comparing the samples separately (p= 0.025) and comparing the groups (p= 2.9e-07). *Bacteroides* also showed a significant rise in abundance at timepoint 2 in the CHX group (p= 0.001). Figure 12 shows linear regression models at timepoint 2, which indicate that the weight difference in this cohort is significantly negatively correlated with *Lactobacillus* (p= 1.6e-05) and positively with *Ruminococcaceae* UCG014 (p=6.7e-05).

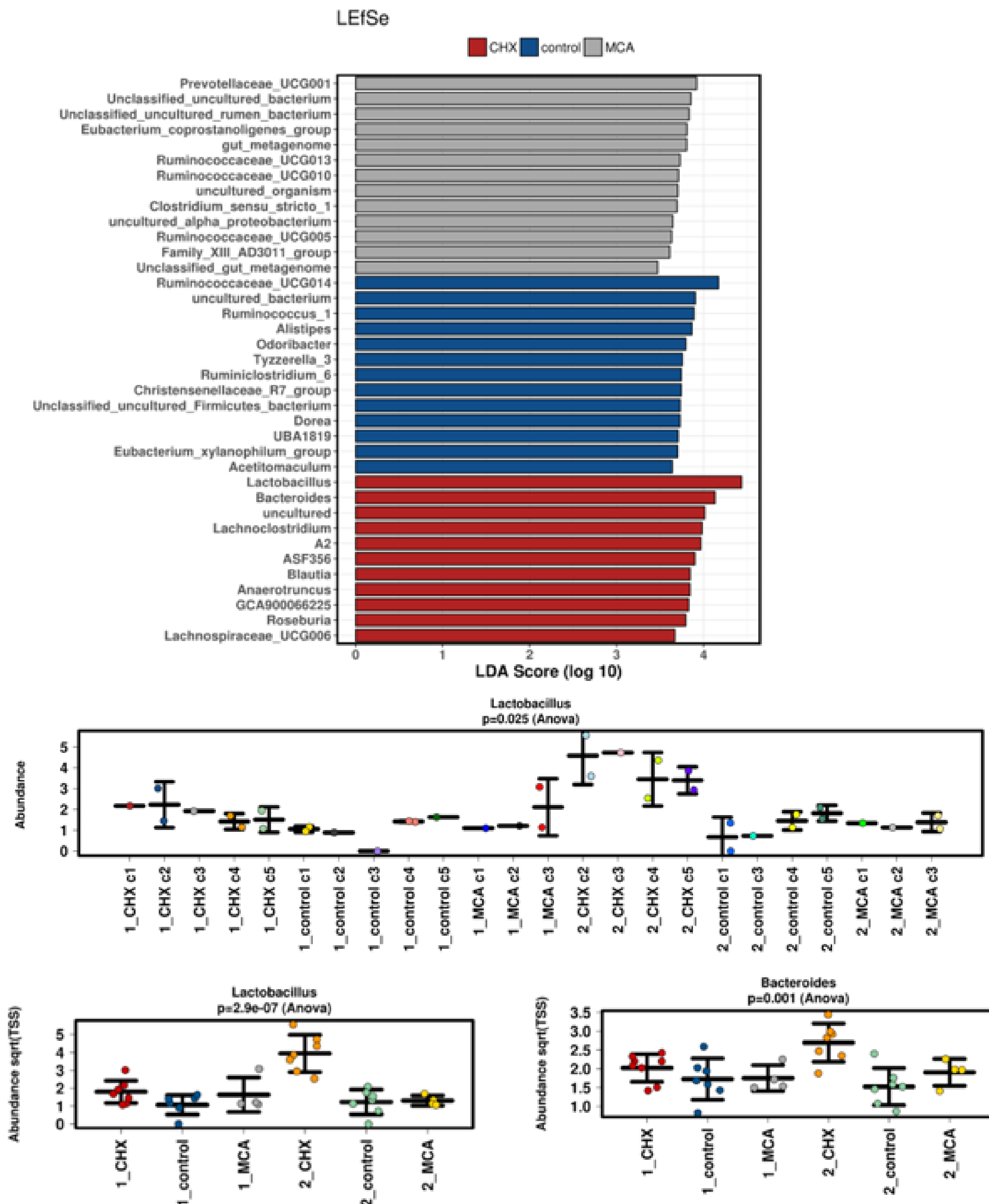


Figure 11 mouse group 2 - LEfSe Analysis on the genus level timepoint 2. *Lactobacillus* abundance in samples separately and comparison between groups. *Bacteroides* abundance of all groups and both timepoints.

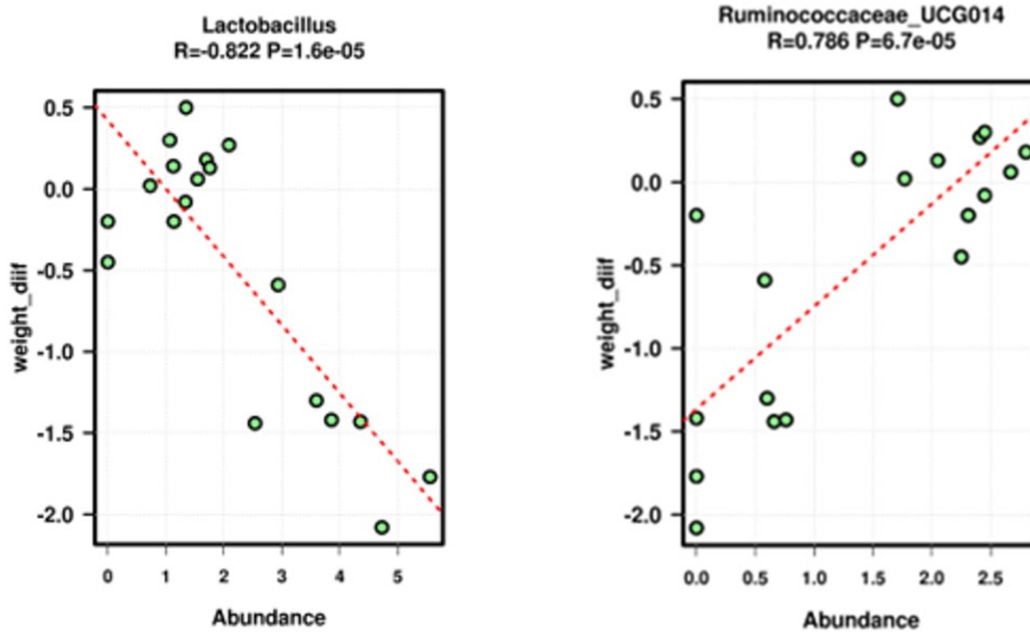


Figure 12 mouse group 2 - Regression analysis based on Pearson of all groups at timepoint 2.

The LEfSe analysis on RSV level for timepoint 2, shown in Figure 13 revealed a large number of individual RSV differentially abundant in the three different groups. The associations with the highest LDA score were: RSV2020 corresponding to *Lachnospiraceae_NK4A136* for MCA, RSV279 corresponding to *Alistipes* for control and RSV51 identified as *Lactobacillus salivarius* for CHX. *Lactobacillus salivarius* RSV51 and RSV72 showed a significant increase (p= 0.00014 and p= 0.00018) in the CHX group at timepoint 2. *Bacteroides* RSV30 was also seen to be significantly higher in abundance in the CHX group (p= 3.6e-05), whilst *Alistipes* RSV279, *Prevotallaceae_UCG001* RSV324 and *Ruminococcaceae_UCG014* RSV2930 were significantly decreased (p= 8e-05, p=1.12-05 and p=0.043).

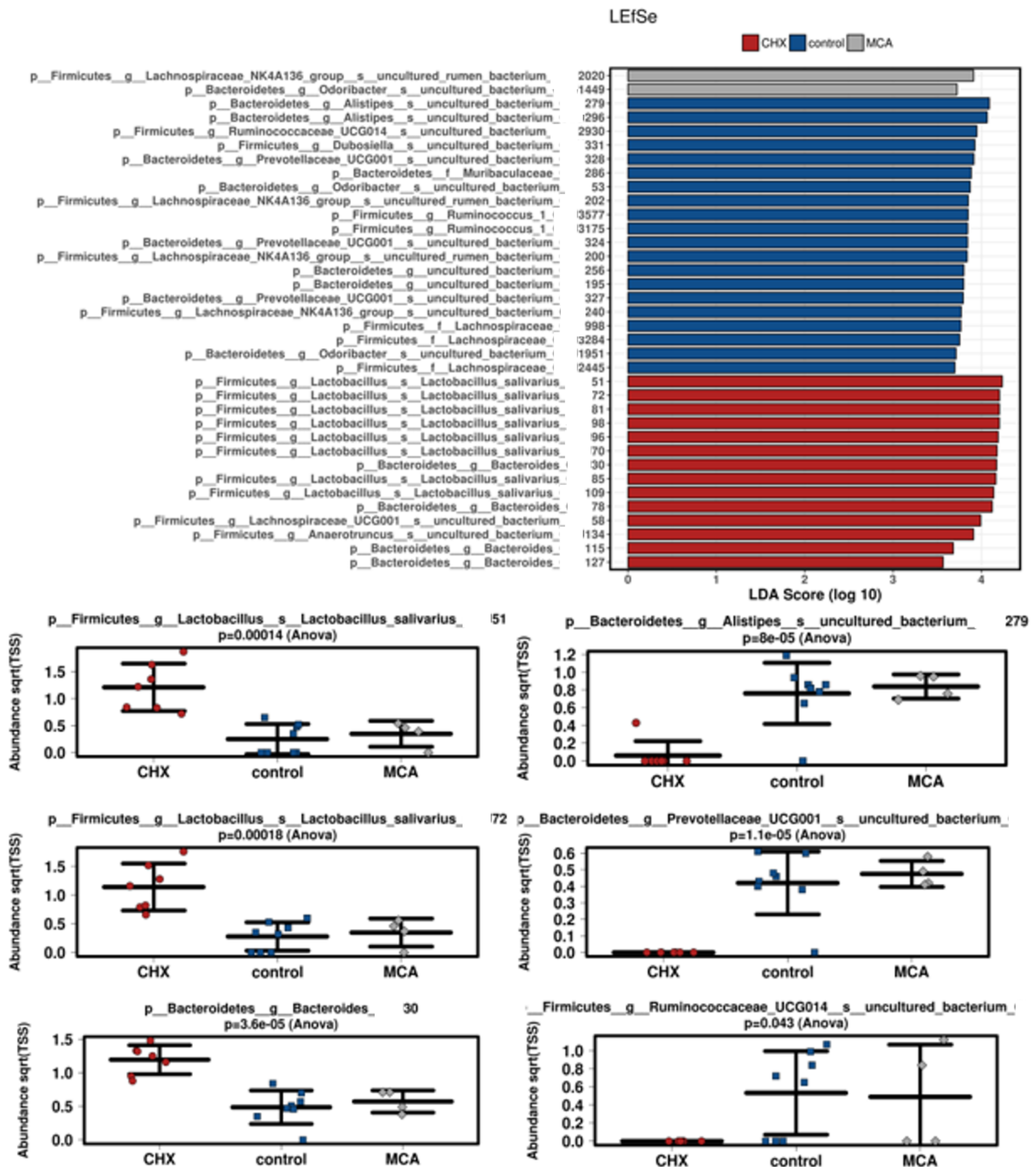


Figure 13 mouse group 2 - LEfSe Analysis RSV-level timepoint 2 all groups. Abundance of *Lactobacillus salivarius* RSV 51/72, *Bacteroides* RSV 30, *Alistipes* RSV279, *Prevotellaceae_UCG001* RSV 324 and *Ruminococcaceae_UCG014* RSV 2930 at timepoint 2 (all groups).

Figure 14 shows the abundance of selected taxa in all groups and comparing both timepoints. *Enterobacteriaceae* and *Lactobacillus reuteri* significantly increased at timepoint 2 in the CHX group (p= 0.00014 and p= 0.033). In *Parabacteroides* and *Lactobacillus gasseri* a rise in abundance was also visible, but not significant.

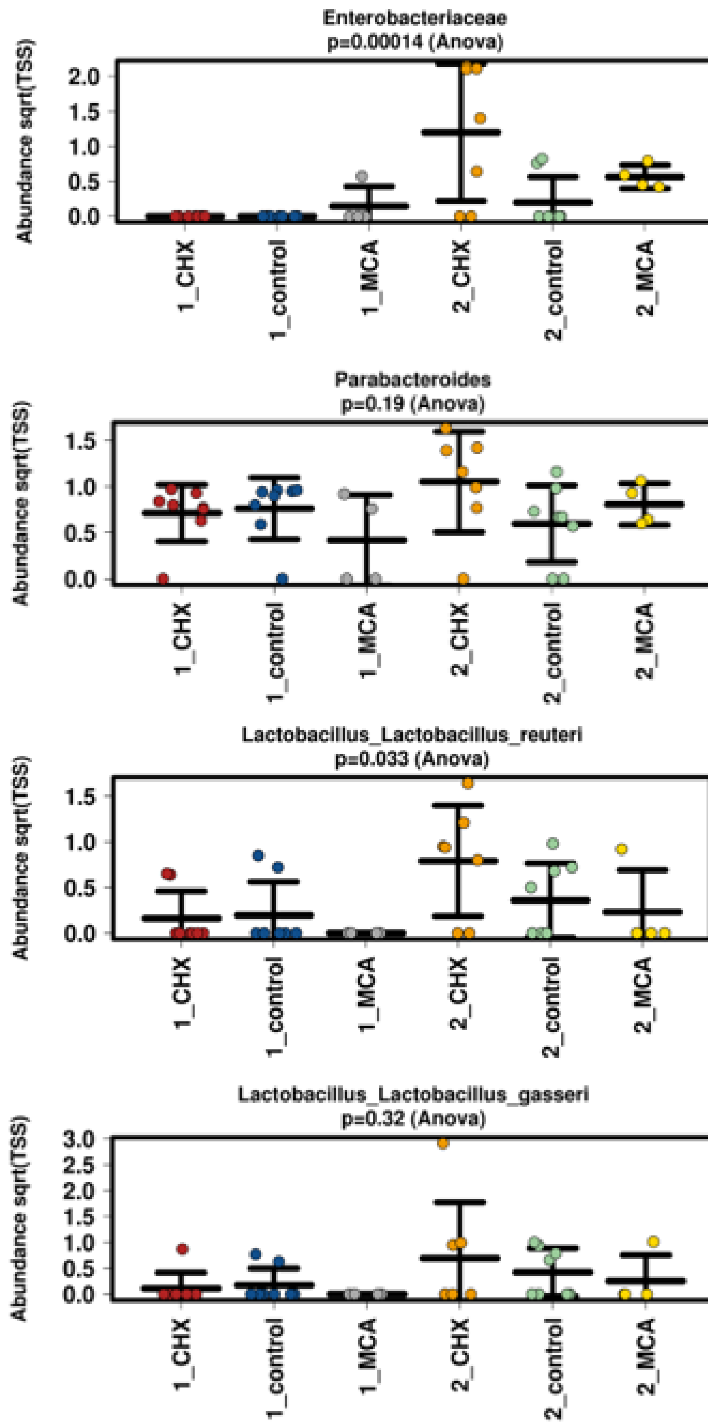


Figure 14 mouse group 2 - Abundance of *Enterobacteriaceae, Parabacteroides, Lactobacillus reuteri* and *gasseri* in all groups at both timepoints.

3.2.4 Summary of mouse group 2

In the CHX/MCA experiment group selection significantly influenced microbial community composition (p= 0.001), moreover, a visible and specific clustering at timepoint 2 was detectable in the CHX group. Also, diversity and richness significantly (p= 0.00011 and p= 0.00017) decreased with progression of cachexia. Furthermore, a significant rise in abundance of *Lactobacillus* (p= 0.025)*,* *Bacteroides* (p= 0.001)*, Enterobacteriaceae* (p= 0.00014)*,* RSV51/RSV72 *Lactobacillus salivarius* (p= 0.00014 and p= 0.00018), and *Lactobacillus reuteri* (p=0.033) in the CHX group were detected at timepoint 2. Whilst *Alistipes* RSV279, *Prevotallaceae_UCG001* RSV324 and *Ruminococcacea_UCG014* RSV2930 showed a significant decline in abundance (p= 8e-05, p=1.12-05 and p=0.043). Furthermore, linear regression models indicate that the weight difference in this murine cohort to be significantly negatively correlated with *Lactobacillus* (p= 1.6e-05) and positively associated with *Ruminococcaceae* UCG014 (p= 6.7e-05).

3.3 Mouse study 3 (antibiotics treatment experiment)

In this study mice were sorted into four groups: cachectic (CHX), cachectic + antibiotic treatment (CHXABX), antibiotic treatment (ABX) and control. CHX group consisted of four, control of four, CHXABX of six and ABX of six mice. They were put into five cages to keep the bias of the cage effect as small as possible. Feces was collected before sacrifice. There were 20 samples obtained from this study cohort in total. Additionally, body weight, food intake and tissue weights were measured. Laboratory observations indicate that antibiotic treatment had no effect on cachexia development and progression, although CHXABX mice showed a higher body weight in comparison to CHX mice. However, neither adipose nor muscle mass were increased, but the postmortem measured mass of the cecum was found to be tremendously elevated. Further collected data, depictured in figure 15, includes the relative body weight (% of initial weight) over time, which showed ABX and control at 115-125 % of initial body weight, CHX with a stable

curve with 100%, followed by a weight loss starting 12 days after tumor inoculation and finally CHX/ABX at 100-110% of initial body weight. Moreover CHX and CHX/ABX mice were found to have a significantly reduced food intake (g/mouse/day) at days 19-24, a decreased tissue weight of inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), quadriceps and gastrocnemius muscle, and furthermore an increased weight of the spleen.

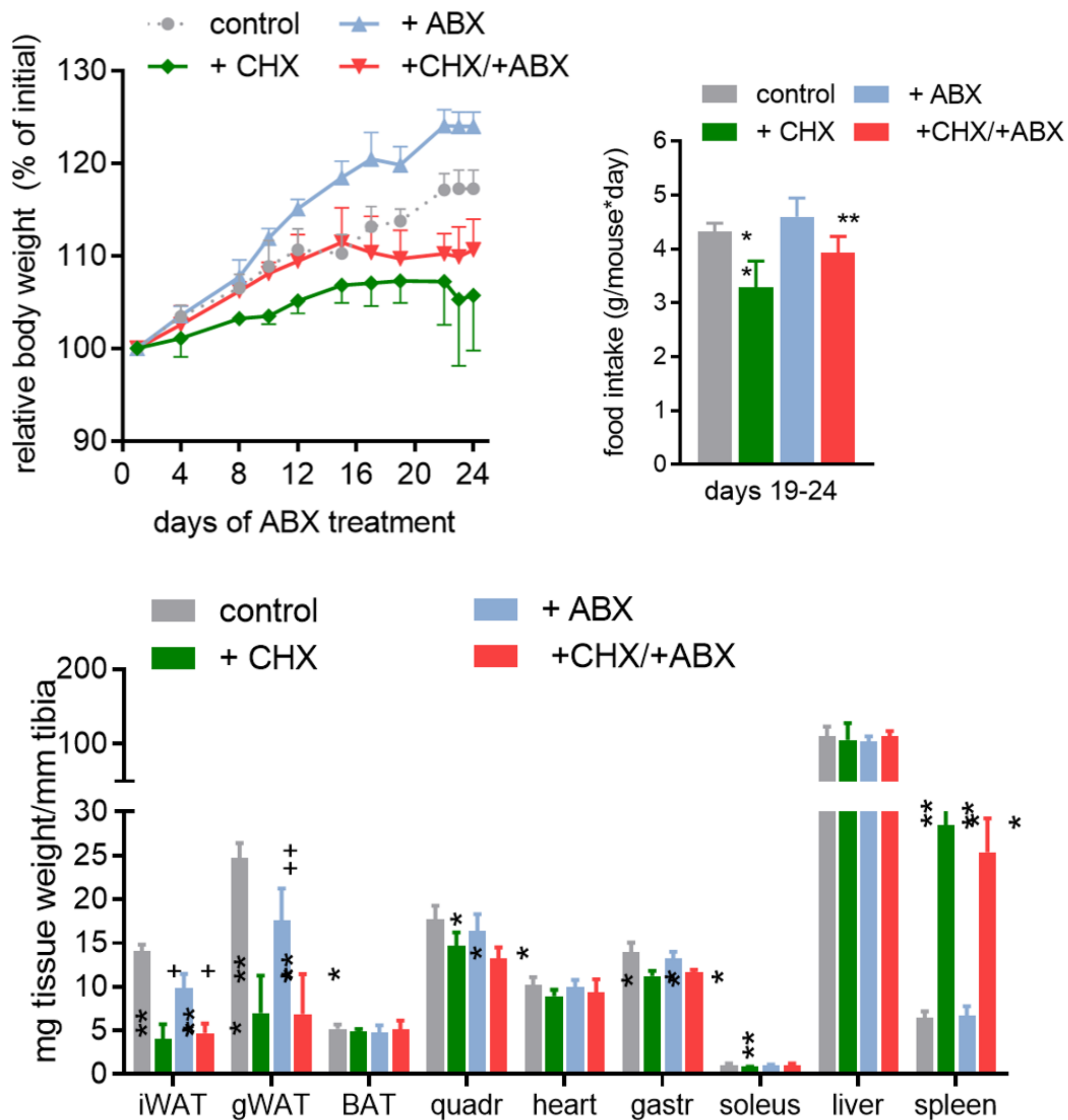
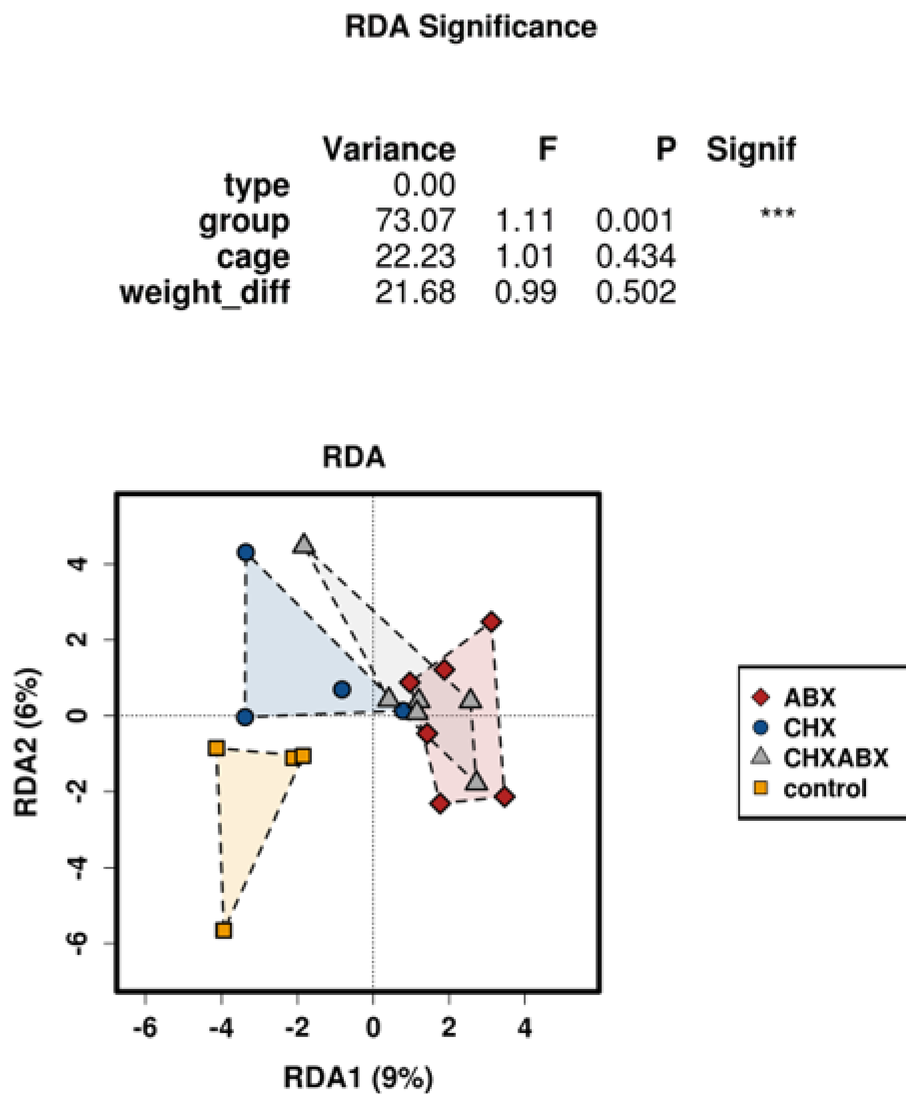


Figure 15 mouse group 3 - observational parameters

3.3.1 Significant influence of the group selection on microbial community composition

To test for environment-microbiota associations Redundancy Analysis RDA of all samples was performed. Figure 16 shows that variance in microbial community composition is strongly influenced by grouping (p= 0.001), whereas cage effect is not significant in this cohort (p= 0.434).



RDA Significance

	Variance	F	P	Signif
type	0.00			
group	73.07	1.11	0.001	***
cage	22.23	1.01	0.434	
weight_diff	21.68	0.99	0.502	

Figure 16 mouse group 3 - RDA Analysis of all samples

3.3.2 Antibiotic treatment reduces diversity and richness in the microbial community

Microbial diversity was measured by Inverse Simpson Index, Richness and Rarefaction Analysis. As illustrated in figure 17 richness is massively reduced in the groups treated with antibiotics and diversity is also lower, but not significant in comparison to the other groups.

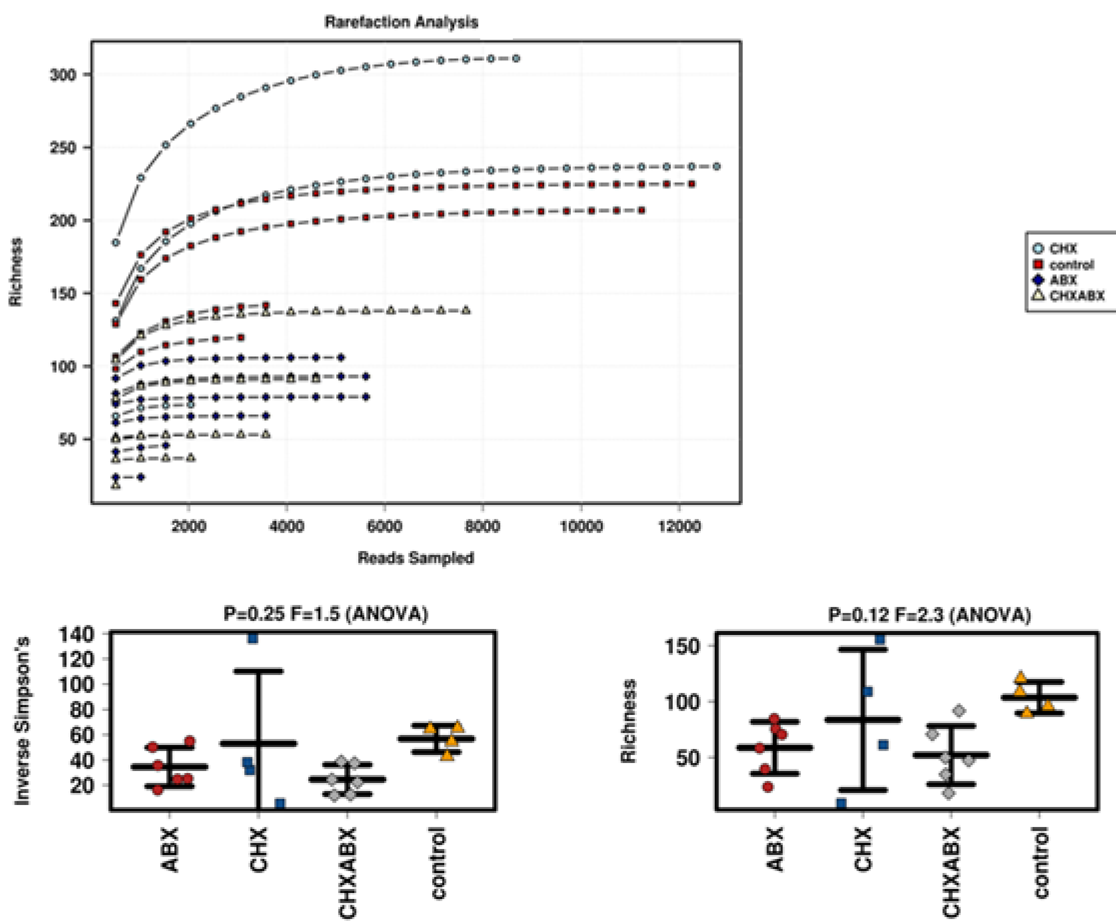


Figure 17 mouse group 3 - Rarefaction Analysis, Inverse Simpson´s and richness of all samples

3.3.3 Tumor- induced cachexia and antibiotic treatment correlate with the relative abundance of microbes creating specific signatures

Figure 18 visualizes the microbial composition of the CHX compared to the control group. On genus level, a clear change in microbial composition was visible. The major differences include a higher abundance of *Lactobacillus*, *Bacteroides*, and *Pseudomonas* and furthermore a decrease in *Ruminococcaceae* and *Dubosiella* in the CHX group.

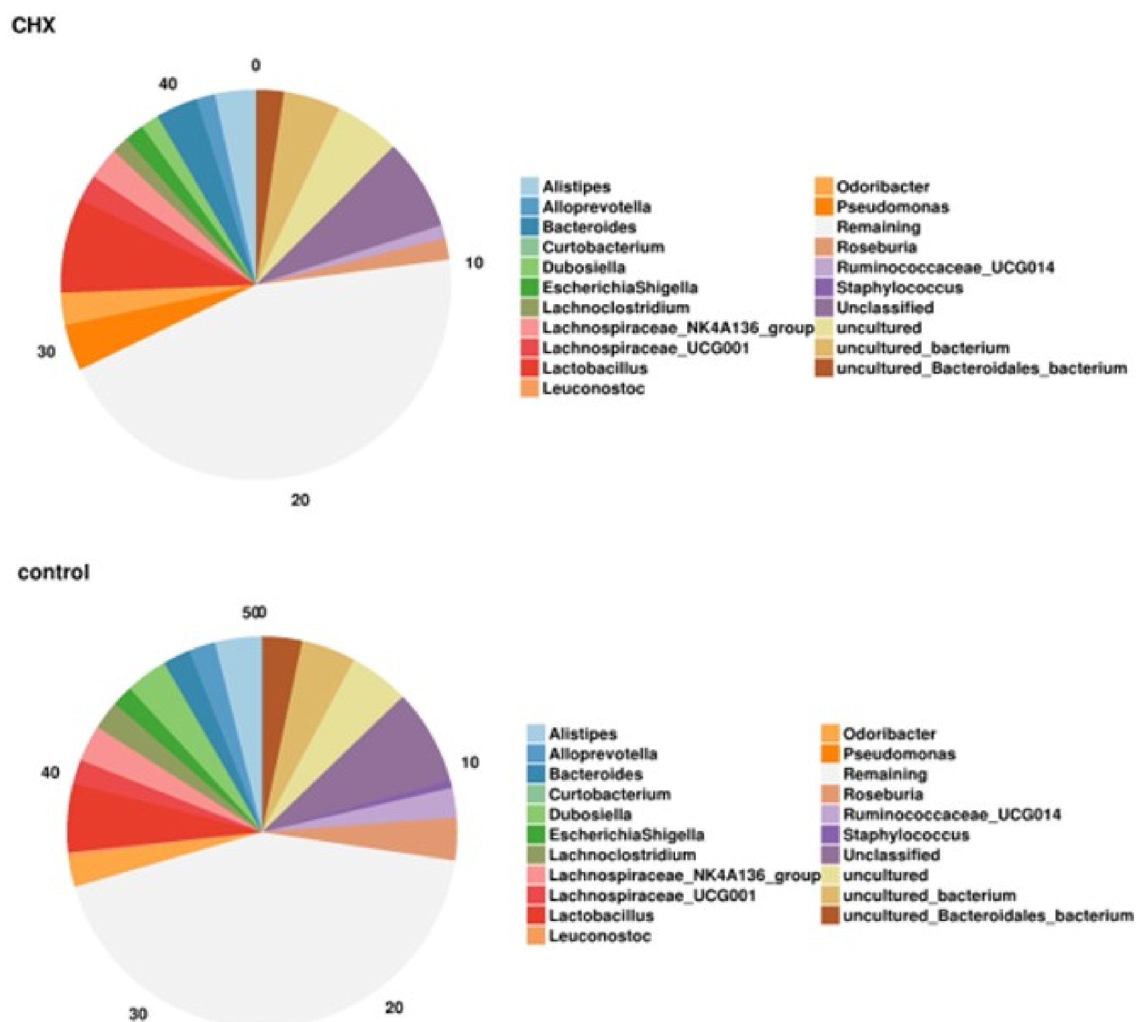


Figure 18 mouse group 3 - Pie Chart on genus level of most abundant taxa in the CHX (above) and control group (below).

The microbial composition of the CHXABX and ABX group was depicted in Figure 19. Differences in taxa abundances became visible when comparing the two groups on genus level. In the CHXABX group an increase in *Odoribacter, Lactobacillus, Lachnospiraceae_NK4A136* and *Alistipes* was found, whilst abundance of *Pseudomonas, Odoribacter, Lachnoclostridium* and *Ruminococcaceae_UCG014* was decreased.

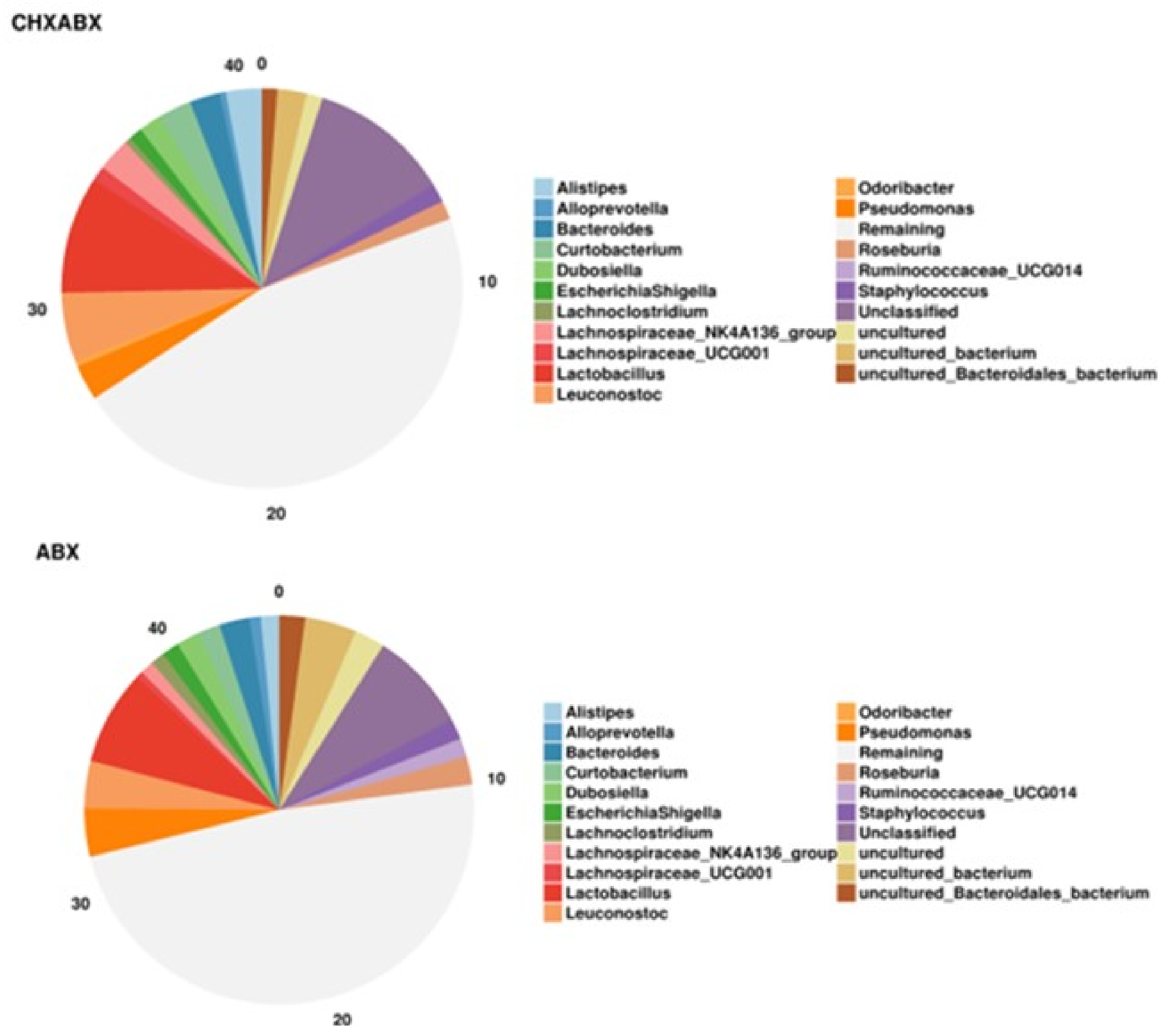


Figure 19 mouse group 3 - Pie Chart on genus level of most abundant taxa in the CHXABX (above) and ABX group (below).

Figure 20 shows the microbial composition of the ABX group compared to control. On genus level a clear shift of microbial community composition became visible. In the ABX group higher abundances of *Pseudomonas* were found, while *Odoribacter, Lactobacillus, Lachnospiraceae_NK4A136, Lachnoclostridium, Alloprevotella, Alistepes, Staphylococcus, Ruinococcaceae_UCG014* and *Rosebundaria* were diminished.

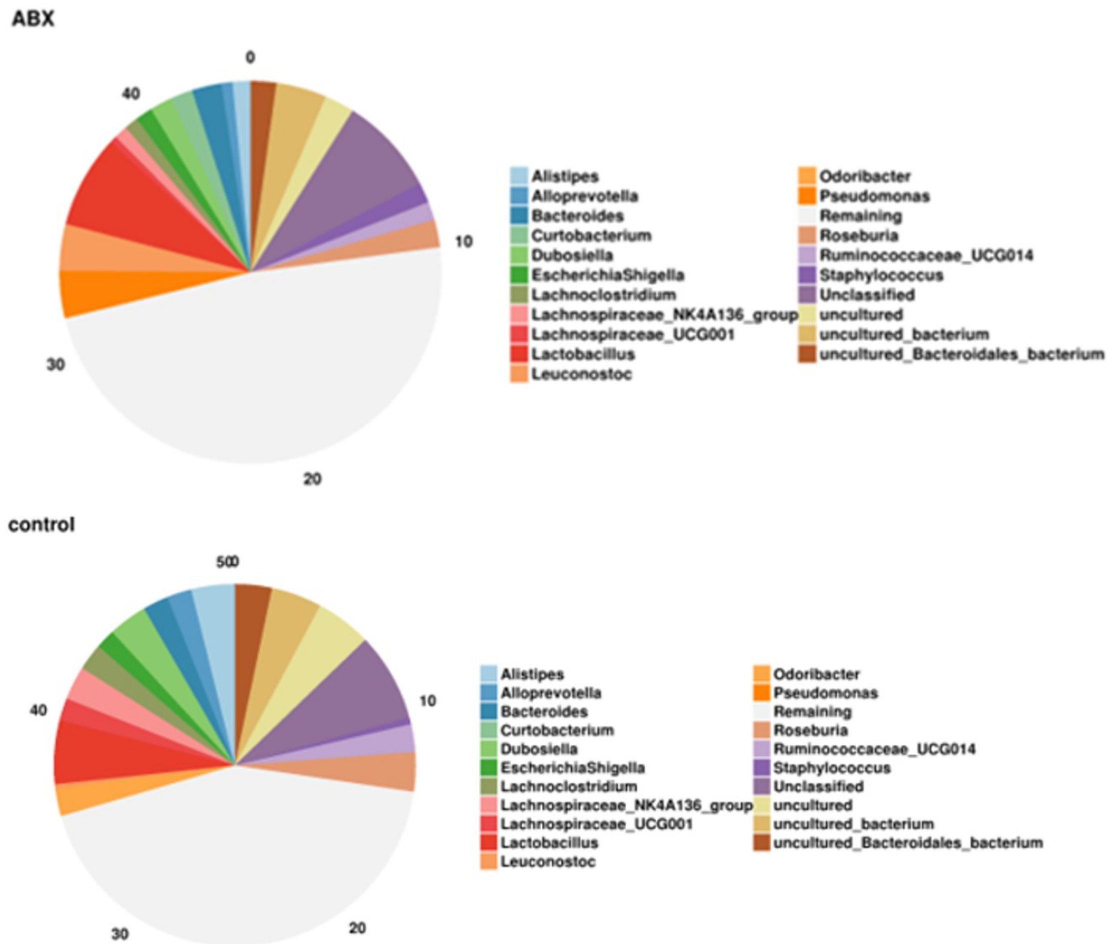


Figure 20 mouse group 3 - Pie Chart on genus level of most abundant taxa in the ABX (above) and control group (below).

3.3.4 Summary of mouse group 3

In this experiment observations indicated that antibiotic treatment had no clinical effects on cachexia development and progression. Although CHXABX mice had a higher body weight in comparison to CHX mice, only the cecal mass was found to be elevated. Analysis of the fecal samples showed change in microbial community composition to be strongly influenced by grouping (p= 0.001) and richness to be massively reduced in antibiotics treated mice. Differences in microbial abundances include an increase of *Lactobacillus*, *Bacteroides* and *Pseudomonas* in the CHX group, whilst *Ruminococcaceae* and *Dubosiella* were diminished. The CHXABX group showed an increase in abundance of *Odoribacter, Lactobacillus, Lachnospiraceae_NK4A136* and *Alistipes*, and a decrease of *Pseudomonas, Odoribacter, Lachnoclostridium* and *Ruminococcaceae_UCG014*.

4 DISCUSSION

The aim of this thesis was to explore the role of the microbiome in tumor induced cachexia in three independent mouse experiments inoculated with cachexigenic or non-cachexigenic cancer cells. Fecal samples, obtained from the murine cohorts at different time points with the progression of cachexia, were analyzed regarding their microbial composition using NGS-based amplicon sequencing. The summarized results of each mouse experiment, as an important part of this discussion, were included in the previous sections for better understanding.

In line with the formulated hypothesis, our results showed changes in microbiota composition in tumor-bearing cachectic mice compared to control groups. More specifically, a shift in community composition and clustering of samples with progression of cachexia was observed, supporting the findings of previous studies (5,7). Additionally, cachectic mice exhibited a decrease in bacterial diversity and richness, which also has been reported for leukemic mice with cachexia (7). The data obtained from our experiments further showed a strong association of the beginning of weight loss in cachectic mice with the decline in bacterial diversity and richness (see Figure 3), which suggests that the microbial composition might mirror the state of progression in tumor-induced cachexia. Interestingly, a pilot study with human subjects has shown a lower diversity and altered microbiota composition in postmenopausal women with breast cancer (186), but to our knowledge there is currently no published research exploring these findings in cachectic patients.

As another key finding evident in all three murine experiments, was that tumor-induced cachexia correlated with the relative abundance of certain microbial taxa. For instance, cachectic mice showed an increase of *Lactobacillus spp.*, in particular certain RSV associated with *Lactobacillus salivarius* were statistically associated with this group (see Figure 13). Remarkably, *Lactobacillus salivarius* is a known bacteriocin producer able to stimulate protective immune responses and modulate gut microbiota (187). Specific strains of *L. salivarius* are used as probiotics in animal health and are also under investigation as a possible

treatment option for chronic diseases, including cancer, in humans (188). Knowledge on the aspect of a potential use in cancer is building on the findings of experiments with rat models, where oral administration of *Lactobacillus salivarius REN* was shown to suppress 1,2-dimethylhydrazine (DMH) – induced colonic carcinogenesis (189). In contrast to our results, comparable studies reported a decrease of *Lactobacillus spp.* in cachectic mice (5,7). Furthermore, scientists demonstrated that a modulation of the microbiota through administration of a synbiotic containing inulin-type fructans and live *Lactobacillus reuteri* 100-23 to leukaemic mice reduced hepatic cancer cell proliferation, muscle wasting and morbidity, and prolonged survival (7). Differences in the settings of the experiments, for instance caging and feeding of the animals, sample type (fecal and cecal) and analyzing methods could potentially contribute to the above mentioned, partly opposed findings. Additionally, we are not able to classify at the strain level and fine distinctions of different *Lactobacillus spp.*, which could possibly help to determine their role in tumor-induced cachexia, remain hidden from us. Another example for changes in the relative abundance of microbes, we observed in cachectic mice, was an increase of *Bacteroides* and *Enterobacteriaceae*, supporting the findings of previous research (7). Moreover, we detected a decrease in abundance of *Ruminococcaceae_UG014* with the progression of cachexia. Importantly, a study indicated *Klebsiella oxytoca* as one of the main Enterobacteriaceae species increased in a murine model of cancer cachexia and moreover found a strong negative correlation of this rise in abundance with *Ruminococcaceae, Lachnospiraceae* and *Porphyromonadaceae* (9), three butyrate-producing families (190). Apart from this, *Klebsiella oxytoca* was also indicated to act as a gut pathobiont and to contribute to intestinal dysfunction (9).

Results from our antibiotic-treatment experiment exhibited, despite a massive reduction of gut bacteria, no change on cachexia onset or severity. Cachectic mice treated with antibiotics, showed similar results, when compared to the other two experiments. Notably, a higher body weight was observed in antibiotics treated compared to non-treated cachectic mice, yet only the postmortem measured cecum was found to be enlarged. A massive increase in cecal weight and size, due to colonic remodeling influenced by a decreased availability of butyrate as an

energy substrate for the cecal enterocytes, was also found in an experiment that assessed the effects of antibiotic-induced microbiome depletion (191). This led us to the assumption that the enlarged cecum, detected in our study, could be a result of the decreased microbial mass caused by the antibiotic treatment.

The conducted experiments, presented in this thesis, have several limitations. Firstly, the results, obtained from the murine studies, are difficult to be exactly compared to another, due to, for example a different selection of cohort sizes and sampling timepoints. Moreover, the reliability of this data is impacted by a notable influence of the cage effect on the microbial composition. As well as that, our findings were not controlled for a potential modification of the specific taxa caused by changes in food intake. Furthermore, the lack of a functional analysis of the microbiome gives us a limited insight concerning its role in tumor-induced cachexia. Lastly, it is beyond the scope of our findings to draw direct implications for research in humans as profound intrinsic differences and environmental factors limit the generalizability of the results from murine studies (192).

To summarize, our findings indicate a strong association of the microbiota with tumor-induced cachexia and therefore highlight the importance of future studies to further explore correlations and detect mechanisms to eventually create a new prevention strategy or treatment option for cachexia in humans. Very few human studies focus on the role of the microbiome in cachexia and thus knowledge is very limited and relies mainly on experimental models. A study of cachectic patients with advanced gastric cancer reported altered tight junctions as a potential gateway for bacterial translocation, which might contribute to the development of cancer cachexia (193). Studies focusing on obesity highlight the role of SCFA on body weight gain (120) and appetite regulation (194) and could thereby be an interesting target of research on cachexia as well. Moreover, a recent study (195) showed significant modifications of the microbiome in a murine model of neuroblastoma-associated cachexia, supplemented with prebiotic oligosaccharide (POS), however no improvement on gut permeability was detected. Pre-/probiotics and FMT could potentially serve as therapeutic options, but more studies are needed to evaluate the safety of their use in patients with cancer-induced cachexia.

As a conclusion, research in this field is just at the beginning and a clear interpretation of the role of the microbiome in tumor-induced cachexia is not yet ascertainable. Studies with human subjects focusing on cancer-induced cachexia before the start of treatment could potentially answer open questions remaining from experimental research. In addition, a more standardized approach for analysis of the microbiome could lead to more comparable results. Equally, future studies should include metagenomics and metabolomics strategies to get a more comprehensible overview of the role of the microbiota in tumor-induced cachexia.

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