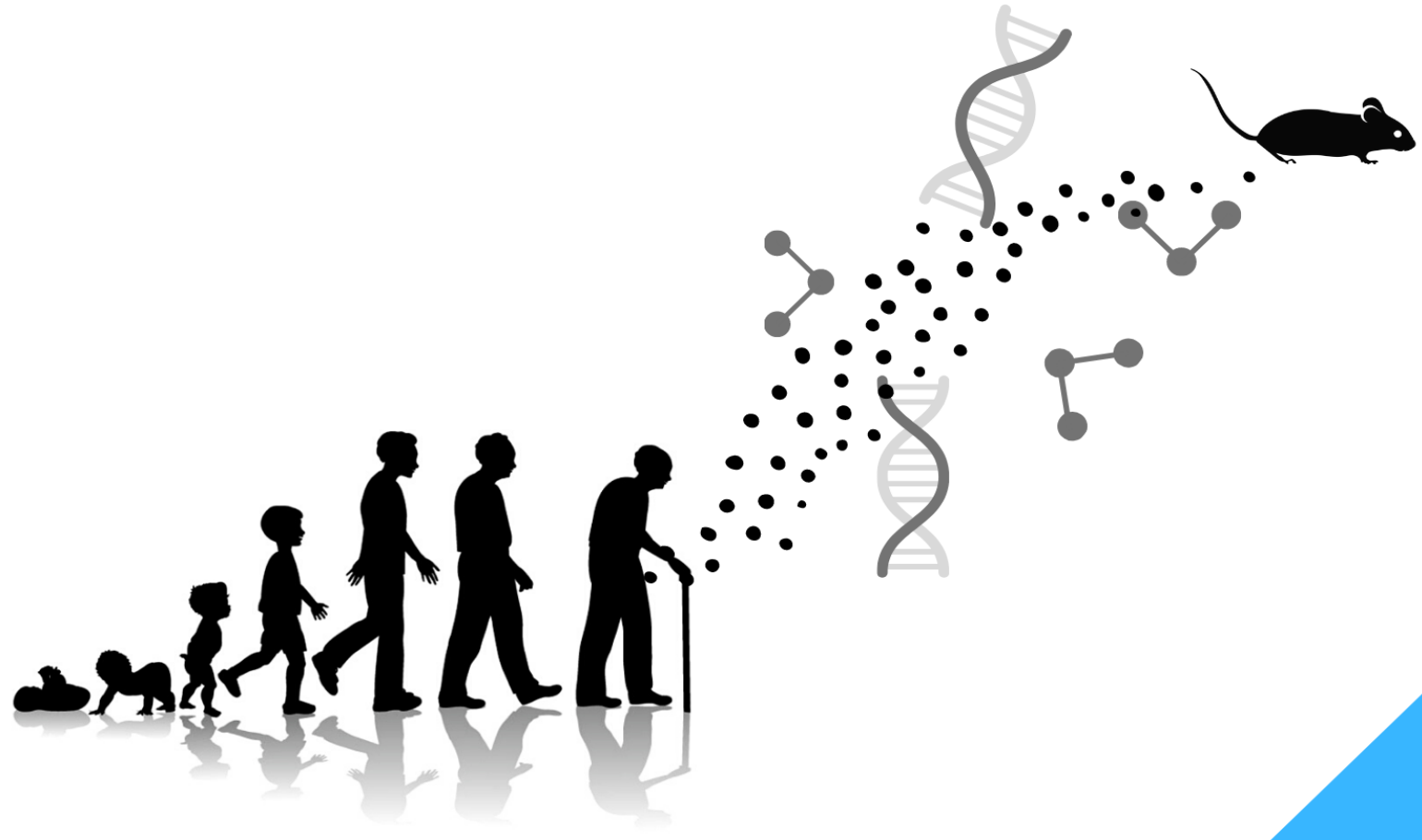
The effect of aging in the murine gut microbiome



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The effect of aging in the murine gut microbiome

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%	Percentage
°C	Celsius degrees
µl	Microliter
µm	Micrometer
µM	Micromolar
ARRIVE	Animal Research: Reporting of In Vivo Experiments
Av.	Average
BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CE-MS	Capillary electrophoresis mass spectrometry
cm	Centimeters
Col1α1	Collagen 1 alpha 1
Defα1	Defensin alpha 1
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium treatment
EU	European Union
FELASA	European Laboratory Animal Science Associations
G	Gram
GC-MS	Gas chromatography mass spectrometry
GDF-15	Growth differentiation factor 15
GF	Germ-free
GIT	Gastrointestinal tract
H&E	Hematoxylin and eosin staining
Hmgb1	High-mobility group box 1
I-FABP	Intestinal fatty acid binding protein
IL	Interkeukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilograms
KO	Knock-Out
LC-MS	Liquid chromatography mass spectrometry
LPS	Lipopolysaccharides
min	Minute
ml	Milliliter
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NMDs	Non metric multidimensional scaling
NMR	Nuclear magnetic resonance
OTU	Operational taxonomic unit
p	Probability value
PCoA	Principal Coordinate Analysis

PCR	Polymerase chain reaction
PERMANOVA	Permutational Analysis of Variance
pH	Potential of Hydrogen
ppm	Parts per million
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid gene
s	Second
SCFAS	Short chain fatty acids
SEM	Standard error of the mean
SIBO	Small intestinal bacterial overgrowth
SIMPER	Similarity percentage Analysis
SOP	Standard Operating Procedure
SPF	Specific pathogen free
spp.	Species
αSma	Alpha smooth muscle actin

CHAPTER I

1 Introduction

1.1 General Introduction

We live in a microbial world as the whole environment around us harbors important numbers of microbes. Microorganisms are ubiquitous [1] and are able to adapt to the harsh environmental conditions of water, soil and our bodies [2]. Thousands of microorganisms live inside and outside of the bodies of humans and animals, and work in a perfect symbiosis to ensure vital processes [3]. The microbiota is known as the assemblage of microorganisms colonizing a specific environment which includes not only bacteria but also fungi, archaea, viruses, and protozoans [4]. Another term frequently used is the microbiome, which is defined as the habitat, with the microorganisms and their genomes, metabolomes and proteomes. [5]. Since the discovery of bacteria by Antonie van Leeuwenhoek in the 17th century, with the first microscopes, bacteria have fascinated humans due to the broad use, omnipresent nature, and their capacity to either produce diseases or protect from them [6]. Bacteria are still the most studied microorganisms, and the bacteria colonizing different body niches from animals and humans have shown interactions with internal factors as host genetics, metabolism and external factors such as geographic location, diet and environment. These interactions might have modeled the evolution of the organisms [7].

1.1.1 The mammalian microbiota

Mammals host different types of bacteria that are associate with the digestive, immune, neuroendocrine health. The microbiota is acquired vertically from mother to offspring and horizontally from shared interactions and the environment [8]. Hosts and their microbiomes have evolved through thousands of years developing a beneficial mutualistic relationship. The term 'holobionts' names this close association of the host and their associated microbial communities living in the same ecological unit [9]. Some authors consider that the holobiont and the hologenome, the genetic information of the host and its microbiota, should be thought as a unit that has evolved and adapted together through thousands of years [7]. As the microbiota reaches its maturity in the young adulthood, humans and mammals have one of the most complex microbial environments in the gut, which contains trillions of bacteria [10]. These bacteria have several functions such as: break down components from diet, produce beneficial metabolites like vitamins and short chain fatty acids (SCFAs), prevent the overgrowth of pathogenic bacteria and the bacterial translocation to other systems and organs [11]. The metabolites produced by these microorganisms are of great importance for keeping an adequate function of the organs specially in the gastrointestinal tract (GIT), as for example the well-known SCFAs help to keep a healthy mucosal layer and feed the colonocytes [11]. This set of microorganisms present in the gut are considered a forgotten organ, as this union between the mammalian organisms and the different bacteria, protozoa, virus has evolved to form a perfect symbiosis providing substances required for the complex biochemical mammalian pathways [12]. All mammals have different associated microbiota, as the living environments are not the same and the GIT have physiological differences [13].

Internal and external factors such as; genotype, diets, lifestyles and drugs are key factors to affect gut microbiota [14]. These factors can be strictly controlled in animal models to identify changes in the microbiota induced by the research questions [15].

Several mammals, like pigs, rats and mice are used as laboratory animals in experimental models for health and disease in microbiome research. However, simpler non-mammal organisms as drosophila, *C. elegans* and zebra fish can also be helpful to research and understand the microbiota under different circumstances [3]. Initial basic microbiome research in these types of models is key, as results can be later translated to humans [16].

1.1.2 The mouse as an animal model for the study of the microbiota

Murine models are valuable research tools that provide insight into the complex human body. Mice are the preferred experimental animal for preclinical research due to their well explored genetic and the ability to control a wide range of factors as when compared to humans [17]. Another reasons why murine models are popular in research addressing microbiome questions are the lower price compared to other mammals and high reproductive rates and short life [15].

However, mice and humans have differences regarding anatomy (see Fig. 1), physiology, immunology and behavior. These dissimilarities should also be taken in consideration when translating microbiome research to humans [16]. Mice and humans have similar ratio of intestinal body surface [15]. Nevertheless, several intestinal sections present differences that are shown in Figure 1.

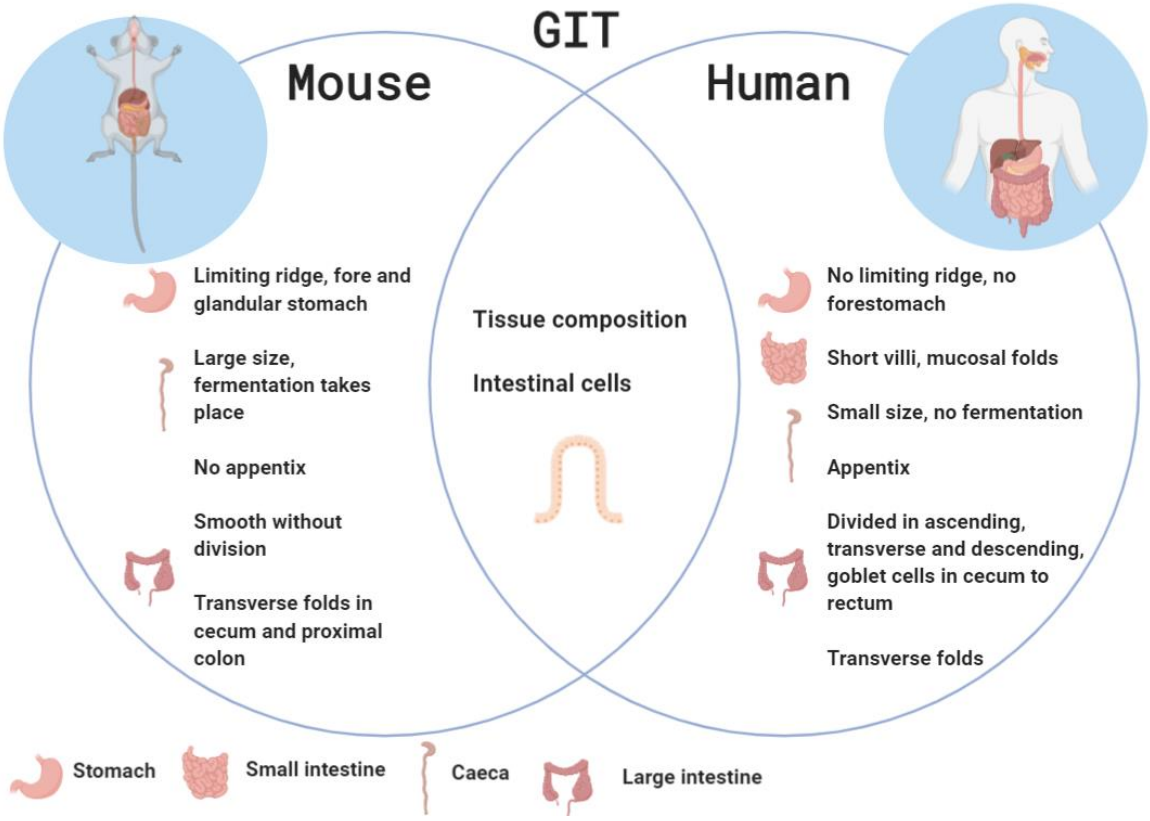

Figure 1 *Similarities and differences from the GTI of human and mouse.*

(adapted from Nguyen, *et al.* 2015)

Mice have longer villi compared to humans, possibly as a compensation to the lack of mucosal folds [15]. The mouse cecum is large and it is where the fermentation takes place, while in humans, cecum is small and does not have a specific function. However, differences are not only at physiological level, as at cellular level the distribution of goblet cells and Paneth cells are not comparable to humans [15]. However, the GIT from mice is similar enough to the human GIT and it helps us understanding the environments of the human digestive system, however the existing differences should be taken in consideration when murine models are used for human studies [15].

In mice experiments, factors as living conditions (cage effect), genetics, age, diet and inter-individual variability can strongly influence the microbiota composition [17].

These features could also play an advantage as different conditions can be tested to analyze the changes that the microbial ecology of the different organs pass when the animals goes through different interventions. During the last years, murine models have emphasized the role of the gut microbiota in several metabolic activities like: the regulation of energy homeostasis, the lipid and amino acid metabolism and the modulation of host fatty acid composition [18]. Some murine models with particular microbial characteristics are useful for microbiome experimental setups, some examples are: specific pathogen free (SPF) mice, germ-free (GF) mice and gnotobiotic mice, the last one refers to mice colonized with a limited bacterial consortium. To study mechanisms from the microbiota or bacteria in specific diseases is common to use mice with specific genetic features such as strains with different disease predisposition and Knock-Out (KO) with inactivated genes [19]. Mice models have helped us acquire knowledge about the microbiota in different diseases and conditions, therefore is a necessary tool that will continue to help us in the research of the different aspects of the microbial ecology of the mammalians organs, tissues and secretions. However, researchers should choose carefully the genetic background and the experimental setups to translate the results to humans [14].

1.1.3 Comparison between murine and human microbiota

The differences in the GIT physiology between mice and humans, influence the variances of the diversity and composition of the microbiota. The most noticeable difference is, as previously described, that the fermentation takes place in the cecum of mice contrary to the colon in humans. Despite these differences, both murine and human guts are dominated by the phyla Bacteroidetes and Firmicutes [15]. At genus level, murine and human microbiota have similar bacterial components and functionalities, but the abundances of each bacterium are different. As for example the

murine intestinal tract has more bacteria of the phylum Deferribacteres, which in human are only found in small amounts in the stomach [20]. Wang *et al.* identified 37 core genera across healthy mice with different ages, sexes and strains. Some of these genera were unclassified. The members of this core group were: *Anaerostipes*, *Anaerotruncus*, *Oscillibacter*, *Clostridium XlVb*, *Bacteroides*, *Barnesiella*, *Alistipes*, *Helicobacter*, *Prevotella*, *Lachnoanaerobaculum*, *Intestinimonas*, *Roseburia*, *Alloprevotella*, *Rikenella*, *Allobaculum*, *Pseudoflavonifractor*, *Marvinbryantia*, *Mucispirillum*, *Odoribacter*, *Acetatifactor Bifidobacterium*, *Olsenella*, *Lactobacillus*, *Enterorhabdus*, *Parasutterella*, and *Turicibacter*, *Parabacteroides*, *Flavonifractor*, *Clostridium XIVa*, *Blautia*, *Anaerofilum*., *Eggerthella*, *Gordonibacter* and *Ruminococcus* [21].

The core microbiota is referred as the microorganisms at different taxonomical levels present in all individuals The host gut homeostasis and health is affected by the core microbiota [22]. Still, there is not enough knowledge of the ecological relationship between the species of the core gut microbiome [23]. The gut pan-microbiome is formed by different combinations of intra-core groups populations and/or higher similarity clusters [24]. In mice and humans, the genotype, the diets, the lifestyles and the drugs impact the composition of the gut microbiota. Both, human and mice have also shown wide inter-individual structural variations [17].Therefore, the phylogenetic core could be partitioned into different structural possibilities that variate with age, gender, lifestyle etc… [24]. Future perspectives include to understand how individual core groups are enriched in particular predicted metabolic functions [24].

Several factors are influencing the microbial composition of lab mice, but not in humans. In a study done by Kovaks *et al*. [25], the mice genotype had a stronger weight on the microbial differences than the sex. The housing is a factor that has also

been studied and identified as a weight factor for microbial differences, showing that each animal facility has a specific mixture of microbes colonizing the mice [26]. Mice that are housed in the same facility, fed with the same diet tend to harbor similar microbial groups at higher taxonomic levels, while inter-individual differences are mostly detected at the level of species or strain [25]. Adequate standardization are required to control the environmental conditions and to ensure reproducibility in the animal facilities [15].

As every scientific model, murine models have limitations. It is important to address these limitations when making conclusions and extrapolating the results to humans. Until now, microbiome research using mice as a model animal has shown a great potential to help us understanding the behavior of the different microbial communities. Further research using murine and alternative models is necessary to approach different research questions and understand the mechanisms underlying the core microbial communities and the relation with different systemic functions.

1.2 The microbiota of the gastrointestinal tract (GIT)

The principal functions of the GIT are the digestion of food, absorption of nutrients and excretion of unwanted substances [27]. The mammalian GIT is composed of the mouth, pharynx, esophagus, stomach, small intestine, large intestine measuring around 6 meters in humans and 56 cm in mice and anus. [27].

The healthy microbiome colonizing the GIT, provides the host with energy, vitamins a protection of the epithelial barrier integrity and immune modulation (Figure 2) [28]. Each niche contains a specific mixture of bacterial strains depending on the function and environment. The microbes from the GIT colonize mostly the intestinal lumen, with less amounts of bacteria detected in the mucus layer that overlays the gut epithelium [29].

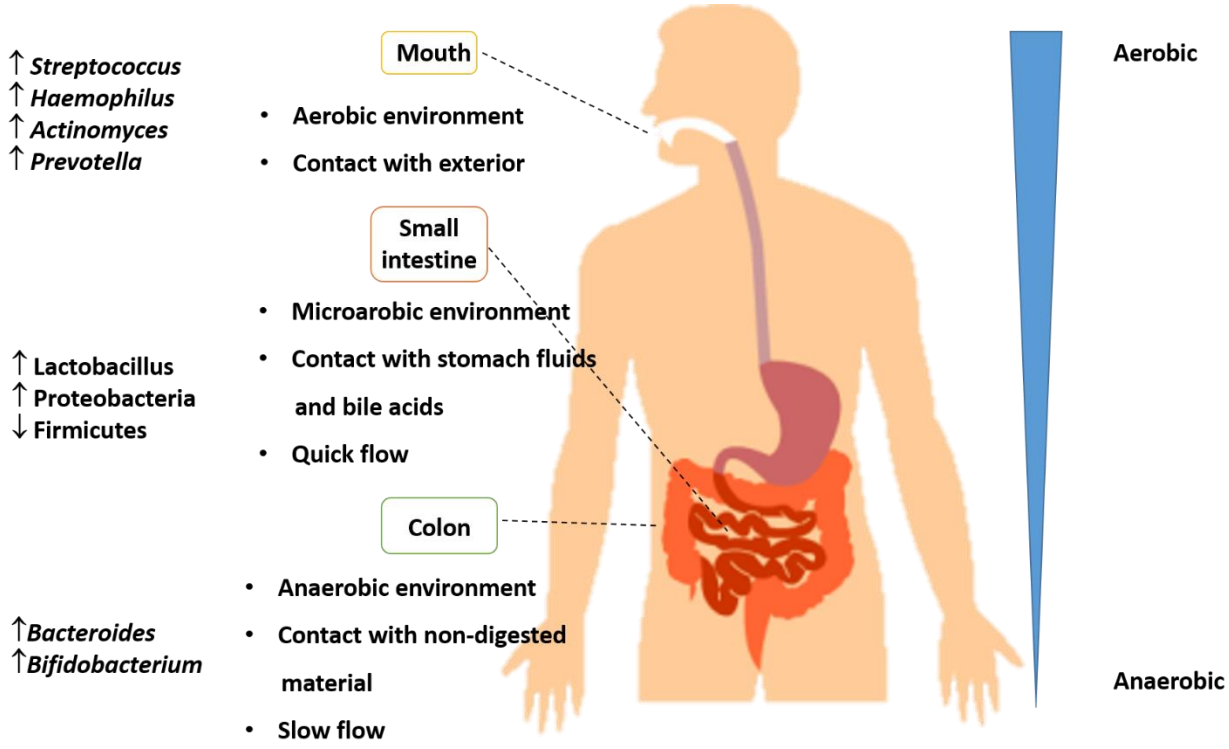

Figure 2 *The microbial environments in the GIT*

1.2.1 Oral cavity

The mouth is the beginning of the digestive system, its main function is to chew food and produce saliva which contains enzymes that initiate digestion. The mouth is colonized by thousands of microorganisms, which are predominantly facultative anaerobes [30]. The bacterial composition of the oral cavity is dynamic and fluctuates regularly, as the environment is in constant change due to the salivary flow, mucosa cell replacement and contact with external factors [30].

In humans and mice, the composition and the bacterial richness are site-dependent and have shown to be different in saliva, supragingival plaque, and buccal mucosa [31]. The oral microsystem is the second most diverse in the body, harboring around 10^{12} bacterial cells from around a thousand species from the genera *Actynomyces*, *Neisseria*, and *Steptococcus* cohabiting with fungi, protozoa, virus and archaea [32]. Previously, the mouth microbiota was usually related to oral diseases like periodontitis,

but recently it has been correlated with GIT diseases like colon cancer. Thus, it can be used as a marker to identify initial changes that could be symptomatic for systemic diseases [33].

Streptococcus dominated the human oral microenvironments, while *Haemophilus* was predominant in the buccal mucosa, *Actinomyces* in the supragingival plaque, and *Prevotella* in the subgingival plaque because of the lack of oxygen [34].

Segata *et al.*, reported that *Actinobacteria* and unclassified *Actinomycetales* had higher abundances on the tooth surfaces, and the microbial ecology associated with tooth surfaces was distinct, but not entirely dissimilar, from other oral surfaces [35]. The subgingival niche showed an increased colonization of the obligate anaerobic genera *Fusobacterium*, *Prevotella*, and *Treponema*. Contrarily, the supragingival plaque was colonized predominantly by facultative anaerobic genera, including *Streptococcus*, *Capnocytophaga*, *Neisseria*, *Haemophilus*, *Leptotrichia*, *Actinomyces*, *Rothia*, *Corynebacterium*, and *Kingella*. Indicating that the oxygen availability might be an important factor modulating the microbial ecology of the different digestive system niches, with great emphasis in the mouth microbiota [35].

1.2.2 Small intestine

The small intestine is responsible for the major digestion and the absorption of nutrients, water and electrolytes [36]. The small intestine is divided into three parts, the duodenum, jejunum and ileum. These segments are covered with villi which enlarge the intestine area that absorb the digested food. Due to the harsh bactericidal environment and the fast transit time with constant flow of acid from the stomach and bile acids, less than 10^2-10^7 bacterial cells/g inhabit the small intestine [37]. In this area of the gut, the oxygen availability is higher than compared to the colon, this leads to an increased abundance of facultative anaerobic bacteria such as *Lactobacillus*

[38]. The proportion of facultative anaerobes decreases from the duodenum to the terminal ileum, as the oxygen concentration decreases [39]. The small intestine is difficult to sample due to the requirement of invasive procedures. Therefore, there are still knowledge gaps regarding the microbiota harboring the small intestine and how it changes through the individual lifespan. In previous literature, the microbiota from the small intestine has been linked with conditions as celiac disease [40] and small intestinal bacterial overgrowth (SIBO) [41]. In the duodenum of obese individuals, the proportion of anaerobic bacteria incremented, compared to normal weight subjects. These previous research indicated that the small intestinal microbiota is affected by diseases and adapts to different dietary patterns [42].

The 'core microbiota' of the small intestine comprises *Streptococcus* and *Veillonella* while *Clostridium*, *Escherichia*, and *Turicibacter* are present in variable amounts and are subject to the inter-individual differences [37]. A comparison between the duodenum and colon biopsies showed that *Proteobacteria* was the more abundant in the duodenum than the colon, and the colon had higher amounts of bacteria belonging to the phylum *Bacteroidetes* [43].

1.2.3 Colon

The caecum, colon (ascending, transverse and descending) and rectum compose the large intestine from mice and humans [15]. A great part of research regarding the microbiota is focused in the colon, as it has the highest bacterial density of the organs from the mammal body having approximately 3.9×10^{13} of bacteria [44]. Longer transit times induce the growth of more microorganism [39]. To study the colon, feces have been broadly used as they are easy to obtain but they are unrepresentative of the gut microbiota as they fail to picture the dynamic gut sites [45]. It is still unclear how the fecal and the colonic microbiome are different but it is an easy, efficient and non-

invasive method to assess the last extreme of the colon composition. One of the roles of the colonic bacteria is to metabolize dietary compounds that cannot be digested on the previous GIT sites [46]. Some of these compounds, such as xyloglucans are broken down specifically by *Bacteroides*, while fructooligosaccharides and oligosaccharides are used by *Lactobacillus* and *Bifidobacterium* [47]. The digestion of these compounds results in the production of SCFAs, such as acetic, propionic, and butyric acids, which are a source of energy for the colonocytes and are absorbed in the colon. Their main functions are the regulation of the intestinal motility, inflammation and have shown to play a part in the prevention of diseases like cancer [48]. The gut microbiota also produces and metabolizes vitamins necessary for the host, like folates, vitamin K, biotin, riboflavin (B2) and cobalamin (B12) [49].

The distal colon has thick and dense mucus, as well as an striated layer with crypts and transverse folds which is optimal for the bacterial attachment and nutrition [50]. The water content affect mucus thickness and microbial composition [51]. Research targeting different colorectal locations from the mucosa and lumen showed that the individual microbial signature drives the colonic microbiota rather than the sampled site [52]. However, the mucosal and luminal sections differed in microbial ecology, as the families *Lachnospiraceae*, *Plancomycetacea, Coriobacteriaceae*, *Bacteroideaceae*, *Ruminococcaceae* and *Family XIII Incertae sedis* differed in abundance between these two sites, on the other hand the diversity did not show any difference [52].

As the colon and fecal composition differs, more studies targeting different colonic environments are required to understand the modulation of the colonic bacteria in different states of health and disease.

1.3 The gut microbiota during the lifespan

During the aging process there are complex changes at physiological, genomic, metabolic, and immunological levels. This results in a lower capacity of maintaining a good health during old age and might develops senescence. The microbiota is also affected by the aging process (Figure 3). The different factors that the host is exposed during lifespan create a particular community of microorganisms that are specific for each individual [53]. Previous evidence suggests that the microbiota is involved with age-related inflammatory processes which are linked to the development of chronic diseases [54].

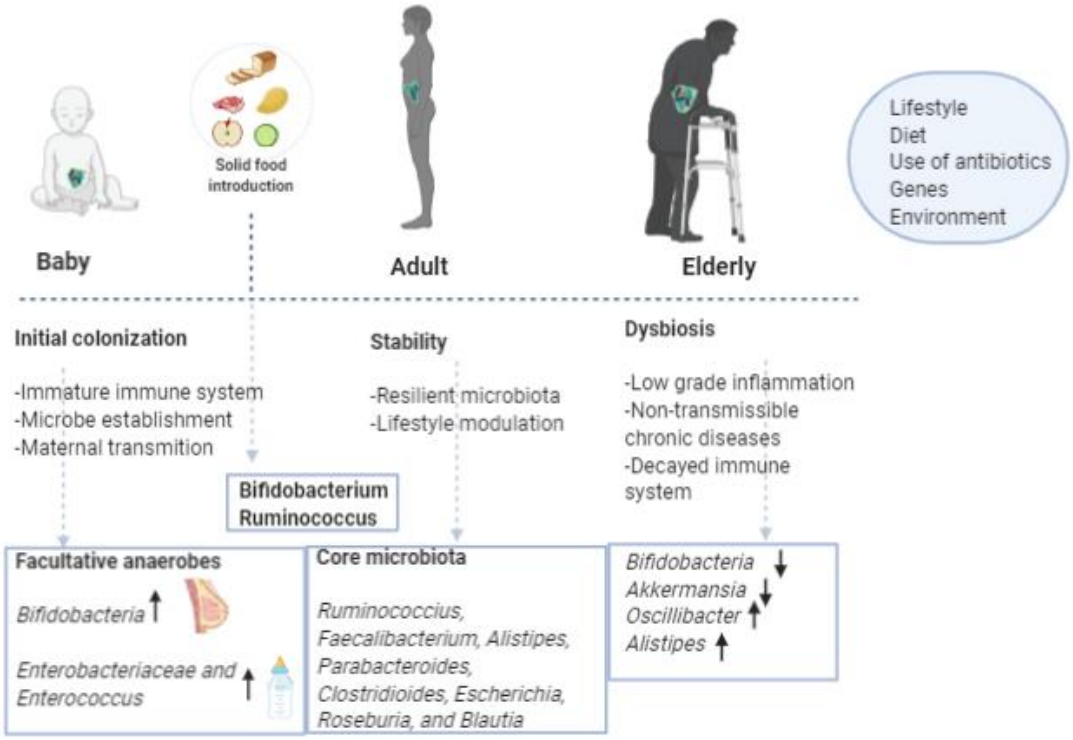

Figure 3 *Development of the microbiota during the individual lifespan.*

1.3.1 Initial years and infancy

During and after birth, all mammals are colonized by external microorganisms that populate the exposed skin, mouth, gut and vagina surfaces from the mother [55]. After the sterility present in the womb, a diverse and dynamic ecosystem develops in the body of the neonate. The microbial ecosystem of the baby is specific for each

individual, as internal and external factors have an influence over the colonizing microorganisms.

At birth, depending to the mode of delivery, the microbiota can resemble either the vaginal or skin microbiota from the mother [56]. In general, the human gut microbiota after birth is dominated by facultative anaerobes [57]. The predominant colonizing bacteria during the first months depends if the neonate is fed with breast milk or formula. When fed with only breast milk, the microbiota becomes dominated by *Bifidobacteria* during the first weeks of life. In contrast, the microbiota of formula-fed human infants is more diverse, with higher counts of members of the *Enterobacteriaceae* and *Enterococcus* families. Around the 4th to 6th month, the high-fat milk-based diet is replaced by a carbohydrate-rich diet and the microbiota develops into a stable adult-like community dominated by bacteria belonging to the phyla Firmicutes, Bacteroidetes, and Actinobacteria with low abundances of Proteobacteria [58, 59]. The microbiota of the intestinal tract from recently weaned babies is generally dominated by *Bifidobacterium* and *Ruminococcus*. In breast feed infants with the introduction of foods there is an increase in the genera *Atopobium*, *Clostridium*, *Akkermansia*, *Bacteroides* and *Lachnospiraceae* and a decrease in *Escherichia* and *Staphylococcus* spp. [60]. Formula-fed babies during weaning exhibit higher abundances of *Bacteroides*, *Clostridium difficile*, *Clostridium perfringens* and *Clostridium coccoides* [60]. Other factors as the use of antibiotics, during this age is related to an alteration of the gut microbial structure and a decrease of obligate anaerobes [61]. There are differences in the microbiota of lab mice puppies when compared to babies. Some of the most important is that *Bifidobacterium* is not consistently detected in neonatal mice and a high relative abundance of early life facultative anaerobic species such as *Escherichia* and *Streptococcus* is frequent in

the mouse gut [62]. The microbiota of the mice diversifies at day 21 with the introduction of coprophagy [62].

In germ free mice, the absence of microbial biofilms in the GIT during the first stages of life impair the mice immunity [63].

The establishment of the microbiota during the first year of life is related to the undeveloped immune system of the neonate [64]. The immature immune system of the neonate during the first year of life, allows the establishment of the microbiota and this occurs in parallel with the maturation of the immune system. The initial colonizers take part on the differentiation and activity of T cells, which are lymphocyte immune cells that protect from pathogens [64]. The settlement of the bacteria in the gut mucosal surface protects against the penetration of pathogens through the intestinal barrier by site and nutrient competition, pH modulation, production of protector metabolites and peptides [64].

In conclusion during the first days and months of life the microbiota is crucial for the development of the immune system and the gut microbial composition depends on the way of delivery and the milk type that the baby is fed. The baby microbiota changes to an adult- like with the introduction of other foods and other factors as the use of antibiotics have also an influence on the microbiome from the baby.

1.3.2 Adulthood

The microbial community developed in the early years remains stable through the adulthood. A universal ‘healthy’ adult human microbiome is still not fully known. As the microbiota shows to be as an individual thumbprint and factors as geography, sex, physical activity, and dietary patterns influence the composition of the human microbiome [65].

The Human Microbiome Project tried to depict what a ‘normal’ human adult microbiota looks like in different parts of the body (18 sites for women and 15 for men) [66]. The oral microbiota generally showed high within-subject diversity. The oral and stool microbial communities had higher diversity, when compared with other parts of the body [66].

A typical murine and human gut ecosystem harbors thousands of species and strains from less than ten bacterial phyla dominated by the Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia [67].

In a study analyzing healthy human reference microbiome list and abundance profile (GutFeelingKB), and a novel Fecal Biome Population Report (FecalBiome) of individuals from different geographic places and ages [67], researchers found 59 most frequent genera. The most abundant genus of the human gut microbiome was *Bacteroides* with *Bacteroides dorei* as the most dominant species and followed by *Bacteroides fragilis*, that is known to be a protective species against colitis from *Helicobacter hepaticus* and probably other pathobionts. Amongst the top genera found in adults are *Ruminococcus*, *Faecalibacterium*, *Alistipes*, *Parabacteroides*, *Clostridioides*, *Escherichia*, *Roseburia*, and *Blautia*. The genus *Bifidobacterium* is also present in higher abundances in the adult human gut, being *Bifidobacterium longum* the most representative species [67].

In lab mice, the composition of the microbiome from the adult mice is influenced by genetic factors related to the strain, and environmental factors related to the facility. At 2 months or 8 weeks of age, mice are considered adults. At this time point most of the intervention experiments that use a murine model take place [20].

In adult mice from inbreed strains several genera were increased in comparison with young infant mice. These genera were *Prevotella*, *Alloprevotella*, *Clostridium XIVb*, *Ruminococcus*, *Alistipes* and an unclassified member of Saccharibacteria. Other genera such as *Olsenella*, *Lactobacillus* decrease in the adulthood [21].

It is well known that several factors can affect even a resilient adult microbiota. Environmental and lifestyle factors can modulate the microbiome and either maintain health or induce diseases [14]. Still, the dietary patterns are in between of the heaviest inducing factors as nutrients interact with the microbiota by direct mechanisms, promoting and inhibiting the growth of bacteria and by indirect effects, modulating the host metabolism and immune system [14].

As in early life, an excess in the use of antibiotics is a factor that can vastly influence the microbial composition. Therefore, there is a close relationship between the adoption of low fiber, high sugar, high fat ‘westernized’ diet with the increment of non-transmissible illnesses [68].

During the last years, the efforts to understand our microbiota have been big. However, there is still a need to know which is the adult composition of the microbiota that promotes health in order to develop strategies to modulate the gut microbial ecology and achieve the health-bearing composition.

1.3.3 Old age

Aging is a complex and long process and it is not clear if the changes in the mammalian gut microbiota influence senescence, increases the incidence of age-related diseases or contrariwise the age-related physiological changes affect the microbiota [69].

In individuals of old age, an important change in the microbiota is the decrease of diversity or bacterial species. The loss of members of the core microbiota could increase the frailty signs. The stability of the core colonizers seems to be affected by age as the variability of the microbial communities increases with time [70]. These changes are related to the diverse external factors that the individual was exposed to during early and adult life.

In order to understand how the microbiota increases the susceptibility of diseases with age, we must understand how the microbiota evolves during aging in healthy subjects.

There are several genera that seem to be more sensitive to the aging process. In elderly individuals there is a decrease of Firmicutes, *Clostridium cluster XIVa*, and *Faecalibacterium prausnitzii* (a group of members of the Clostridium cluster IV) [71]. Both humans and mice have reported lower amount of *Bifidobacterium* when age increases. Old individuals also present increments in *Clostridium*, *Lactobacillus*, *Enterococcus*, *Bacteroides*, *Eubacterium*, Clostridiaceae and Enterobacteriaceae [71].

A study related to frailty observed higher abundances of *Oscillibacter* and *Alistipes* genera and a reduction of Eubacteriaceae, *Faecalibacterium*, and *Lactobacillus* in more frail individuals [72].

In murine experiments, young and old mice have higher similarity within each other than the middle age group. Fecal murine microbiota had an increment of Rikenellaceae family. *Alistipes* genus belong to this taxon and has previously been linked to microbiome from elderly humans. No significant decrease in *Lactobacillus* was observed in old mice and the genus *Akkermansia* was present in very low abundances in the old group compared to young and middle age mouse [73].

Functional profiling of the mouse microbiome, also change with aging. Old mice had underrepresented bacterial encoded functions cobalamin (B12) and biotin (B7) biosynthesis, and bacterial genes associated with DNA repair [73].

Through aging a low-grade chronic inflammation called ‘inflammaging’, is developed, and has been linked to the lipopolysaccharides (LPS) from the gut gram-negative bacteria. Recent research found an increment in the production and permeability of these LPS in old humans which can accelerate the endotoxemia and the general inflammation [74].

The inoculation of microbiota derived from aged mice into young GF mice promoted inflammation. This effect was attributed to the lower levels of *Akkermansia* and higher levels of TM7 bacteria and Proteobacteria in the donor microbiota [75].

With the age, the microbiota is individually shaped and associated to the external factors that each individual has been exposed during life [76]. During senescence, the immune system tends to decay which affects the ability to eliminate pathogens, increases molecular damage and, as the cells slow down the replacement, organs are frail and work in lower rates [77].

Old individuals have a higher prevalence to get diseases from pathobionts, which increase in abundance with aging. Pathobionts are innocuous under normal conditions, but in an altered environment or health status they can cause disease. Several bacteria such as Filamentous bacteria, the species *Helicobacter hepaticus, pylori, Proteus mirabilis, Klebsiella pneumonia* and *Clostridium difficile* showed evidence to behave as pathobionts. *Helicobacter hepaticus* is prevalent in mice from commercial and academic institution and plays a role in the development of diseases like: hepatitis, hepatocellular carcinoma, typhlitis and colitis, especially in strains of

immunodeficient mice [78]. Aging can influence the immune frailty and fail to protect the organism against pathobionts [76]. These colonizers could increase the degradation in the mucin layer and increment the probability of bacterial translocation [79].

There is a close relation between the microbial community and the diseases related to an advanced age. However, it is still not clear how the multiple changes during adulthood affect the microbiota and how it shifts it into a disease-inducing microbial community. Understanding via murine models how the GIT microbiota shifts during the lifetime and results in a disease- bearing microbial ecology will help us targeting the microbiota of elderly individuals and to improve their life quality.

1.4 Different approaches for study of the microbiota

Several methodologies have been developed through the years to study the gut microbial communities (Figure 4). During the 70s, cultivation gave us the insight of the bacterial components of microbiota [80]. The advances in the DNA/RNA extraction methods along with the computing analysis technologies has improved the field of the microbiota research. This development can be measured as the publications in the field continually increase [5]. The sequencing of molecules containing genetic information, has improved in the last years making it easier to identify and quantify microorganisms [81]. Identifying the different ecosystems present in the body of humans and animals is an important step towards understanding the roles and relationships of the different microorganisms in the aging process and the development of diseases and several approaches can be used to give a broader picture of microbial distribution.

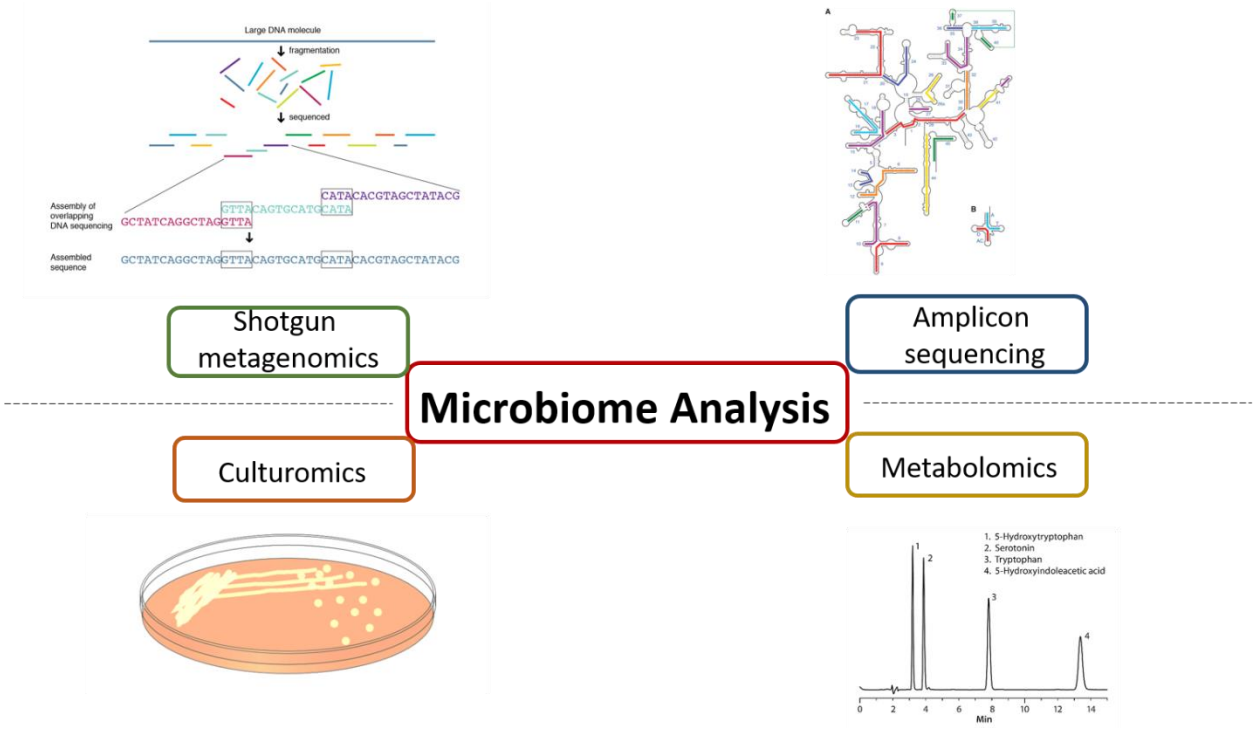

Figure 4 *Visual representation of the different approaches for the analysis of the microbiome*

1.4.1 Amplicon sequencing

Through the development of new next- generation sequencing (NGS) technologies, studies targeting the microbiome had more possibilities of taxonomic assignation. Amplicon sequencing refers to genomic DNA fragments sequenced massively at the same time and producing large amounts of sequencing data.

For the identification of members from the archaeal and bacterial community the 16S ribosomal RNA (rRNA) gene is used. This is a hypervariable regions of a subunit from the ribosomal gene and has allow us to identify the microbial composition very diverse niches as soil, ocean, animals and the different sites of the human body [82]. To target any of the regions, the sequencing of around 200 to 400 base pairs is required using specific primers that align with the conserved region [83].

The 16S rRNA gene contains nine hypervariable regions and between each region a conserved region. The 16S rRNA gene sequencing has offered an alternative to anaerobic microbial culturing, as several bacterial strains are anaerobes and cultivation is sometimes not possible. This gene is used for the study of bacterial phylogeny and taxonomical classification as it is present in almost all bacteria and it is large enough for bioinformatics purposes [84].

Raw sequence data, referred as 'reads' require processing through a bioinformatics pipeline. Low-quality sequences are discarded and clusters of sequences named operational taxonomic units (OTUs) for Mothur and Qiime or DADA2 are created. These sequences frequently clustered at 97% similarity and a representative sequence is used to discriminate from the different bacterial taxa using available databases such as RDP and SILVA and, when samples have a high percentage of similarity a name at a taxonomic level (genus, family, or higher taxon) is given to the cluster or OTU. Reads are normalized and transformed to bacterial abundances. For the statistical analysis, alpha and beta diversity are used to display the complexity related to the abundance and the presence of bacterial taxa

Nowadays Next Generation Sequencing, is the preferred methodology due to the speed, price and facility. However, there are limitations as some microbial members that have not been isolated yet lack classification.

1.4.2 Bacterial cultivation (Culturomics)

The initial knowledge of the microbiota was obtained using cultured based methods [80]. For several years anaerobic cultivation was forgotten and very few studies were using this technique because of the great interest of the scientific community in the new sequencing technologies, that were revolutionizing the microbiota field [85]. However, we realized that these new technologies also had limitations, as for example

many of the bacteria could not be assigned to any taxonomy and the low abundant bacteria could not be identified by these methods [86]. These sequencing limitations resulted in an increase interest in bacterial isolation by cultivation, in order to know which microorganisms are present in the different body niches and to be able to characterize them. The efforts of the bacterial cultivation approach have already given results, as now it is known that almost half of the bacteria from human feces are sporulated and some species require others to survive due to cross-feeding [87]. For the cultivation of microorganisms from gut samples different agar and liquid media is used under anaerobic conditions, and several dilutions are done until a colony is totally isolated. MALDI-TOF mass spectrometry is the reference method to identify bacterial species [88]. Identifying more bacteria using cultivation will improve metagenomic analyses, genome variation analyses, functional characterization and pan-genome analyses [89].

1.4.3 Shotgun metagenomics

Shotgun metagenomics is a high-throughput sequencing approach that allows to profile taxonomic composition and functional potential of microbial communities from whole genome sequences and not only 16S rRNA gene [90]. Shotgun metagenomics has some advantages compared to the 16S rRNA gene approaches which introduce biases related to the broad-range PCR. As shotgun metagenomics allows unbiased microbiome profiling by using high sequencing depth and the taxonomic assignation is more accurate at species level besides the sequencing includes broader regions of the genome [91]. However, this approach also has limitations, as to analyze the information gathered can be quite strenuous, it is less sensitive and, similar to target amplicon sequencing, many organisms have no reference sequence [92]. The bioinformatic analysis of shotgun metagenomics has three phases. In the first phase,

the primary data are processed and filtered. In this step, low-quality bases and chimeras are removed, for this sequencing approach we must discard duplicate reads, sequences from host and other microorganisms (viral, archaeal, protozoal). The second phase involves generating various derivative data sets which means comparing the reads with the different databases available such as: GenBank or KEGG and Basic Local Alignment Search Tool (BLAST) [93]. In the third phase, the data is used to produce figures and graphs representing the microbial communities such as abundance curves, biodiversity plots, and other ecological and statistical descriptors of community structure [94]. Shotgun metagenomics helps us to identify the different microorganisms living in specific environments and their functionality.

1.4.4 Metabolomics

Metabolites are the final products of the metabolism of microorganisms colonizing the human intestinal tract, that are constantly absorbed from the lumen of the intestine and transported to the circulatory system [95]. Previously, specific metabolites as SCFAs and polyamines have been targeted together with the study of the microbiota. However, there is still not much information about the complete idea of the total metabolites (metabolome), especially in the intestinal lumen. Some of the methodological approaches to study the metabolites, are gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), NMR, Fourier-transform ion cyclotron resonance mass spectrometry, and capillary electrophoresis mass spectrometry (CE-MS) [96]. Previous experiment in mice have concluded that as expected, intestinal microbiota highly influences the colonic luminal metabolome [95]. Still, there is not enough information of the impact of these metabolites in the gut and the whole system. Other fluids and secretions, such as blood and urine have also a specific metabolome that could be related to the

microbiota [97]. The research of the metabolomics is required to understand the biological mechanisms behind the microbiota, in different diseases and health status.

The microbiota is a complex mixture of microorganisms that are niche dependent. Different physiological factors that are site dependent modulate this composition. Still there is no consensus of what a ‘healthy’ microbiota looks like in each site due to the interpersonal variability but several studies have focused on finding the core colonizers of the GIT. During the lifespan, the microbiota changes in composition and in old age, many individuals present dysbiosis that promotes the development of diseases. Murine models are tools that helps us analyze the changes in the microbial communities in different health and disease stages.

1.5 Scope and work hypothesis

During the last years, research stated clearly that the microbiome changes through the lifespan. This could be related to the development of diseases or the maintenance of health at old age. Most studies using mice as a model organism for human studies focused in the characterization of microbial communities of fecal pellets. Microbial communities of fecal samples have shown not to be representative of the different niches of the digestive tract. As well, this research has targeted the DNA of the bacteria. The DNA represents the bacteria that are active, dormant and dead. On the other hand, RNA indicates which bacterial cells are active bacterial cells. The development of NGS technologies allowed the access the genetic information of the colonizing bacteria present in the different microenvironments of the digestive tract. However, there are several factors that can be modified in the experimental conditions, from the sampling methods to the selected 16S rRNA gene variable region and the different bioinformatical pipelines. This lack of consensus makes difficult to compare data from the different research published.

Therefore, the aim of this project is to analyze the microorganisms from the tissue of the different sections of the digestive tract using the mice as a model for human studies and to identify the effect of aging in the gastrointestinal tract.

The specific objectives, were to identify possible changes in the microbiome regarding the different types of sampling (Chapter 2), to study how the changes of the microbial communities are associated with the intestinal barrier function and the liver inflammation (Chapter 3) and to identify if the active part of the microbial community is affected by aging in the different niches of the digestive tract from mice (Chapter 4).

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CHAPTER II

2 Changes in oral microbial ecology of C57BL/6 mice at different ages associated with sampling methodology

2.1 Abstract

The mouth is an important niche for bacterial colonization. Previous research used mouth microbiota to predict diseases like colon cancer and inflammatory bowel disease (IBD). It is still unclear how the sampling methodology influences microbial characterization. Our aim was to determine if the sampling methods, e.g., cotton swab or tissue biopsy, and the age influence the oral microbial composition of mice. Microbial DNA was extracted using a commercial kit and characterized targeting the 16s rRNA gene from mouth swabs and tissue biopsies from 2 and 15 months old C57BL/6 male mice kept in the same SPF facility. Our results show statistical different microbial community of the different ages, type of sampling, and the two fixed factors age x type of sample (p-value <0.05). At the genus level, we identified that the genera *Actinobacillus*, *Neisseria*, *Staphylococcus*, and *Streptococcus* either increase or decrease in abundance depending on sampling and age. Additionally, the abundance of *Streptococcus danieliae*, *Moraxella osloensis*, and some unclassified *Streptococcus* was affected by the sampling method. While swab and tissue biopsies both identified the common colonizers of oral microbiota, cotton swabbing is a low-cost and practical method, validating the use of the swab as the preferred oral sampling approach.

2.2 Introduction

Gastrointestinal microbiota has a strong relation with metabolism and the modulation of individual health [1]. For the last decades, microbiota research has especially focused on stool and colon, the latter being considered the niche with the highest bacterial density with estimates of 3.9×10^{13} bacterial cells [2]. The human oral cavity is colonized by more than 700 bacterial species, making it the second most diverse site after the colon [3]. The combination of the mucosal shredding surfaces from the tongue, internal cheeks, and the hard tooth surfaces creates different environments for the adherence of bacteria in the mouth [4]. Subsequently, the biofilms in the oral cavity vary in bacterial composition and abundance, making the mouth a polymicrobial niche [5]. Several aspects can influence the composition of the oral microbiota, as the mouth is in direct contact with the exterior. Additionally, internal physiological factors, such as aging, could also play a role in the microecological structure [6]. During aging, the salivary flow and composition changes, the cellular exchange modifies, and the loss of dental pieces is frequent [7,8], being those aspects that could also influence the adherence and growth of different bacterial species.

For human samples, the Human Microbiome Project has specifications on how to collect oral samples in the different environments of the human mouth [9,10]. However, mouth samples from mouse models are broadly used to understand the behavior of the microbiota and to associate with diseases and the effect of dietary treatments in the gut [11,12]. In mouse models, the protocols for sampling the oral microbiota are not standardized and the reduced space in the oral cavity is difficult for the sampling. Several forms of sample collection have been used in mouse models including cotton

swabbing, extraction of dental pieces, and tissue biopsy [13,14]. Cotton swabbing is an inexpensive and practical method for the collection of oral microbiota without the need to cut tissues, use sedatives, or sacrifice the animal; however, it is not clear if it is the adequate method to characterize the oral microbiota. The aim of this study was to determine whether there is a difference in the oral microbiota based on the sampling approaches and if the age of mice influences the bacterial composition in the oral cavity.

Materials and Methods

Male C57BL/6 mice were bred in the Central Experimental Animal Husbandry (ZET) at the University Hospital Jena, Jena, Germany. The animals were housed in groups of 8 mice in standard 820 cm2 cages (Type III 1290D Tecniplast, Varese, Italy), according to EU guidelines (100 cm2 / mouse) with access to food and water ad libitum. Mice were checked daily and the cage was changed weekly, in the case of necessity, the cage was changed more often according to dirtiness to avoid cyclical bias [15]. The mice were fed a standard diet with pellets from ssniff (V1534-300, 10 mm pellets, 9 kj% fat, 24 kj% protein, 67 kj% carbohydrates, for a detailed composition see: http://www.ssniff.de/documents/01-1%20%20DE%20RM%20&%20low%20phyt.pdf, ssniff Spezialdiäten GmbH, Soest, Germany). Resin-free granules of cottonwood were used as bedding (LASbedding PG3, B.LBPG3.10A, granules 3–6 mm, LASvendi GmbH, Soest, Germany). The mice were subjected to strict hygienic controls according to official standards and veterinary regulations. The hygiene status according Federation of European Laboratory Animal Science Associations (FELASA) was recorded by regular deductions and documented by means of health certificates by the specialized staff of ZET. This study was carried out in strict accordance with the recommendations of the European Commission on

the protection of animals used for scientific purposes, and all procedures were performed according to the ARRIVE guidelines [16].

Samples from three to seven healthy C57BL/6 male mice aged between 2 and 15 months, respectively, were collected by swabbing the oral cavity and removal of tissue from the mouth immediately after sacrifice by cervical dislocation [number of approval: twz25-2017, date of approval: 06 April 2017, administration: animal protection at University Hospital Jena, Indication of the killing of vertebrates for scientific purposes according to §4 (3) Animal Welfare Germany (from 18 July 2016)]. Samples were immediately snap frozen in liquid nitrogen and stored at −80 °C. The total DNA was extracted following the Trizol protocol (Trizol, Sigma Aldrich, Darmstadt, Germany) with a preliminary step of bead beating (30 s, 5.5 m/s) in a FastPrep instrument (MP Biomedicals). DNA extracts were stored at −20 °C.

Library preparation was performed by targeting the V1-V2 region of the 16S rRNA gene according to the Illumina protocol described by Kaewtapee *et al*. (2017) [17] with a pre-PCR that amplified the region of interest. The master mix was prepared using PrimeSTAR® HS DNA Polymerase kit (TaKaRa, Beijing, China), 2 µl of DNA template, 0.2 µM of primer, and 0.5 U Taq primer star HS DNA (TaKaRa, China) in a 25 µl volume for the pre and second PCR and 50 µl for the third.

PCR reactions were held at an initial denaturation temperature of 95 °C for 3 min, followed by 10 cycles for the pre and second PCR and 20 cycles for the third PCR following the protocol: 98 °C denaturation for 10 s, annealing of 55 °C for 10 s, and an extension of 72 °C for 45 s with a final extension of 72 °C for 2 min. Libraries were standardized and purified using SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) and sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Sequencing reads were processed using MOTHUR as indicated on the MiSeq SOP [18]. Quality filtering was performed and chimeras were identified and removed by UCHIME. Sequences were aligned against the database Silva version 132. Sequences from chloroplasts, mitochondria, archaea, and eukaryotes were deleted before the OTU clustering at 97% identity. The cut-off for bacterial taxonomy was followed, as described by Yarza *et al*. (2014) [19]. Data were submitted to the European Nucleotide Archive under the accession number PRJEB32736. Sample reads were standardized by total and a comparison between samples was made by creating a sample-similarity matrix using the Bray-Curtis similarity coefficient (Primer 7) [20]. The differences between the microbial community structure associated with the sampling method and age were identified using Permutational Analysis of Variance (PERMANOVA). For the visual hierarchical clustering and ordination of the community structures, a two-dimensional Principal Coordinate Analysis (PCoA) was created. To assess bacterial diversity, Shannon's Diversity was calculated. The similarity percentage analysis (SIMPER) was used to identify the OTUs contributing to the observed differences in the oral microbiota sampling. The differences in the abundance of specific OTUs between the treatments were determined with the unpaired Welch's t-test with a cut-off p-value < 0.05. Figures were produced using the web-based tool MicrobiomeAnalyst [21]. Further statistical analyses were calculated using R and SPSS.

2.3 Results

A total of 26,366 ± 13,296 sequencing reads were obtained per sample after quality filtering. Reads were clustered into 821 Operational Taxonomic Units (OTU) that were assigned to 210 genera with 20 genera detected on average abundances higher than

0.5%. The core microbiota comprised of 153 OTUs, which were shared between all samples regardless of the type of sampling or age of the animals (Figure 5). Swab samples obtained from 2 months old mice had more unique OTUs than tissue biopsies (141 vs. 97) corresponding to a sum of 28% of the total OTUs. These numbers were lower in 15 months old animals kept in the same facility, as 25 unshared OTUs were detected in swab-samples and 41 in tissue biopsies, which together correspond to a sum of 8% of the total OTUs.

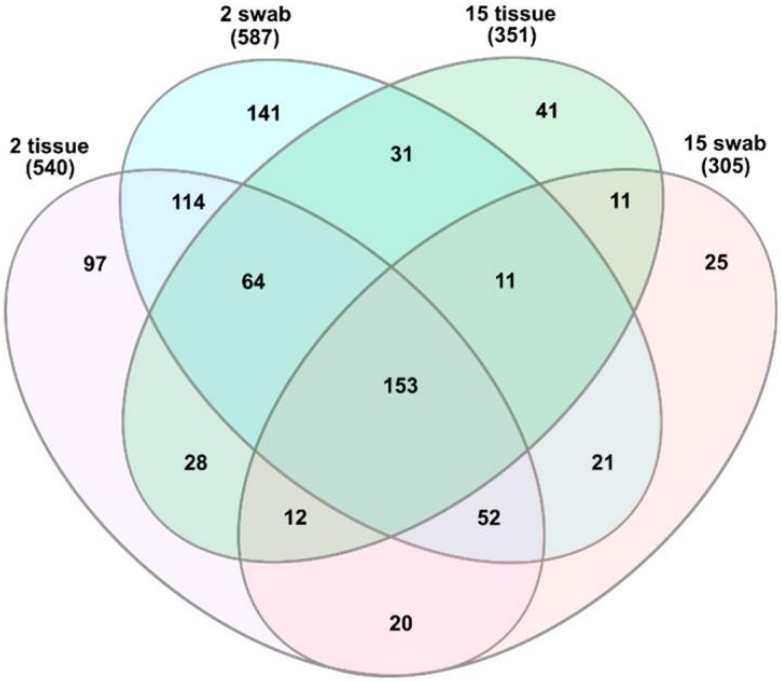

Figure 5 *Venn diagram showing the shared and unshared Operational Taxonomic Units between all groups.*

The two-dimensional PCoA revealed a clustering in the community similarity structure among the different methods of sampling and age groups (Figure 6). Statistical differences were tested using Permutational Analysis of Variance (PERMANOVA) and differences in the microbiota composition were observed between the two age groups (p-value 0.001), sampling methods (oral swab and tissue biopsy) (p-value 0.001), and the interaction of both factors (p-value 0.01).

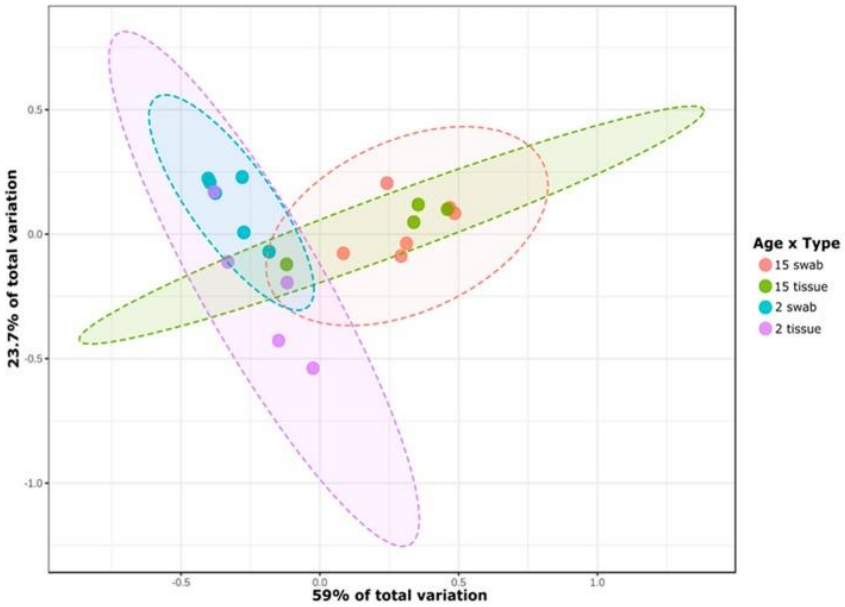

Figure 6 *PCoA plot based in Bray Curtis distance matrix.*

Each point represents one sample from the different groups. Sample spread was statistically different based on Permutational Analysis of Variance (PERMANOVA) analysis (p-value < 0.05). Age is represented in months.

Swab sampling showed the highest average similarity between samples when compared to tissue biopsies. In young mice swab samples were 66% similar, while similarity among 15 months old mice was 65%. Tissue average similarities were 39% in 2 months old mice and 46% in 15 months old mice, respectively.

Shannon's diversity index [22] neither showed statistical significance regarding sampling methods nor for the interaction between both factors. In contrast, statistical significant differences were observed between both young and old mice (p-value 0.00), independently of the sampling type with the diversity of microorganisms being higher in 15 months old mice than in young animals (Figure S1).

Firmicutes was the principal phylum detected in the 2 months old mice, while in 15 months old mice, Proteobacteria was predominant. Swab samples of 2 month old mice showed a higher abundance of Firmicutes compared to the tissue biopsies obtained from the same mouse (49% and 33%, respectively) (p-value 0.02). At 15 months of

age, none of the bacterial phyla showed statistical differences between both types of sampling. Phyla pattern was similar between swabs and tissues (Figure 7a).

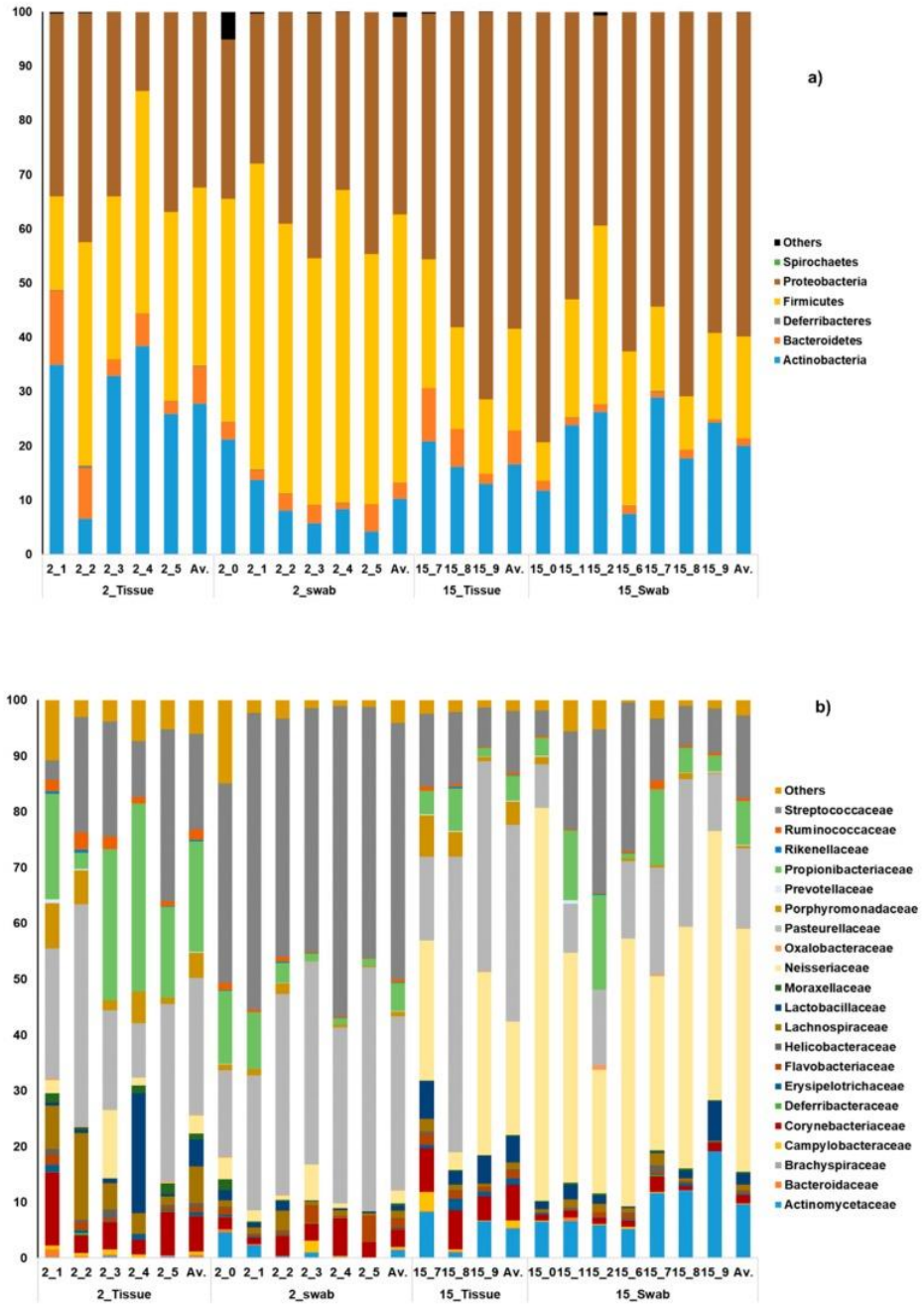

Figure 7 *Bacterial community composition at phyla (a) and family (b) level.*

Age is represented in months (ex. 2_tissue: Tissue biopsies of 2 months old mice). (a) The average relative abundance of each phyla (a) and family (b) detected in each sample of cotton swab and tissue biopsies from each age group are shown

alongside each other. The average (Av.) of each set of samples is shown in the last column of each group.

Actinobacteria, the third most abundant phylum present in the samples, was detected in higher abundance in tissue biopsies obtained from 2 months old mice (28%) when compared to the swab samples obtained from the same animals (10%) (p-value 0.03) (Figure 7a).

At the family level, statistical differences were detected among the swab and tissue biopsy samples obtained from 2 months old mice for the families Porphyromonadaceae, Propionibacteriaceae, Ruminococcaceae, and Streptococcaceae (p-value ≤ 0.05). In samples obtained from 15 months old animals, the abundance of the families Corynebacteriaceae and Flavobacteriaceae was significantly different (p-value ≤ 0.05) (Figure 7b).

When comparing swab samples of young and old mice differences in the family Actinomycetaceae (p-value ≤ 0.05) were found, while the abundance of Neisseriaceae, Pasteurellaceae, and Streptococcaceae (p-value ≤ 0.05) differed in biopsies obtained from young and old mice (Figure 7b).

At genus level, *Streptococcus* was one of the most abundant groups of microorganisms in the oral samples of young mice, however, in samples of 15 months old mice, *Neisseria* was detected in higher abundance than *Streptococcus* (Figure 8). When comparing biopsies and swab samples obtained from 2 months old mice, we observed variances in the relative abundance of *Propionibacterium*, *Streptococcus*, *Clostridium XlVa*, and an unclassified member of Ruminococcaceae (p-value ≤ 0.05). In contrast, in samples derived from 15 month old animals, *Corynebacterium* (p-value

≤ 0.05) was the only genus that showed statistical differences being more abundant in the swabs than in the biopsies (Figure 8a).

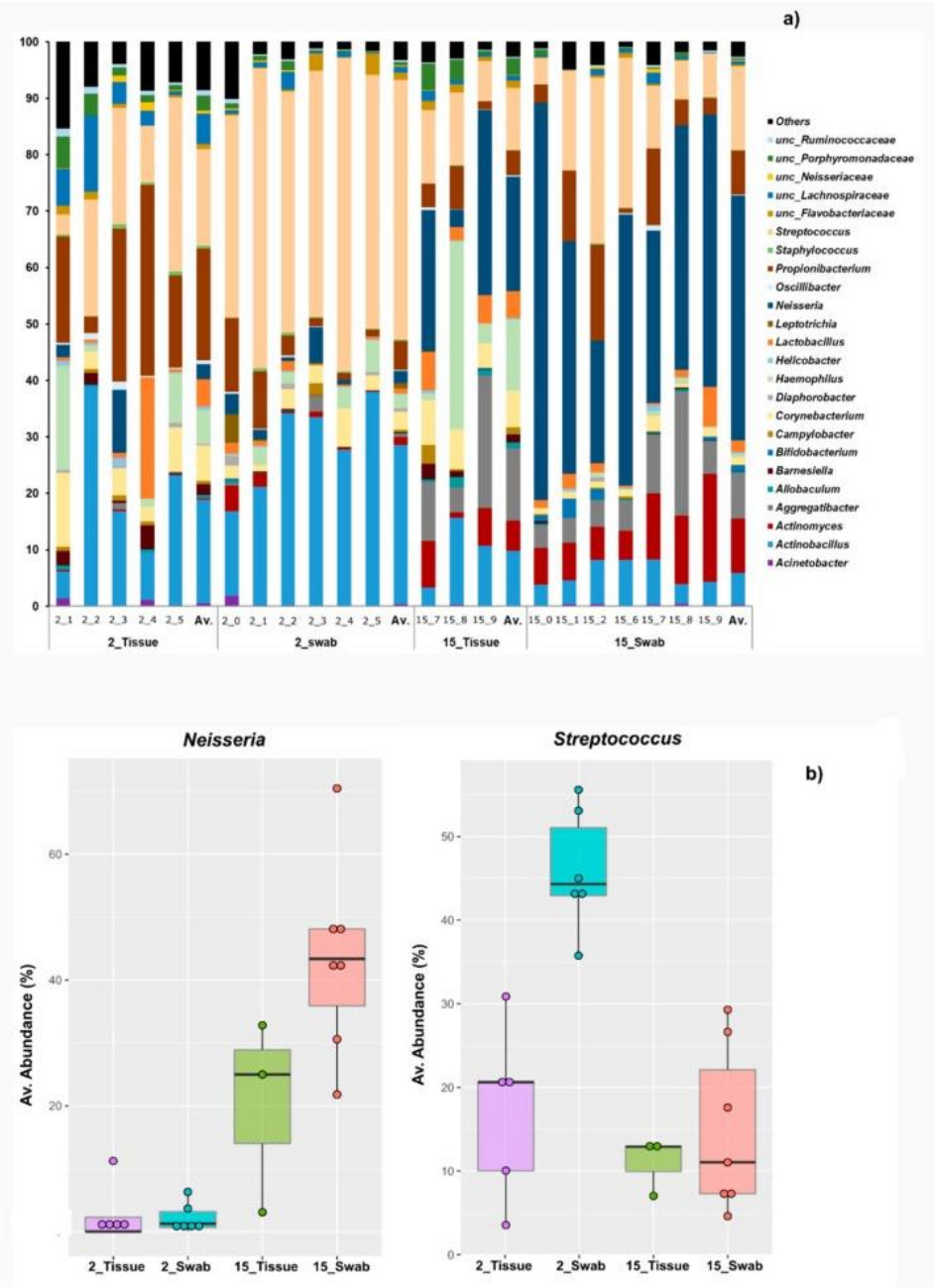

Figure 8 *Bacterial community composition at genus level.*

Age is represented with months (ex. 2_tissue: Tissue biopsies of 2 months old mice). (a) The average relative abundance of each genus detected in each sample of cotton swab and tissue biopsies from each age group are shown alongside each other. The average (Av.) of each set of samples is shown in the last column of each

group. (b) Box plots representing the average abundance of the genera Neisseria and Streptococcus, each dot represents one sample. For the means, standard deviation of the mean (SEM), variance, and 95% confidence intervals of each genus go to Table S1 (Supplementary Materials).

The comparison of the cotton swab microbial community from the two different mouse age groups showed a statistical difference in the common mouth colonizers *Actinobacillus*, *Actinomyces*, *Aggregatibacter*, *Neisseria*, *Staphylococcus*, *Streptococcus*, and an unclassified member of Clostridiales (p-value ≤ 0.05) (Figure 4). The microbial community determined in tissue biopsies samples revealed more stability across different ages; however, *Propionibacterium*, *Streptococcus*, and an unclassified member of Ruminococcaceae revealed statistical significance between the sampling groups at 2 months of age (p-value ≤ 0.05) (Table 1). At 15 months of age, Neisseria and an unclassified member of the Porphyromonadaceae family showed statistically different abundances.

Table 1 *Genera showing differences between sampling types.*

Phyla	Genera	Cotton Swab				Tissue Biopsy				*p*-Value
		2 Months		15 Months		2 Months		15 Months		
		Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	
Actinobacteria	*Propionibacterium*	5.1 *	−0.3–34.2	7.8	1.9–13.7	19.8 *	5.2–34.3	4.4	1.9–13.7	0.05
Firmicutes	*Streptococcus*	45.9 *	38.3–53.5	14.8	5.6–23.9	17.1 *	4–30	10.9	5.6–23.9	0.00
Firmicutes	unc_*Ruminococcaceae*	0.3 *	0.4–1.4	0.2	0.1–0.3	0.9 *	0–0–6	0.2	0.1–0.4	0.02
Proteobacteria	*Neisseria*	2.1	−0.3–4–6	43.3 *	29.1–57.5	2.7	−3.3–8.7	20.2 *	29.1–57.5	0.03
Bacteroidetes	unc_*Porphyromonadaceae*	0.6	0.03–1.3	0.3 *	−0.03–0.7	2.6	0.1–5.2	2.9 *	−0.03–0.7	0.05

* Compared samples with statistical difference.

At the species level, several bacteria showing statistical difference between cotton swab and tissue biopsies could not yet be assigned to a specific species and therefore remained named as unclassified bacteria. In 2 month old mice, *Cutibacterium acnes* was detected in higher abundance in the tissue biopsies (19.7%) in comparison to swab samples (5%), while *Streptococcus danieliae* was more abundant in swab (34%) than in the tissue samples (8.8%) (p-value ≤ 0.05) (Figure 9) Both of these species contributed to the dissimilarity observed between the two age groups. In 2 month old mice, several low abundant unclassified bacteria belonging to *Streptococcus* had statistical differences compared to the older 15 month old mice. Likewise, other low abundant species followed the same pattern: *Erysipelotrichaceae bacterium*, *Moraxella osloensis*, and *Streptococcus henryi* (p-value ≤ 0.05).

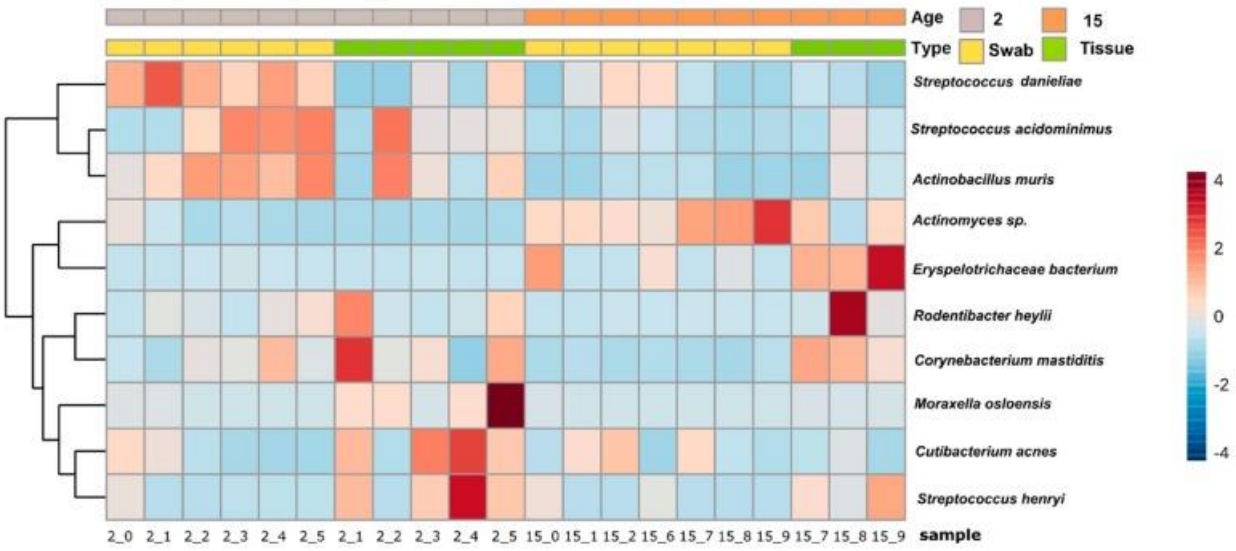

Figure 9 *Hierarchical clustering heatmap showing the variation of taxonomic abundances of identified species with statistical differences between the sampling type and age.*

The color range is represented as a Z-score representing the number of standard deviations above or below the mean.

2.4 Discussion

This study demonstrated that different oral sampling approaches influence the resultant composition of the microbiota present in the oral cavity. In the present study, we showed that oral swabs and tissue biopsies differed regarding their microbial ecology. Our findings point out that aging impacts the oral microbiota by modifying the composition and diversity of the oral niche. This research is subjected to the sample size limitations; however, we could identify statistical differences in the microbial communities for different sampling methodologies that were possibly related to the aggregated microbial community of the diverse mice oral microenvironments. Whereas the oral swab could collect bacteria present in higher abundances in the saliva, tongue, and shedding tissue surfaces, biopsies could be a better screening of the bacteria attached to the oral mucosal areas and thus in closer contact with the

host. The overall diversity of the oral cavity samples from 15 months old mice was higher than the diversity of younger animals. Generally, higher microbial diversity is related to a good health status [23]. In line with our findings in the younger mice, in humans, the gut bacterial diversity of newborns remains low and increases with age exponentially during the first three years and continues to increase until adulthood at a lower rate, yet in old age individuals the microbial diversity tends to decrease [24,25]. In this study, we also hypothesized that several intraoral conditions such as salivary flow, lip, or cheek movement and chewing forces could have an impact in the microbial ecology composition [26]. Such factors are influenced by the changes occurring during aging, thus inducing shifts in the bacterial community as at 15 months of age. C57BL/6 mice can be considered between middle and old age at 15 months of life as at this age, this strain has already developed some senescent changes [27]. However, in other mice strains at 15 months, they are considered old because of the behavioral and physical changes that can be detected [28,29].

The higher abundance of Streptococcus found in the swab samples obtained from 2 month old mice could result from adhesins present in the Streptococci bacteria, which interact with salivary agglutinins that bind bacteria to saliva-coated surfaces and influence the formation of biofilms [30]. The establishment of polymicrobial biofilms starts in the oral cavity from the first days of life [31] when different bacteria colonize mucosal tissue, teeth, tongue, and anaerobic pockets. In human samples, these biofilms have shown to be niche dependent, heterogeneous, and diverge across the ages [32]. The biofilm formation depends on several factors such as co-adhesion, pH, oxygen, and nutrients. Most of the significant bacterial differences between the biopsy and the swab samples were common oral colonizers, implying that the biofilm

composition diverges in the oral cavity niches of this mouse model. We consider that the differences observed between the oral sampling approaches of the microbiota could be associated with the attached bacteria in the tissue biopsies.

Similar to findings in humans, we revealed high inter-individual differences in several usual mouth colonizers, such as Streptococcus and Neisseria [33], the last one identified as part of the core microbiota of the dental plaque and saliva [32]. Those differences were not only inter-individual, but also between the sampling types. One of the advantages of mice models is that lifestyle, diet, and personal environment are factors without weight in the results when compared to human studies. Therefore, we can conclude that age and sampling procedures are elements that influence the identification and quantification of the oral bacterial communities in mice models and those are factors that could also impact the human oral microbial niches. As we found clear differences related to the sampling approaches and the age effect on oral microbiota, further research is required to understand how the bacterial ecology in the mouth fluctuates through the aging process and to establish a protocol that can be comparable between research groups.

2.5 Conclusions

This study suggests that the sampling method is a factor to consider when determining bacterial abundances and diversity in the oral cavity. Indeed, we showed that in oral swab samples, bacterial abundances and diversity differ from those found in biopsies of the oral tissue. As the oral cavity in mice and human is colonized with biofilms, oral swabs could depict the overall composition of the oral niches, while the tissue biopsies could be representative of the soft tissue. Nevertheless, as a similar bacterial composition was found in the cotton swab and the biopsies, we consider it as adequate to use an oral cotton swab for sampling in order to assess the microbiota of the oral cavity as it is a cost-effective and practical approach to collect bacteria from the different oral microbial microenvironments in mice models.

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CHAPTER III

3 Changes of intestinal microbiota and barrier function are associated with the development of aging-associated inflammation and liver degeneration

3.1 Abstract

Background: The number of people above the age of 60 years is raising world-wide being associated with an increase in the prevalence of aging-associated impairments and even diseases. Recent studies suggest that aging is associated with alterations in bacterial endotoxin levels and that these changes may add to low-grade inflammation, the so-called 'inflammaging', and aging-associated liver degeneration. However, mechanisms involved, and especially, the interaction of intestinal microbiota and barrier in the development of aging-associated inflammation and liver degeneration have not been fully understood.

Objective: The aim of the present study was to determine if intestinal microbiota composition changes with age and if these alterations are associated with changes of markers of intestinal barrier function and the development of inflammation and liver degeneration.

Methods: Blood, liver, small and large intestinal tissue of male 2-, 15-, 24- and 30-months old C57BL/6 mice fed standard chow were obtained. Intestinal microbiota composition, expression levels of antimicrobial peptides in small intestine and markers of intestinal barrier function were measured. Furthermore, indices of liver damage, inflammation and expression levels of lipopolysaccharide binding protein (*Lbp*) as well as of toll-like receptors (*Tlr*) 1-9 in liver tissue were assessed.

Results: Diversity of bacterial families and intestinal microbiota composition were significantly altered in small intestine of 15-, 24- and 30-months old mice, respectively, when compared to young animals while similar alterations were not found in colon. Concentrations of NO_x were significantly lower in small intestine in 15-, 24- and 30-

months old mice compared to young mice while mRNA expression of the antimicrobial peptides *defensin alpha 1* and *lysozyme 1* was unchanged. In contrast, in liver tissue, older age of animals was associated with increasing inflammation and the development of fibrosis in 24- and 30-months old mice. Numbers of inflammatory foci and neutrophils in livers of 24- and 30-months old mice were significantly higher compared to 2-months old mice. These alterations were also associated with higher endotoxin levels in plasma as well as an increased mRNA expression of *Tlr1*, *Tlr2*, *Tlr4*, *Tlr6* and *Tlr9* in livers in older mice.

Conclusion: Our data suggest that alterations of intestinal microbiota and barrier function are associated with an induction of several Tlrs and beginning hepatic inflammation in older mice and increase with age.

3.2 Introduction

Results of demographic studies suggest that globally the number of old aged individuals is steadily increasing (United Nations, WHO 2015). Indeed, the WHO has projected in its report published in 2015, that by the year 2050, the number of persons aged 60 years and older will outnumber children under the age of 15. Results of epidemiological studies further suggest that older age is among the key risk factors for the development of many diseases including Alzheimer's disease and metabolic diseases like type 2 diabetes and non-alcoholic fatty liver disease (for overview see [1-2]). However, even in settings of ´healthy` aging, it has been shown that aging is associated with a loss of function of cells, tissues, organs, and finally death [3]. Aging is also considered as a state of low-grade inflammation, occurring in the absence of overt infection and often defined as 'inflammaging' [1]. For instance, it has been shown that even in healthy elderly, markers of inflammation like interleukin 6, C-reactive protein and tumor necrosis factor alpha are elevated [4]. Despite a continuously increasing number of studies, the question how, when and by what means, aging intrinsically compromises tissue and cell function, and subsequently, leads to 'inflammaging' has not yet been fully clarified.

It has been reported that senescence in humans is associated with decreased α-diversity of gut microbiota [5-6]. Furthermore, results of more recent studies suggest that similar to the findings in metabolic diseases, e.g. type 2 diabetes and metabolic liver diseases, changes of intestinal microbiota composition and diversity along with impairments of intestinal barrier function may be critical in the development of aging-associated organ degeneration, low-grade inflammation and even non-healthy aging in humans [7-10]. Indeed, supporting the hypothesis that changes in intestinal barrier function, and subsequently, an increased translocation of bacterial toxins may be critical in the development of 'inflammaging', we recently showed that even in settings

of ‘healthy’ aging, liver degenerates, e.g. hepatic inflammation and fibrosis develop, and that this is associated with increased bacterial endotoxin levels and an induction of Tlr4-dependent signalling cascades in mouse liver [11]. In the same study, it was also shown that a loss of the lipopolysaccharide binding protein (LBP) was associated with a lessening in markers of senescence and inflammation in liver [11]. However, when these changes are first prevalent and molecular mechanisms involved have not yet been fully understood. Starting from this background, the aim of the present study was to determine if and when intestinal microbiota in small and large intestine of mice changes and if this is associated with changes of markers of intestinal barrier function and the onset and progression of inflammation and aging-associated liver degeneration in mice.

3.3 Materials and Methods

Animal experiments

Male C57BL/6 mice were housed in the Central Experimental Animal Husbandry (ZET) at the University Hospital of Jena, Jena, Germany. Animals had free access to standard chow (V1534-300, Ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum* at all times. At the age of 2, 15, 24 and 30 months, mice (n=5-7 per group) were sacrificed by cervical dislocation (number of approval: twz25-2017, administration: animal protection at University Hospital Jena, indication of the killing of vertebrates for scientific purposes according §4 (3) Animal Welfare Germany (from 18 July 2016)). Blood was collected by cardiac puncture. Liver and small as well as large intestinal tissue were fixed in PBS-buffered formalin or snap frozen for further analyses.

Microbiota composition in small and large intestinal tissue

Total DNA was extracted from small and large intestinal samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and as previously described [12]. The genomic DNA was used for the construction of Illumina libraries targeting the V1-V2 region using PrimeSTAR® HS DNA Polymerase kit TAKARA kit in a 3 PCR protocol described previously [13]. Samples were sequenced using a 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform. The sequencing of 16S rRNA gene from 32 small intestine and colonic samples yielded 1,043,176 clean reads, with an average of 32,599 ± 3,669 reads per sample. Raw sequencing data were processed by MOTHUR pipeline, following the MiSeq SOP [14]. Sequences were quality filtered and chimeras were removed. Silva version 123 was used for the alignment step. Chloroplasts, mitochondria, archaea and eukaryotic sequences were eliminated, and the sequences were clustered at 97 % identity into operational

taxonomic units (OTUs). Reads were taxonomically assigned following the recommendation of Yarza *et al*. [15].

Histological evaluation of liver and small intestinal tissue

For histological evaluation, sections of paraffin-embedded liver and small intestinal tissue (4 µm) were stained with hematoxylin and eosin (Sigma-Aldrich, Steinheim, Germany). Liver sections were scored as previously detailed [16]. Neutrophils were stained with a commercially available Naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich, Steinheim, Germany) and counted (magnification 200 x) using a microscope (Leica, DM6 B, Leica, Wetzlar, Germany) as previously described [17].

Immunofluorescence staining of p16-positive nuclei

To determine p16-positive cells, liver sections (4 µm) were incubated with anti-CDKN2A/p16^{INK4a} primary antibody (Abcam, Cambridge, UK) and secondary antibody conjugated to AlexaFluor 594 (Invitrogen, Darmstadt, Germany) as previously described [11]. P16-positive nuclei in liver tissues were quantified with ZEISS ZEN 2.3 imaging software (Zeiss, Jena, Germany) and presented as percentage of positive to negative tagged nuclei.

Endotoxin, Griess assay and ELISA

Plasma bacterial endotoxin levels were measured with a commercially available limulus amebocyte lysate (Charles River, Ecully, France) as previously described [18]. Nitric oxide (NO_x) concentration in small intestinal tissue was measured using Griess reagent assay (Promega, Mannheim, Germany). Plasminogen activator inhibitor 1 (PAI-1) concentrations in plasma were measured using a commercially available ELISA kit (LOXO, Dossenheim, Germany).

RNA isolation, cDNA synthesis and real-time PCR

RNA was extracted from liver and small intestinal tissue with TRIZOL (peqGOLD Trifast, Peqlab, Erlangen, Germany) and mRNA was reverse transcribed into cDNA (Promega, Madison, WI, USA). Real-time PCR was used to measure expression of respective genes in liver and small intestinal tissue as detailed before [11]. Primer sequences are listed in Table 2.

Table 2 *Primer sequences.*

	Primer sequences	
	Forward (5´- 3´)	**Reverse (5´- 3´)**
αSma	ctg aca gag gca cca ctg aa	cat ctc cag agt cca gca ca
Col1a1	acg tgg aaa ccc gag gta tg	ctt ggg tcc ctc gac tcc ta
Defα1	tgc ctg ctc atc cta atc cat c	agg aca agg gca aag agt agg
F4/80	tgg ctg cct ccc tga ctt tc	caa gat ccc tgc cct gca ct
Hmgb1	cgc gct ggc tgg aga gta at	gag gca cag agt cgc cca gt
iNos	ccc ctg gaa gtt tct ctt caa agt c	gat tct gga aca ttc tgt gct gtc c
Irf3	aac cgg aaa aga agt gtt gcg	gca ccc aga tgt acg aag tcc
Irf7	aca ggg cgt ttt atc ttg cg	tcc aag ctc ccg gct aag t
Lbp	ggt ggc gtc gtc act aat gt	ctc act tgt gcc ttg tct gg
Lyz1	gct gac tgg gtg tgt tta gc	tga tcc caca gg cat tct tag
Myd88	ccc tag ggc aga ggg gaa ga	atg cct gtg tgt gcag agg ag
Tlr1	cgc ctg gac cca gag ttt gt	cgc acc cag gaa ggt cag tt
Tlr2	ctc cac aag cgg gac ttc gt	ggc tcc agc aaa aca agg a
Tlr3	cgc cct cct ctt gaa caa cg	gga acc gtt gcc gac atc at
Tlr4	agc cat tgc tgc caa cat ca	gct gcc tca gca gga ctt c
Tlr5	agc ctc cgcctc cat tct tc	tca cgg cct ctg aag ggg ttc

Tlr6	ccc aaa gac ctg cca cca ag	cgc cat agg gca gca aga ga
Tlr7	ccc atg tga tcg tgg act gc	cat tgg ctt tgg acc cca gt
Tlr8	tct cct cca tgc ccc gac t	tgc cat tgt ggc tca ggt t
Trl9	atc acc act gtg ccc cga ct	gag att gctcag gcc cag ga
18S	gta acc cgt tgaacc cca tt	cca tcc aat cgg tag tag cg

αSma, alpha smooth muscle actin; Col1α1, collagen 1 alpha 1; Defα1, defensin alpha 1; Hmgb1, high-mobility group box 1; iNos, inducible nitric oxide synthase; Irf, interferon regulatory factor; Lbp, lipopolysaccharide binding protein; Lyz1, lysozyme 1; Myd88, myeloid differentiation primary response 88; Tlr, toll-like receptor.

Western blot

Protein levels of intestinal fatty acid binding protein (I-FABP) in plasma, used as a marker of intestinal permeability [19], and growth differentiation factor 15 (GDF-15), known as a marker of cellular senescence [20] were measured by Western blot analysis. 40 µg protein of plasma of mice was separated on an 8 % SDS polyacrylamide gel and transferred onto PVDF membranes. After blocking, membranes were incubated with the primary antibody anti-I-FABP (abcam, Cambridge, UK) or anti-GDF-15 (Santa Cruz, Dallas, Texas, USA) and secondary antibody (anti-rabbit, Cell Signaling Technology, Danvers, USA). Intensities of bands were detected with Super Signal West Dura Kit (Thermo Fisher Scientific, Waltham, MA, USA) and densitometric analyses were performed with ChemiDoc XRS System.

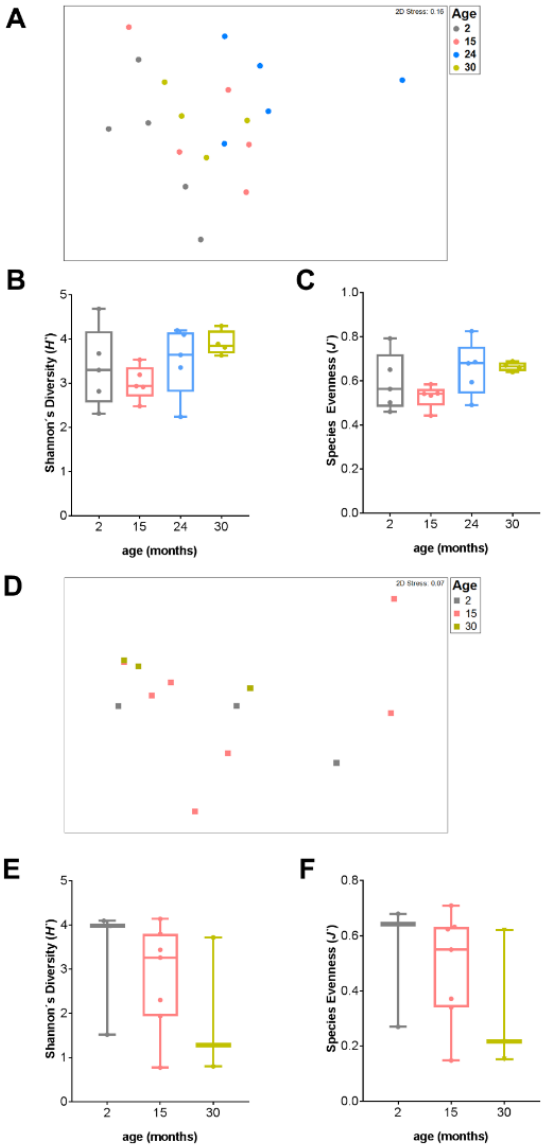
Statistical analyses

For analyzing microbiota composition, sequencing reads were standardized by total using Primer-7 software. A similarity matrix was calculated using the Bray-Curtis similarity coefficient and a non-metric multidimensional scaling (NMDS) plot was created. Permutational Analysis of Variance (PERMANOVA) was used to assess the differences between the sample groups. Data obtained when analyzing tissue and blood samples were statistically analyzed using one-way ANOVA after a Grubbs test was applied to identify outliers. Log-transformation was performed when data were not equally distributed. Data are presented as means ± standard error of the means (SEM).

3.4 Results

Alterations of intestinal microbiota composition in mice of different age

To determine if and when alterations of intestinal microbiota composition are prevalent in aging mice, 16S rRNA gene amplicon sequencing was performed in tissue samples obtained from small intestinal tissue of mice aged 2, 15, 24 and 30 months and of colon of mice aged 2, 15 and 30 months. Samples extracted from colon of 24-months old mice were not suitable for analysis due to the low number of reads obtained after sequencing. Accordingly, they were not included in further analysis. NMDS plot revealed a clustering of the small intestine samples of mice within the same age group (P=0.006) but the same was not observed in large intestine (Figure 10). Shannon´s diversity and species evenness indexes showed no statistical difference between age groups in either small or large intestine (Figure 10). In small intestine, at phylum level, Firmicutes and Bacteroides comprised more than 90 % of the total abundant bacteria. Despite differences in relative abundance, microbiota of mice at different ages was taxonomically assigned to 28 genera in small intestine (Figure 11).



Supplemental Figure 1

Figure 10 *Global community structure, bacterial diversity and evenness of small and large intestine in mice of different ages.*

NMDS of global bacterial community structure of small (A) and large intestine (D), Shannon diversity index and species evenness of small (B, C) and large intestine (E, F) in 2-, 15-, 24- and/or 30-months old mice. Data are presented as means ± SEM, n=5-7. NMDS, non-metric multidimensional scaling.

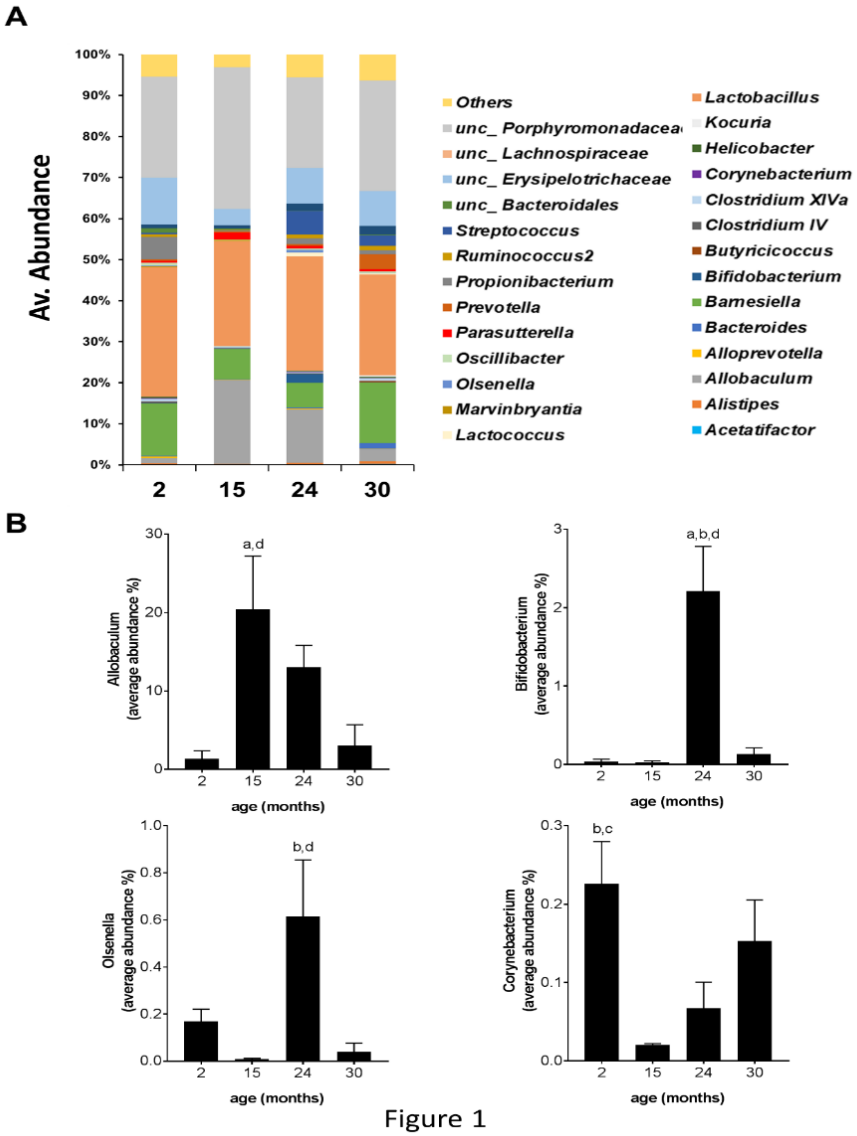


Figure 1

Figure 11 *Microbiota composition in small intestine in mice of different age.*

(A) Relative abundance of bacterial genera and (B) genera that are statistical different in small intestine of 2-, 15-, 24- and 30-months old mice. Data are presented as means ± SEM, n=5-7, $^{a}p < 0.05$ *compared to 2-months old mice,* $^{b}p < 0.05$ *compared to 15-months old mice,* $^{c}p < 0.05$ *compared to 24-months old mice,* $^{d}p < 0.05$ *compared to 30-months old mice.*

The genus *Lactobacillus* dominated the small intestinal niche at all ages followed by *Allobaculum* and an unclassified member of the *Porphyromonadaceae* family. Four of the genera in the small intestine differed significantly with relation to age. Specifically, in 15-months old mice, the average abundance of *Allobacullum* was higher (20.4 % ± 6.7) than in 2- (1.3 % ± 1.0) and 30-months old mice (2.9 % ± 2.6). *Bifidobacterium* had a significant increment in 24-months old mice (2.2 % ± 1.2) compared to all other ages studied where the average abundance ranged between 0.13 - 0.03 %. The genus *Olsenella* was significantly more abundant in 24-months old mice (0.6 % ± 0.2) than in 15- (0.01 % ± 0.00) and 30- (0.04 % ± 0.00) months old mice, respectively. The abundance of genus *Corynebacterium* being generally present at low abundances in proximal intestinal tissue, was statistically higher at 2 months of age (0.23 % ± 0.05) when compared to 15- (0.02 % ± 0.00) and 24-months old (0.15 % ± 0.03) mice (Figure 11). In colon, differences alike were not found between the different age groups. Indeed, only the relative abundance of *Allobaculum* was found to differ statistically in colon between 2-months and 30-months old mice (Figure 12). As differences regarding intestinal microbiota composition between the different age groups were more prominent in small than in large intestine, further measurements and analysis focused on small intestine.

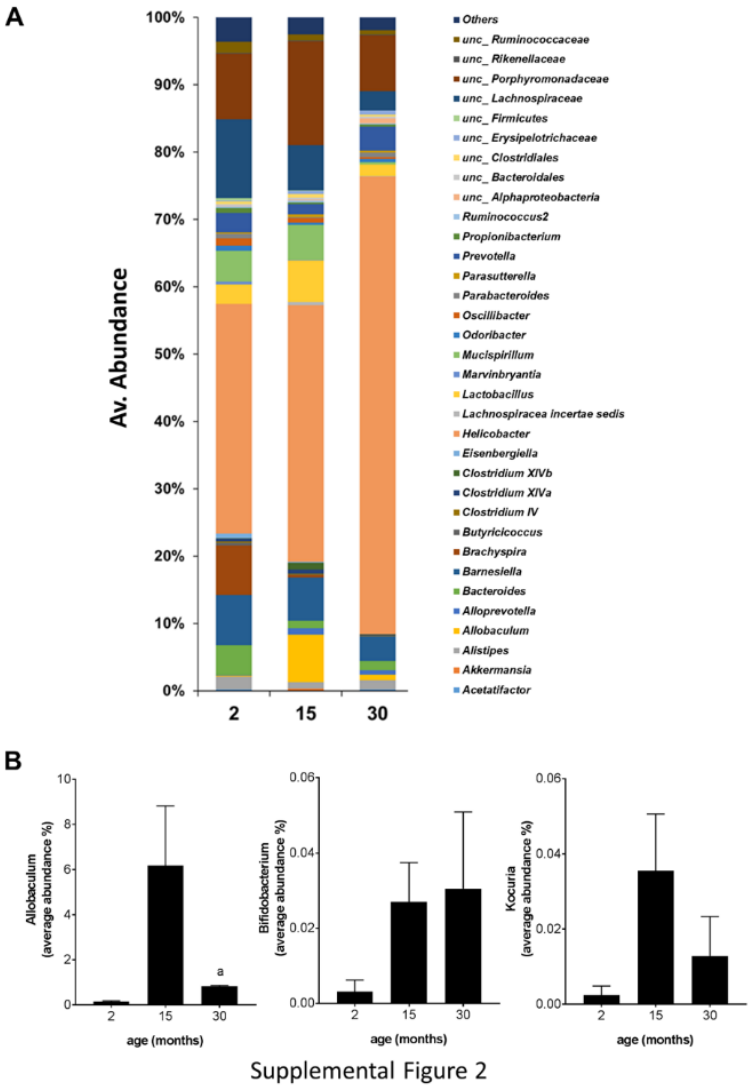


Figure 10 *Microbiota composition in large intestine in mice of different age.*

(A) Relative abundance of bacterial genera and (B) genera that are statistical or by trend different in small intestine of 2-, 15- and 30-months old mice. Data are presented as means ± SEM, n=5-7, $^{a}p < 0.05$ *compared to 2-months old mice.*

Antimicrobial peptides, NO_x concentration and markers of inflammation in small intestinal tissue in mice of different age

Expression levels of the antimicrobial peptides *defensin alpha 1* (*Defα1*) and *lysozyme 1* (*Lyz1*) mRNA in small intestinal tissue did not differ between age groups of mice (Table 3). NO_x concentrations in whole intestinal tissue were significantly lower in 15-, 24- and 30-months old mice when compared with 2-months old mice. Somewhat contrasting these findings, *iNos* mRNA expression was not different between groups; however, data varied considerably (Table 3). Expressions levels of *F4/80* mRNA used as a marker of macrophages, were significantly lower in small intestinal tissue of 15- and 24-months old mice than in 2-months old mice while again, similar differences were not found for the comparison of 30- and 2-months old animals as data varied considerably (Table 3).

Table 3 *Antimicrobial peptides, NOx concentration and marker of inflammation in small intestinal tissue in mice of different age.*

	age (months)			
	2	**15**	**24**	**30**
***Defα1* mRNA expression (% of younger mice)**	100 ± 12.2	104.4 ± 30.1	66.1 ± 12.6	43.1 ± 14.4
***Lyz1* mRNA expression (% of younger mice)**	100 ± 18.1	167 ± 60.1	58.2 ± 16.2	60.9 ± 13
NO_x (µM/mg protein)	62.9 ± 4.7	36 ± 5.6[a]	29.7 ± 7.7[a]	33.3 ± 9[a]
***iNos* mRNA expression (% of younger mice)**	100 ± 27.1	153.2 ± 60.4	250.6 ± 65.4	206.3 ± 72.9
***F4/80* mRNA expression (% of younger mice)**	100 ± 8.1	46.5 ± 14.1[a]	46.6 ± 10.9[a]	95.3 ± 19.9

Data are presented as means ± SEM, n=5-7, [a]$p < 0.05$ compared to 2-months old mice. Defα1, defensin alpha 1; iNos, inducible nitric oxide synthase; Lyz1, lysozyme 1; NO_x, nitric oxide.

Markers of intestinal permeability in mice of different age

To determine if alterations found in intestinal microbiota composition and NO_x in small intestine of mice at different age were associated with changes of intestinal permeability, plasma levels of I-FABP and bacterial endotoxin as well as *Lbp* mRNA expression were assessed. Bacterial endotoxin levels were significantly higher in 15- and 30-months old mice than in 2-months old mice, and by trend, in 24-months old animals while plasma levels of I-FABP did not differ between age group (Figure 13).

Expression levels of lipopolysaccharide binding protein (*Lbp*) in livers were higher in 24- and 30-months old mice compared with 2-months old mice; however, as data varied considerably in 30-months old mice and the n in this group was only 5, differences were only different by trend (p=0.11: 30-months compared with 2-months old mice, Figure 13).

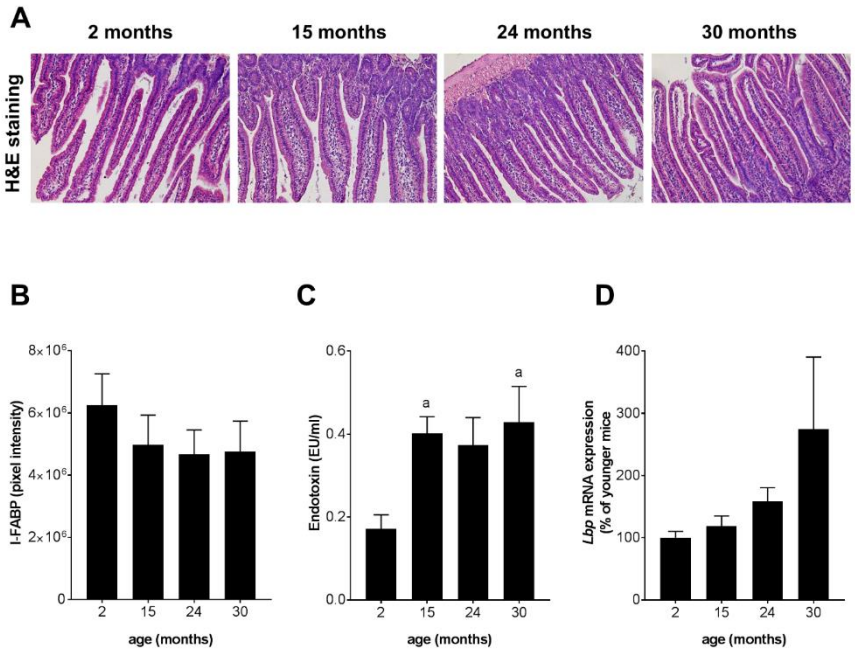


Figure 11 *Markers of intestinal permeability in mice of different age.*

(A) Representative pictures (magnification 200 x) of hematoxylin and eosin staining (H&E) of intestinal small intestine, (B) protein expression of I-FABP and (C) bacterial endotoxin concentration in plasma as well as (D) mRNA expression of Lbp *in liver tissue of 2-, 15-, 24- and 30-months old mice. Data are presented as means ± SEM, n=5-7,* [a]p *< 0.05 compared to 2-months old mice. I-FABP, intestinal fatty acid binding protein; Lbp, lipopolysaccharide binding protein.*

Expression of Tlrs and markers of Tlr signaling cascades in liver tissue in mice of different age

Hepatic mRNA expressions of *Tlr1* were significantly higher in 24- and 30-months old mice when compared to 2- and 15-months old mice (Figure 14). Expression of *Tlr2* and *Tlr4* mRNA were both significantly higher in 30-months old mice compared with 2-, 15- and 24-months old mice (Figure 14). In contrast, *Tlr5* mRNA in liver was significantly lower in 30-months old mice compared with 15-months old mice (Table 4). Both, *Tlr6* and *Tlr9* mRNA expressions were significantly higher in livers of 30-months old mice compared with 15-months old mice and in addition, *Tlr6* mRNA was significantly higher in 30-months old mice compared with 2-months old mice (Figure 14). No differences were found for *Tlr3*, *7* and *8* mRNA expression in liver between age groups (Table 4). Expression of myeloid differentiation response 88 (*Myd88*) was significantly higher in 30- months old mice in comparison of 2- and 15-months old mice (Figure 14). Interferon regulatory protein 7 (*Irf7*) expression was significantly higher in 30-months old mice compared to 2-, 15- and 24-months old mice while expression of *Irf3* did not differ between groups (Figure 14). Expression of high-mobility-group box 1 (*Hmgb1*), known as an inducer of Tlr2, 4 and 9, but also discussed as a marker of senescence [21], was also significantly higher in 30-months old mice when compared with 2- and 15-months old mice (Figure 14).

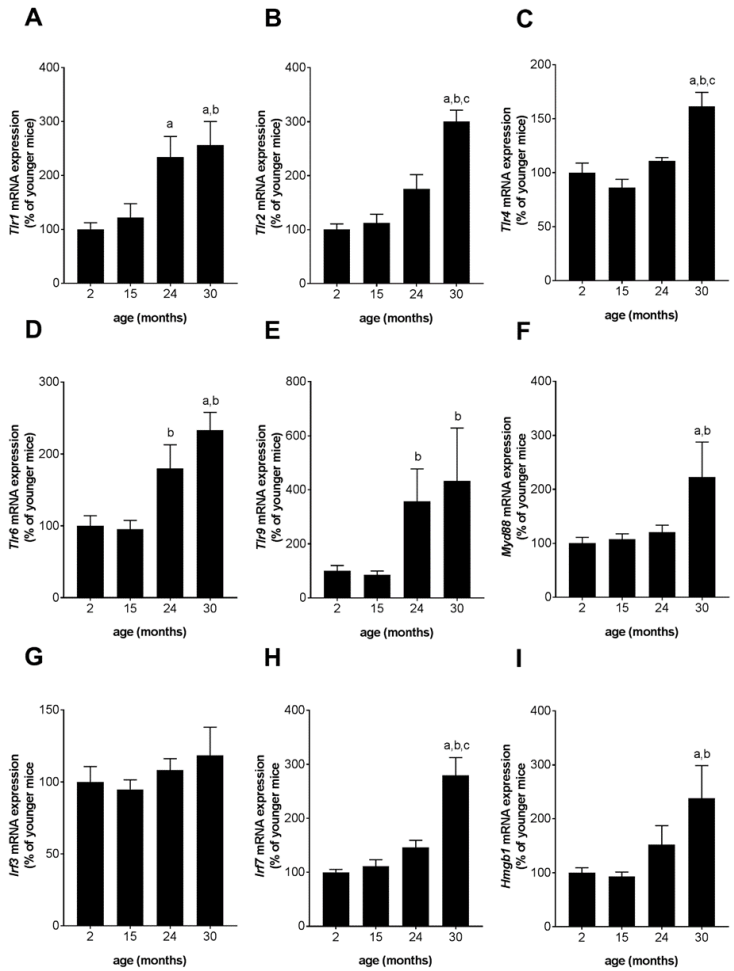


Figure 3

Figure 12 *Expression of Tlrs and markers of the Tlr signaling cascades in liver tissue in mice of different age.*

Expression of (A-E) Tlr1, Tlr2, Tlr4, Tlr6 *and* Tlr 9 *as well as (F)* Myd88, *(G, H)* Irf3, Irf7 *and (I)* Hmgb1 *mRNA expression in liver tissue of 2-, 15-, 24- and 30-months old mice. Data are presented as means ± SEM, n=5-7,* $^{a}p < 0.05$ *compared to 2-months old mice,* $^{b}p < 0.05$ *compared to 15-months old mice,* $^{c}p < 0.05$ *compared to 24-months old mice. Irf, interferon regulatory factor; Hmgb1, high-mobility-group-protein B1; Myd88, myeloid differentiation primary response 88; Tlr, toll-like receptor.*

Table 4 *Expression of Tlrs in liver tissue in mice of different age.*

	age (months)

	2	15	24	30
***Tlr3* mRNA expression (% of younger mice)**	100 ± 18.6	112.9 ± 8.9	126.2 ± 13.2	155.4 ± 36.7
***Tlr5* mRNA expression (% of younger mice)**	100 ± 27.2	131.7 ± 14.4	98.8 ± 13.2	48 ± 8.5[b]
***Tlr7* mRNA expression (% of younger mice)**	100 ± 21	90.6 ± 20.2	121.8 ± 28.8	201.8 ± 52.1
***Tlr8* mRNA expression (% of younger mice)**	100 ± 18.7	104.6 ± 16.2	146.1 ± 34.5	180.7 ± 43.1

Data are presented as means ± SEM, n=5-7, [b]$p < 0.05$ compared to 15-months old mice. Tlr, toll-like receptor.

Markers of senescence and inflammation in livers of mice of different age

The changes found at the level of the gut and the increase in bacterial endotoxin as well as the induction of the expression of different Tlrs in liver were associated with significantly higher PAI-1 and GDF-15 in plasma as well as p16 levels in liver tissue of mice aged 15, 24 and 30 months when compared to young animals. This was also associated with significantly higher scores of inflammation and numbers of neutrophils in liver tissue in 24- and 30-months old mice compared with 2- and 15-months old mice, respectively. Interestingly, while older mice were markedly heavier, no fat accumulation was found in liver tissue (Table 5, Figure 15). Beginning fibrosis was observed in older mice with mRNA expression of alpha smooth muscle actin (*αSma*) being significantly higher in 30-months old animals compared with 2-, 15- and 24-months mice while collagen 1 alpha 1 (*Col1α1*) was only significantly higher in 30-months old mice compared to 15-months old mice (Table 5).

Table 5 *Body weight and fibrosis marker in mice of different age.*

	age (months)			
	2	**15**	**24**	**30**
Body weight (g)	25.9 ± 0.6	39.5 ± 1.9[a]	30.2 ± 1.5[b]	33.0 ± 1.2[a,b]
***αSma* mRNA expression (% of younger mice)**	100 ± 17.6	103.3 ± 21.4	96.3 ± 10.8	228.8 ± 37.9[a,b,c]
***Col1α1* mRNA expression (% of younger mice)**	100 ± 14.2	57.1 ± 6.4	115 ± 27.4	139.7 ± 31.8[b]

Data are presented as means ± SEM, n=5-7, [a]$p < 0.05$ compared to 2-months old mice, [b]$p < 0.05$ compared to 15-months old mice, [c]$p < 0.05$ compared to 24-months old mice. αSma, alpha smooth muscle actin; Col1α1, collagen 1 alpha 1.

Liver inflammation

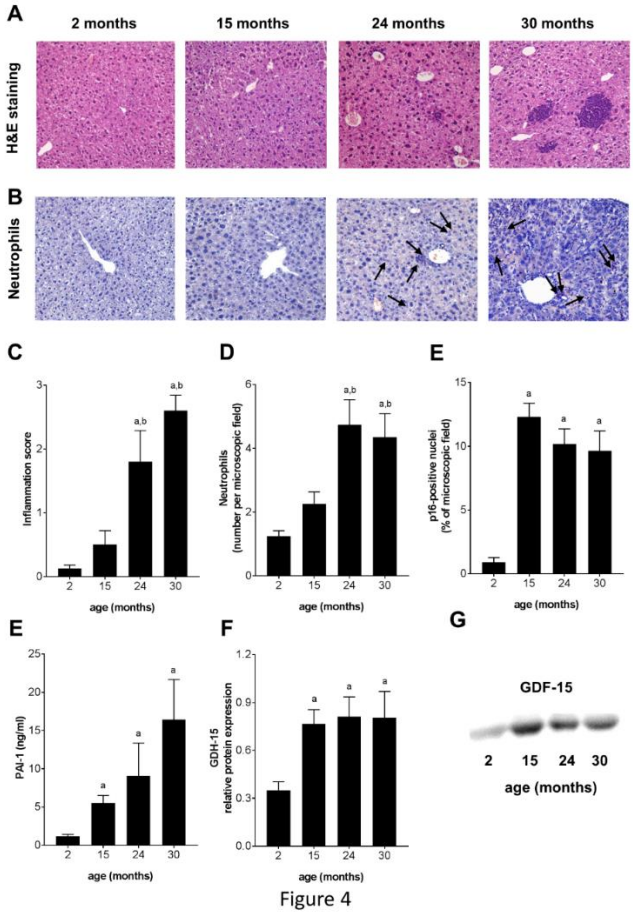


Figure 13 *Markers of senescence and inflammation in liver tissue in mice of different age.*

Representative pictures (magnification 200 x) of (A) hematoxylin and eosin staining (H&E), (B) neutrophils, (C) quantitative analysis of non-alcoholic fatty liver disease activity score and (D) number of neutrophils as well as (E) p16-positive nuclei in liver tissue, (F) PAI-1 concentration, (G) GDF-15 relative protein expression in plasma as well as representative pictures of GDF-15 of 2-, 15-, 24- and 30-months old mice. Data are presented as means ± SEM, n=5-7, $^{a}p < 0.05$ *compared to 2-months old mice,* $^{b}p < 0.05$ *compared to 15-months old mice. GDF-15, growth differentiation factor 15; PAI-1, plasminogen activator inhibitor 1.*

3. 5 Discussion

In recent years, it has been suggested that aging and the so-called 'inflammaging' might be related to changes in intestinal homeostasis, and that herein, alterations of intestinal microbiota composition may be critical (for overview see [22-23]. In support of this hypothesis, results of animal and human studies in humans suggested that gut microbiota composition dramatically changes during aging and these alterations have been linked to host health and life-span [24-25]. Furthermore, Langille *et al*. observed taxonomic and functional changes in the fecal microbiome of old mice that correlate with age and frailty [26]. In the present study, prevalences of *Allobaculum*, *Bifidobacteria*, *Olsenella* and *Corynebacterium* in small intestine were found to be different between young and older aged mice in small intestine, while in colon, alterations alike were not found. Changes in the abundance of the genus *Allobaculum*, *Bifidobacteria* and *Corynebacterium* but not *Olsenella* during aging in mice have been reported by others, too [27-28]. However, while recently metabolites produced by *Corynebacterium durum* were even reported to extend lifespan in *C. elegans* [29], in the present study, prevalence of bacteria of the genus *Corynebacterium* were lower in older aged mice than in young once. Also, in the study of Thevaranjan *et al*. it was shown that fecal microbiota transplantation of a defined consortium of bacteria was associated with a restoration of intestinal permeability in old mice [27]. Furthermore, while others reported changes in fecal or cecal microbiota, in the present study, intestinal microbiota was predominately altered in small intestine. Results of our study also suggest that some of the changes observed seem to be rather transient, e.g. an increase of the prevalence of *Allobaculum* at 15- and 24- and of *Bifidobacterium* and *Olsenella* at 24-months of age, while the prevalence of the genus *Corynebacterium* was lowest at 15-months of age and gradually seemed to increase again until the age of 30 months. Furthermore, it was also shown that germ-free mice had an extended

lifespan, further suggestion, that indeed, intestinal microbiota may impact aging. If changes, e.g. in relative abundance of certain bacterial genus at a certain age, are critical in the onset of 'inflammaging' and senescence needs to be assessed in future studies. Somewhat contrasting the findings in stool samples of humans [5] and fecal and cecal samples, respectively, of mice [28, 30], neither α-diversity represented by Shannon's diversity index nor species evenness indexes were altered between different age groups of mice. Differences might have resulted from differences in species, e.g. free-living humans vs. experimental mice kept under SPF conditions but also experimental set up (one ´old` age vs. different ages) and detection methods used. In line with the findings of others in humans and mice [31], expressions of antimicrobial peptides were not altered in small intestinal tissue of aging mice in the present study. Interestingly, mRNA expression of *F4/80* and NO_x concentration were lower in older mice than in young ones in the present study, suggesting that contrary to the findings of us and others in other tissue like liver [11] showing higher numbers of macrophages and increased iNos expression in old age, in small intestine, number of macrophages and NO production decreases with age in the present study. However, somewhat surprising, in 30-months old animals, expression of *F4/80* was not altered when compared to young animals. Also, *iNos* expression was similar between age groups as expression varied considerably between animals. Results of others suggest that NO_x generated by the host might be critical in modulating intestinal microbiota composition [32]. Also, both, macrophages and NO have been shown to play a pivotal role in epithelium integrity and maintenance of intestinal homeostasis through altering cellular redox status [33]. In line with the findings of the present study, changes in markers of intestinal permeability, e.g. an increase in bacterial endotoxin levels and permeation of FITCs dextran in older aged mice and humans have been

shown by others before, too [7, 11, 27, 34]. Interestingly, in the present study, intestinal morphology was not altered while other showed that old age was associated with marked alterations in intestinal morphology, e.g. number of goblet cells, intestinal wall thickness, villus height and crypt depth [35]. However, data in this respect are contradictory. Indeed, others also found no overt changes in intestinal morphology in healthy old aged mice and humans, respectively [36-37]. Contrasting the findings of the present study and those of others, Wilms *et al*. found no changes in markers of intestinal permeability in elderly compared to younger individuals [38]. Differences between the study of Wilms *et al*., and the present study might have resulted from difference in age. Also, if only a loss of intestinal barrier integrity and changes in intestinal microbiota are critical in the increase of bacterial endotoxin found in the present study or if clearance of bacterial toxins and herein especially bacterial endotoxin is also altered with age, contributing to the increase of bacterial endotoxin levels found in the present study, needs to be determined in further studies.

Taken together, our results further support the hypothesis that aging even in settings of `healthy´ aging is associated with changes of intestinal microbiota composition and markers of intestinal immune defence, e.g. number of macrophages and NO production but not with changes of antimicrobial peptides. Implications of these alterations, and herein especially, of the changes in prevalence of specific intestinal bacterial genus for intestinal homeostasis need to be determined in further studies. However, results of recent studies suggest that targeting intestinal microbiota through faecal microbiota transfer or feeding a human-origin probiotics cocktail may improve intestinal barrier function during aging [27, 39].

Alterations of small intestinal microbiota and markers of intestinal permeability are associated with aging-associated liver degeneration and increased markers of senescence

In the present study, alterations in microbiota composition in small intestinal tissue and increased bacterial endotoxin levels were associated with an induction of *Lbp* and *Hmgb1* expression in mice at older age. In line with these findings, expression of *Tlr1*, *2*, *4*, *6* and *9* and their adaptor proteins *Myd88* as well as *Irf7* also gradually increased with age, too. Furthermore, the increase in Tlrs and related signalling proteins was associated with inflammation and beginning fibrosis in liver as well as a higher markers of senescence, e.g. GDF-15 and PAI-1 [40-41] protein in plasma as well as p16 in liver tissue of older mice. These findings are in line with previous findings of our group and those of others showing a positive association between markers of intestinal permeability and inflammatory markers in blood as well as liver tissue [11, 34]. In the present study, expressions of *Tlr3*, *7* and *8* were similar between age groups; however, expression of *Tlr3*, *7* and *8* varied considerably. *Tlr5* expression was decreased in liver tissue of 30-months old mice compared to 15-months old animals. This is in line with the findings of Liang *et al*. reporting that *Tlr5* expression was significantly lower in macrophages isolated from > 18-months old Balb/cByJ mice compared with their young counterparts [42] suggesting that aging-associated alterations of innate immunity are likely to be dynamic, i.e. both a decrease and an increase as well as no changes of immune activity can occur during the aging process [43-44]. Taken together, results of the present study support previous findings of us and others, that aging is associated with an induction of some Tlrs in the liver as well as liver degeneration further supporting the hypothesis that changes in intestinal barrier

function, and subsequently, increased permeation of bacterial toxins, viruses and other ligands of Tlrs are associated with aging-associated decline.

3.6 Conclusion

In summary, our data suggest that changes in intestinal microbiota composition and barrier function are markedly altered in old aged mice and that this is associated with an induction of several Tlrs in liver and liver degeneration. Our results also suggest that these alterations, at least in part, are more pronounced with older age of mice. However, further studies are needed to determine the molecular mechanism involved and if targeting these alterations are associated with an extension of healthy life span. Also, if alterations alike are also involved in aging-associated degeneration and ‘inflammaging’ in humans remain to be determined.

3.7 References

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CHAPTER IV

4 The active microbial community of mice gastrointestinal tract through the lifespan

4.1 Abstract

Each segment of the gastrointestinal tract has associated microbial communities that vary depending of the physiological conditions and the functions of digestion, absorption and waste disposal. The mouth is in direct contact with the exterior, the small intestine interacts with the biliary and gastric secretions and in the colon, fermentation takes place and the oxygen availability is low. Previous research indicated that physiological changes occurring during the lifespan, influence the composition of the microbiome. It is still not clear if these changes influence the development of diseases in old age. Our aim was to analyze the active microbial community of the oral, duodenal and colonic niches in mice of different ages (2, 9, 15, 24 and 30 months). We observed that the samples differed by site (p-value 0.0001) and the combination of both AgexSite (p-value 0.05). These differences were observed at different taxonomical levels, and were more evident after 15 months of age. At genus level we observed changes in Lactobacillus abundance that were related to age and site, while Helicobacter showed fluctuations across the age. At species level, we could also identify members of this species *Helicobacter hepaticus* and *Helicobacter ganmani*. The process of aging affects the microbial composition of the different GIT sites and influence the prevalence of pathobiontic species.

Key words: GIT microbiota, active microbial community, niche dependent microbiome, murine models

4.2 Introduction

The gastrointestinal tract (GIT) of humans and animals is a biological environment colonized by thousands of bacteria from different species [1]. Each section of the GIT

has a specific composition. This spatial stratification is due to the different microenvironments, enzyme production, flow, oxygen availability, and other factors that favor the growth of different bacterial taxa [2]. The bacterial communities can adapt to the environment that they are exposed to [3]. First segments of the GIT, mouth, esophagus, stomach and small intestine, have higher availability of oxygen, which modulates the microbiota to include more aerobes [4]. The microbial ecology of the mouth and the small intestine are not as diverse as the colon [5]. However, most of the dietary compounds are absorbed in this section and it is in direct contact with bile acids and gastric juices [5]. The biological functions of the different segments from the GIT, model the microbial environments and differentiate the communities from each niche.

An imbalance of the microbiome, also called dysbiosis is related to a broad number of diseases. For example, in the mouth can cause periodontal disease, in the duodenum leads to lactose intolerance and celiac disease and in the colon can go from obesity to cancer [6, 7]. Some of these pathologies are chronic conditions of which the incidence increase with age [8]. Previous research has indicated that aging influences gut microbiome [9]. Aging is a complex process that consists of biological, physiological, environmental, psychological, behavioral, and social changes. The loss of microbial diversity is common in aged individuals [10]. It is still unclear how the microbiota shifts through life and supports the development of diseases. Age related dysbiosis could be linked to several biological changes, however external factors as medication used and environmental changes can also influence the progression to a different microbial community. In very old individuals, conditions such as loss of dental pieces, dry mouth, acid reflux, gastritis, bacterial overgrowth (SIBO), can either change the diet or directly influence the progression to a different microbial ecology of

the GIT segments [11]. Aging research in humans is challenging, as it is often complicated to access samples from the same individual during all its lifespan. Using murine models have advantages due to the shorter lifespan and GIT similarities to humans [12].Target amplicon sequencing of the 16S rRNA gene has been a methodology broadly used for the understanding of the microbial communities in the gastrointestinal tract of different species [13]. DNA is often used to depict the total microbial community, however, by using it does not let us know which bacteria are still active. To target the active bacteria, RNA should be used and further sequenced [13]. In previous studies, the active and total microbial communities have shown a similar structure but different composition, not only in the bacterial communities but also in the archaeal ones [13]. The study of the active microbiota can give us a qualitative understanding of the relationship/competition between microorganisms and the overall functionality [14]. Therefore, targeting the active microbial communities could help us understand the complex dynamics of the microorganisms related to the aging process. In this chapter, we used a murine model to analyze the active bacterial communities from different GIT sites in different stages of lifespan.

4.3 Methods

Animal management

C57Bl6 male mice were housed in groups of 8 in SPF conditions at the animal facility of the University Hospital Jena, Germany. Housing followed EU guidelines with access to food (sniff pellets, ssniff Spezialdiäten GmbH, Soest, Germany) and water *ad libitum*. Groups between 3-6 mice from the ages 2, 9, 15, 24 and 30 months of age were sacrificed by cervical dislocation. A small piece of tissue sample (approx. 0.5 cm) was taken from the mouth snap frozen in liquid nitrogen and immediately stored

at -80°C. Tissue from approximately 1 cm long was taken from duodenum and colon and were stored in RNA later at -80°C to ensure no degradation.

Sample preparation

From the tissue samples, total RNA was extracted using a modified Trizol protocol (Trizol, Sigma Aldrich) that included a bead beating step (30 secs, 5.5 m/s) in a FastPrep instrument (MP Biomedicals). Remaining DNA was depleted with a DNAse kit and RNA pellet was precipitated with ethanol. All samples were tested for DNA remnants before transcription. RNA was transcribed to cDNA using SuperScript® III (Invitrogen) and the random hexamers from the kit were used. The resulting cDNA was used to prepare the libraries targeting the V1-V2 region of the 16S rRNA gene following the protocol of Kaewtapee *et al*. (2017). Libraries were standardized and purified using SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) and sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Bioinformatic and statistical analysis

Raw reads were quality filtered, assembled, and aligned using mothur pipeline (Kozich *et al*. 2013) [15]. Sequences were excluded if they have any primer or barcode mismatch and an N character. A total of 24269 ± 13564 reads were obtained per sample . Reads were aligned to the Silva database version 132 and clustered at 97% identity into Operational Taxonomic Units (OTUs). Chimeric reads were identified and removed with UCHIME. Sequences from chloroplasts, mitochondria, archaea and eukaryotes were removed. The taxonomy assignation was done using the cut-off limits for bacterial taxonomy as described by Yarza et. al, 2014 [16].

Non metric multidimensional scaling (NMDs) were performed using Primer-7 after calculating the Bray-Curtis dissimilarity. Alpha diversity indices (Shannon, Species Evenness) were calculated using Primer-7. For the multiple comparisons between samples at the different taxonomical levels, t-test and Tuckey HST was used to find significantly different means using the JMP software.

4.4 Results

In this chapter, we analyzed the active fraction of the microbial community of the oral and intestinal sites from the C57BL/6 mouse at different ages (2, 9, 15, 24 and 30 months). The beta diversity was calculated using Bray–Curtis dissimilarity based on the taxonomic abundance profiles (Figure 16). PERMANOVA analysis showed statistical significance for body site (p-value 0.0001) and the combination of the factors age and body site (p-value 0.05) (Figure 16A). A statistical trend was observed for the factor age (p-value 0.06).

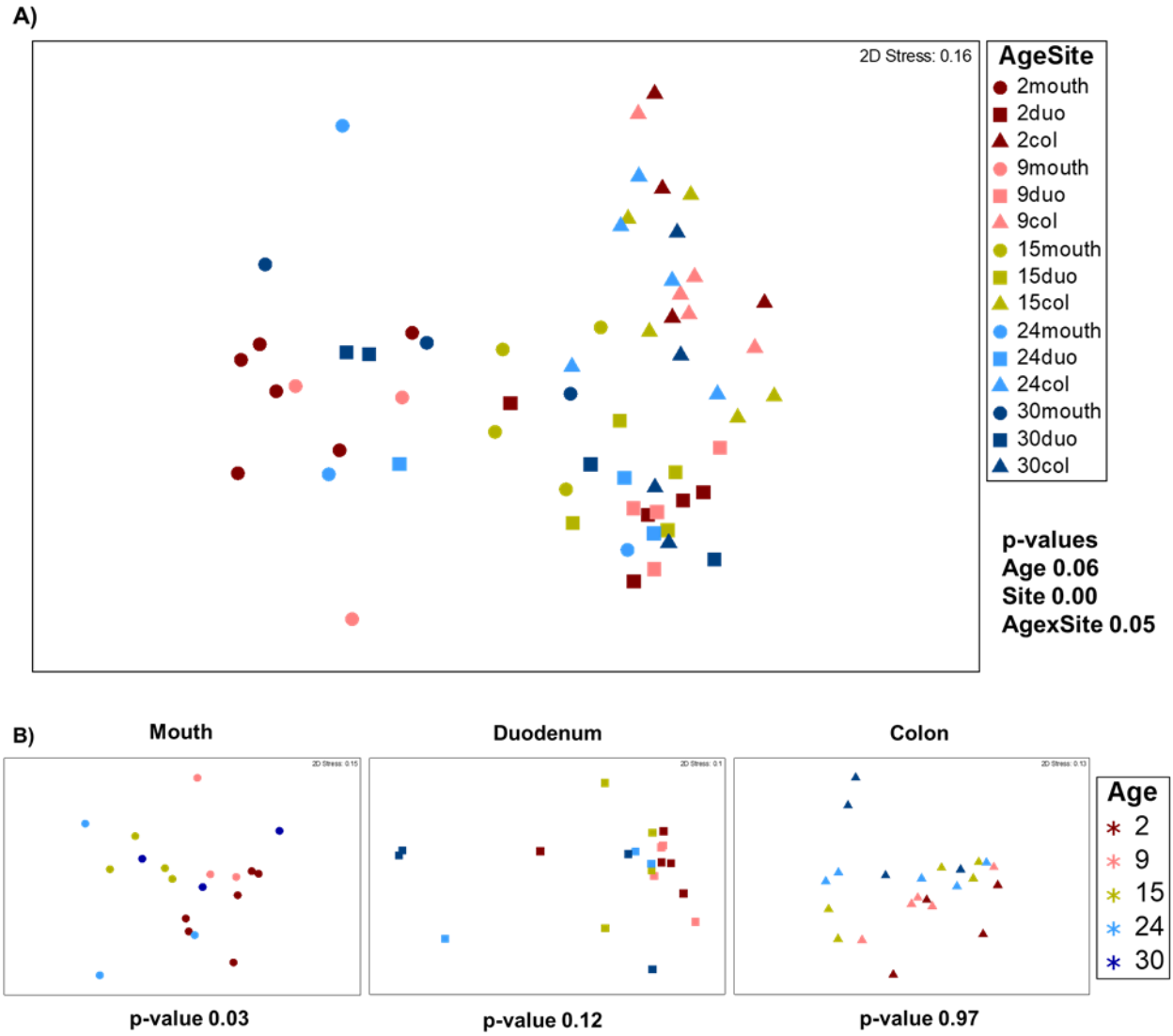

Figure 14 *Non metric multidimensional scaling (nMDS) ordination of the active microbial communities.*

Showing the A) overall community distribution and B) the community distribution from the murine mouth, colon and duodenum samples at age 2, 9,15, 24 and 30 months. Symbols present sites and colors age.

We could not identify statistically significant differences when we compared diversity measurements of Species Evenness and the Shannon's diversity within groups of samples from the same age or samples from the same sites at different ages (Figure 17). Probably due to the high variability between samples of the same group. However, we observed an increased variability in colonic samples Shannon's diversity and the Species Evenness. The Species Evenness considers the proportions for each of the OTUs present in the samples and the Shannon's diversity considers richness and the evenness. Both of these indexes showed that there was more variability between the samples of the colon, than the mouth or duodenum

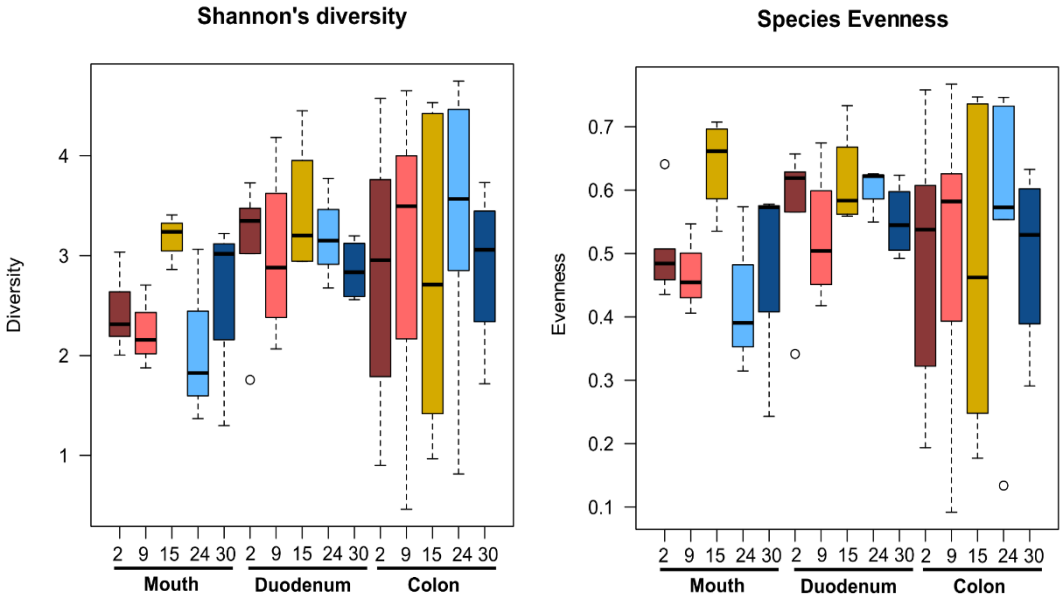

Figure 15 *Boxplot showing Shannon's diversity and Species Evenness from the samples.*

Age is represented in months.

Firmicutes, Proteobacteria, Bacteroides, Actinobacteria, Spirochaetes, Deferribacteres and Verrucomicrobia were present in all the samples being considered the core microbiota (Figure 18). At 2 months the mouth average abundance of

Actinobacteria was statistically higher compared to the abundances present in the duodenum and the colon. A similar effect was not observed at different ages either due to the higher variability between samples or to the higher similarity between sites.

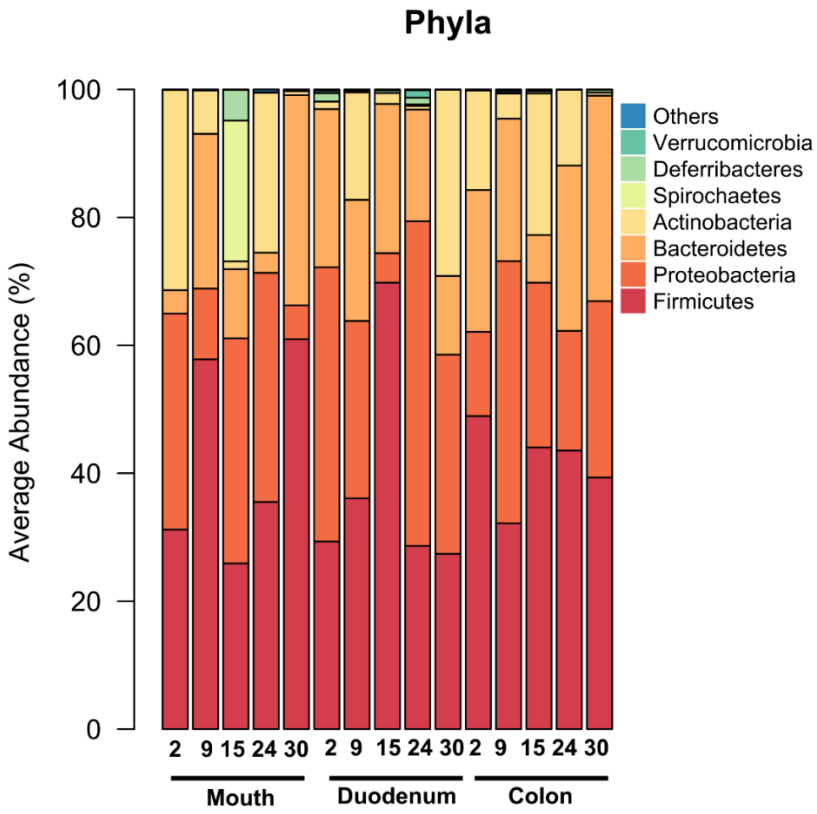

Figure 16 *Principal phyla present in the different intestinal ages and sites. Age is represented in months.*

Bacteroidetes abundance was statistically different between the duodenum and the mouth at 2 months of age. At 9 months of age, Bacteroidetes also showed statistical differences between the mouth compared to the colon (p-value 0.03) and the duodenum (p-value 0.01). However, the colon and the duodenum had no differences related to this phylum at this age. The active community of Bacteroidetes showed

significant differences in samples from colon at 2 and 30 months (p-value 0.04) of age. Changes in Bacteroidetes abundance throughout the lifespan were observed mostly in the mouth. Mouth samples from 15 months old mice showed a shift in Bacteroidetes being statistically different from the younger ages of 2 and 9 months (p-value 0.01).

Firmicutes showed statistical differences when comparing the mouth and the duodenum at both 2 (p-value 0.03) and 15 (p-value 0.00) months of age. As well, a statistical significance was identified in the comparison of colon and duodenum at both 9 (p-value 0.04) and 15 (p-value 0.00) months of age. Within the same site, Firmicutes showed a trend in the duodenal samples when comparing the ages of 15 and 30 months (p-value 0.07).

At the genus level, 49 genera were detected with an average abundance higher than 1%. The most abundant genera were *Lactobacillus*, *Helicobacter*, *Propionibacterium* and *Streptococcus* (Figure 19).

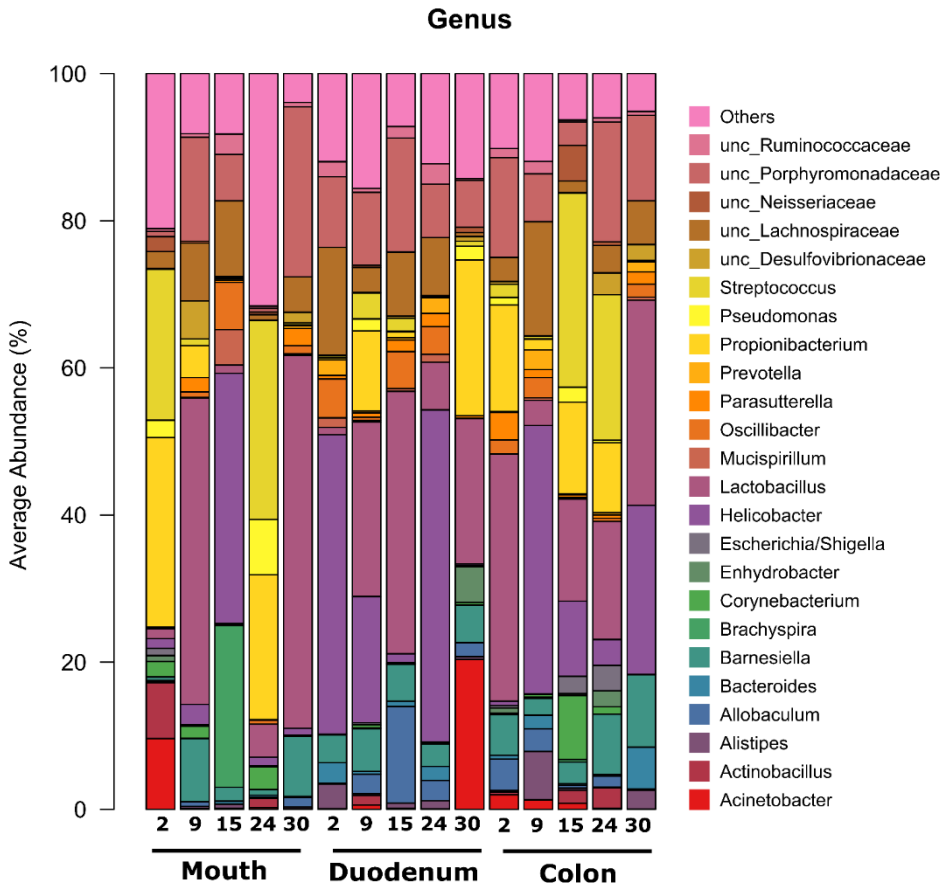

Figure 17 *Top 25 genera present in the different intestinal ages and sites. Age is represented in months.*

In the mouth, we observed shifts from age 2 to 15 months in *Barnesiella*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Oscillibacter*, *Parasutterella* and *Staphylococcus* (Table 6). In the oral cavity of the 2 months old mice, we observed higher abundances of *Corynebacterium* when compared to the 24 months old mice, lower abundances of *Micrococcus* when compared to the 30 months old mice (Table 6) and shifts in *Ralstonia* were identified when compared with the 24 (p-value 0.05) and 30 months old mice (p-value 0.01). These changes were not detected in other ages. For example, the mouth active community of 9 months old mice, did not show any differences at genus level in comparison to the 24 months old mice. When we

compared the 9 months old mice with the 30 months old, the *Clostridium IV* was the only genus showing a statistically significant difference (p-value 0.00).

In the duodenum, differences at genus level related to age were not frequently found. The duodenal samples from mice of younger ages, at 2 and 9 months, had higher abundances of *Acetatifactor* when compared with 30 months old mice (p-value 0.04). In the murine duodenum at 2 months old, the genus *Clostridium XlVa* tends to be higher compared to 30 months old mice (p-value 0.06). At 15 months *Ruminococcus* was more abundant than at 2 (p-value 0.07) and 30 (p-value 0.08) months old mice.

Table 6 *Average abundance in percentage (%) and standard error of the mean (SEM) from the most abundant genera found in the samples.*

Age	2 months			9 months			15 months			24 months			30 months		
Site	Mouth	Duo	Colon	Mouth	Duo	Colon	Mouth	Duo	Colon	Mouth	Duo	Colon	Mouth	Duo	Colon
Acetatifactor	0.4 ± 0.4	0.1 ± 0.0	0.7 ± 0.6	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
Acinetobacter	9.6 ± 8.7	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	20.4 ± 20.2	2.0 ± 2.0	1.6 ± 1.2	0.8 ± 0.7	0.2 ± 0.1	0.0 ± 0.0
Actinobacillus	7.6 ± 3.8	0.1 ± 0.1	0.0 ± 0.0	1.3 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	1.3 ± 0.5	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.1 ± 0.1	1.8 ± 0.8	2.8 ± 2.3	0.0 ± 0.0
Actinomyces	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 4.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Akkermansia	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	1.2 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Alistipes	0.2 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	3.4 ± 1.6	0.2 ± 0.2	0.7 ± 0.4	1.1 ± 0.6	0.4 ± 0.3	0.2 ± 0.2	4.6 ± 2.9	0.3 ± 0.2	0.1 ± 0.0	2.6 ± 1.0
Allobaculum	0.1 ± 0.1	0.6 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	1.3 ± 0.9	0.1 ± 0.1	2.7 ± 1.2	13.1 ± 10.1	2.8 ± 1.5	1.9 ± 1.8	4.3 ± 1.3	2.2 ± 1.5	0.4 ± 0.3	1.5 ± 1.4	0.2 ± 0.1
Alloprevotella	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	1.0 ± 0.5	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
Bacteroides	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.3 ± 0.3	0.2 ± 0.1	2.8 ± 1.2	0.4 ± 0.3	0.7 ± 0.5	1.9 ± 0.9	0.0 ± 0.0	0.5 ± 0.4	1.8 ± 0.6	0.2 ± 0.1	0.2 ± 0.1	5.7 ± 4.2
Barnesiella	0.5 ± 0.1	8.6 ± 2.2	1.9 ± 1.0	0.8 ± 0.7	8.2 ± 0.8	3.8 ± 1.2	5.8 ± 1.1	5.0 ± 2.0	3.1 ± 1.1	5.1 ± 4.3	5.6 ± 3.2	1.9 ± 0.6	2.9 ± 2.3	8.2 ± 4.5	9.9 ± 3.3
Brachyspira	0.0 ± 0.0	0.1 ± 0.1	22.0 ± 12.7	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.1	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
Clostridium IV	0.3 ± 0.2	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.3 ± 0.1	1.0 ± 0.4	0.0 ± 0.0	0.3 ± 0.2	0.4 ± 0.3	0.0 ± 0.0	0.5 ± 0.3	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Clostridium XlVa	0.6 ± 0.6	1.0 ± 0.4	1.3 ± 1.0	0.1 ± 0.0	0.3 ± 0.2	1.3 ± 0.5	0.2 ± 0.1	0.9 ± 0.7	1.1 ± 0.4	0.0 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Corynebacterium	2.1 ± 0.6	1.6 ± 1.5	0.2 ± 0.2	3.1 ± 2.1	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.3	0.1 ± 0.1	0.4 ± 0.2	8.7 ± 7.3	1.0 ± 0.6	0.1 ± 0.0
Eisenbergiella	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.2
Enhydrobacter	0.8 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.9 ± 4.8	0.7 ± 0.7	0.0 ± 0.0	0.2 ± 0.2	2.2 ± 2.1	0.0 ± 0.0
Escherichia/ Shigella	1.0 ± 0.5	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.3	0.1 ± 0.1	2.4 ± 1.4	3.4 ± 2.7	0.0 ± 0.0
Haemophilus	2.4 ± 1.6	0.1 ± 0.1	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	0.8 ± 0.6	0.0 ± 0.0
Helicobacter	1.3 ± 1.3	2.8 ± 2.4	34.0 ± 9.7	1.2 ± 1.0	0.9 ± 0.5	40.7 ± 14.4	17.2 ± 10.7	1.2 ± 0.6	45.1 ± 17.8	0.2 ± 0.1	0.6 ± 0.2	44.9 ± 15.9	10.2 ± 5.5	3.5 ± 1.1	23.0 ± 17.1
Lactobacillus	1.3 ± 0.6	41.7 ± 11.3	1.1 ± 0.6	4.5 ± 1.6	50.7 ± 15.5	1.0 ± 0.4	23.7 ± 8.4	35.7 ± 9.4	6.5 ± 2.7	19.7 ± 17.0	33.5 ± 12.8	3.2 ± 0.6	13.9 ± 11.4	16.1 ± 11.1	27.9 ± 14.2
Methylobacterium	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.1 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.4 ± 0.4	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Micrococcus	0.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.8	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Mucispirillum	0.0 ± 0.0	0.1 ± 0.0	4.8 ± 2.3	0.0 ± 0.0	0.2 ± 0.1	1.3 ± 0.8	0.2 ± 0.1	0.4 ± 0.3	1.1 ± 0.8	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.4 ± 0.3
Neisseria	0.9 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.7	0.1 ± 0.1	0.0 ± 0.0	2.7 ± 2.1	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Olsenella	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.6 ± 0.6	0.5 ± 0.3	0.7 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Oscillibacter	0.2 ± 0.1	0.8 ± 0.2	6.4 ± 4.6	0.5 ± 0.3	1.1 ± 0.7	5.3 ± 1.5	0.5 ± 0.1	5.0 ± 2.9	3.8 ± 1.7	0.0 ± 0.0	1.9 ± 1.1	2.5 ± 0.6	0.1 ± 0.1	0.4 ± 0.1	1.8 ± 1.0
Parabacteroides	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	1.4 ± 0.6	0.1 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	1.1 ± 0.3	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.2
Parasutterella	0.0 ± 0.0	2.0 ± 1.0	0.3 ± 0.1	0.0 ± 0.0	2.3 ± 2.0	0.5 ± 0.2	0.6 ± 0.1	1.6 ± 0.8	1.8 ± 0.8	0.3 ± 0.3	3.7 ± 1.2	0.8 ± 0.5	0.3 ± 0.2	0.5 ± 0.3	1.7 ± 0.4
Pedobacter	1.7 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.4 ± 1.4	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Prevotella	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.3	2.1 ± 0.9	0.3 ± 0.2	0.3 ± 0.2	2.2 ± 1.2	0.0 ± 0.0	0.1 ± 0.1	2.1 ± 1.0	0.2 ± 0.1	0.3 ± 0.2	1.4 ± 0.7
Propionibacterium	25.8 ± 7.3	4.3 ± 4.1	0.1 ± 0.0	19.7 ± 9.0	0.1 ± 0.1	0.3 ± 0.1	10.9 ± 5.0	0.8 ± 0.6	0.1 ± 0.0	21.2 ± 20.0	14.5 ± 14.3	1.7 ± 0.9	12.5 ± 8.2	9.5 ± 5.3	0.0 ± 0.0
Pseudomonas	2.4 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	7.5 ± 5.2	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.8	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 1.8	1.0 ± 1.0	0.1 ± 0.0	2.0 ± 1.7	0.4 ± 0.3	0.0 ± 0.0
Ralstonia	0.4 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.6 ± 0.6	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Ruminococcus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
Ruminococcus2	0.0 ± 0.0	0.9 ± 0.5	0.3 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	2.9 ± 2.9	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
Sphingomonas	1.1 ± 0.5	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.2	0.0 ± 0.0
Staphylococcus	1.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.3	0.0 ± 0.0	0.9 ± 0.9	0.8 ± 0.5	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0
Streptococcus	20.5 ± 9.5	0.9 ± 0.6	0.2 ± 0.1	27.0 ± 12.3	0.3 ± 0.1	0.3 ± 0.1	3.5 ± 1.3	1.8 ± 1.6	0.1 ± 0.1	0.7 ± 0.2	1.8 ± 1.2	0.4 ± 0.2	26.4 ± 19.1	19.8 ± 9.6	0.2 ± 0.1
o_Actinomycetales	1.1 ± 0.7	0.3 ± 0.3	0.1 ± 0.1	0.7 ± 0.5	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0
o_Bacteroidales	0.0 ± 0.0	0.8 ± 0.4	0.5 ± 0.2	0.0 ± 0.0	0.6 ± 0.3	0.4 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2
f_Bdellovibrionaceae	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.6	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
c_Clostridia	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.5 ± 0.4	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
o_Clostridiales	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	0.9 ± 0.5
f_Desulfovibrionaceae	0.1 ± 0.0	5.2 ± 2.5	0.1 ± 0.0	0.0 ± 0.0	1.4 ± 1.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.6 ± 0.6	0.4 ± 0.3	0.0 ± 0.0	0.1 ± 0.0	3.0 ± 2.1	2.2 ± 2.0
f_Erysipelotrichaceae	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.7 ± 0.4	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.2
f_Lachnospiraceae	2.3 ± 0.7	7.9 ± 3.2	10.3 ± 5.8	0.7 ± 0.1	4.8 ± 3.6	14.7 ± 4.2	3.4 ± 0.8	8.7 ± 5.0	7.9 ± 3.2	0.6 ± 0.3	3.3 ± 0.8	15.3 ± 5.0	1.6 ± 0.7	3.7 ± 2.2	6.0 ± 2.6
f_Neisseriaceae	2.0 ± 1.0	0.2 ± 0.2	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	4.8 ± 4.6	0.5 ± 0.3	0.0 ± 0.0
f_Porphyromonadaceae	0.7 ± 0.3	14.2 ± 4.7	6.3 ± 3.3	0.5 ± 0.1	23.1 ± 6.1	9.6 ± 2.9	9.9 ± 3.0	15.5 ± 6.3	7.3 ± 2.2	6.4 ± 5.9	13.6 ± 5.0	4.7 ± 2.3	3.2 ± 2.5	16.3 ± 13.3	11.6 ± 3.5
f_Ruminococcaceae	0.4 ± 0.1	0.5 ± 0.1	2.8 ± 1.8	0.3 ± 0.1	0.6 ± 0.3	2.1 ± 0.6	0.5 ± 0.1	1.6 ± 0.9	2.8 ± 1.3	0.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.5	0.3 ± 0.1	0.5 ± 0.2	0.6 ± 0.2
Vampirovibrio	0.9 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 1.6	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0

Lactobacillus was present in higher abundances in the duodenal samples (Figure 20). Statistical differences were observed when compared to the colon in the mice at ages 2 (p-value 0.02), 9 (p-value 0.05) and 15 months (p-value 0.05). While the mouth presented differences with the duodenum at 2 (p-value 0.02) and showed a trend at 9 months of age (p-value 0.06). At older ages, 24 and 30 months, *Lactobacillus* did not show statistical differences when comparing mouth, duodenum and colon at the same age. In the colon, *Lactobacillus* was detected in higher abundance at 24 months compared to the younger ages of 2 and 9 months old (p-value 0.05). In the colon, the genus *Lactobacillus* was statistically higher at 24 months of age than at 2 (p-value 0.03) and 9 (av. 0.9 %, p-value 0.01) months of age.

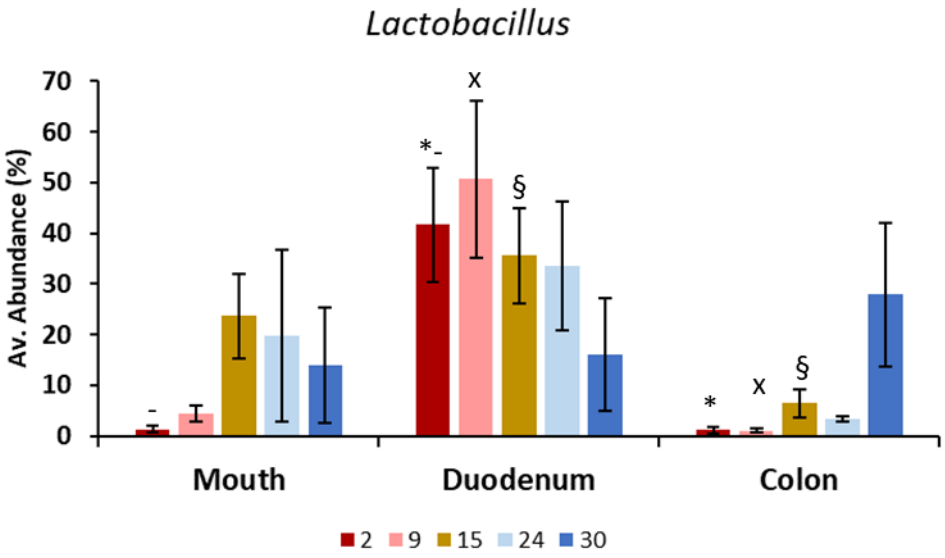

Figure 18 *Average abundances of Lactobacillus by site.*

Ages are represented by number of months in different colors. Same symbols above the graphs represent statistical significance between those groups.

Barnesiella showed statistical differences between sites in the 2, 9 and 15 months (Figure 21). At 2 months of age was more abundant in the duodenum in comparison to the mouth and the colon (p-value <0.05). In the oral cavity, age related differences were observed at 15 months which showed shifts when compared with 2 (p-value 0.02) and 9 months (p-value 0.01).

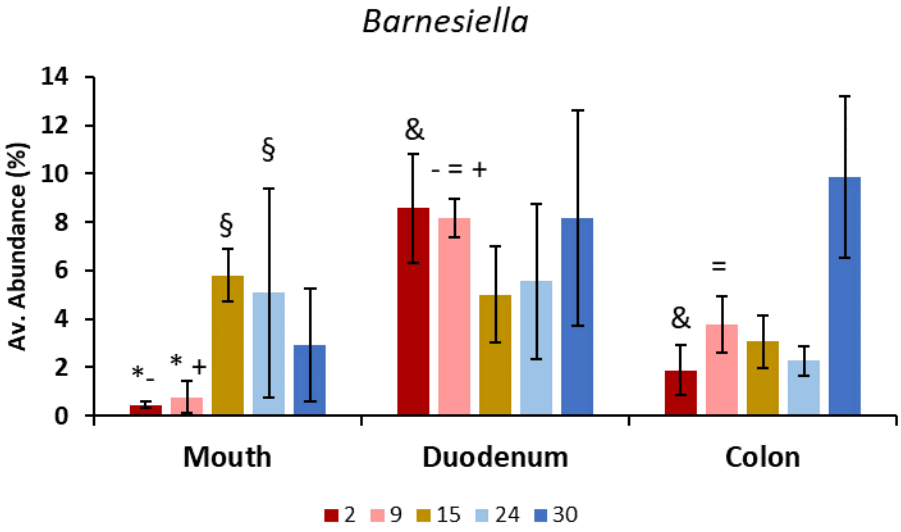

Figure 19 *Average abundances of Barnesiella by site.*

Ages are represented by number of months in different colors. Same symbols above the graphs represent statistical significance between those groups.

The colonic samples were highly dominated by species of the genus *Helicobacter*. At 2 and 9 months old mice *Helicobacter* was statistically higher in the colon when compared to the duodenum and the mouth (Figure 22). These differences were also observed at 15 months of age between the colon and the duodenum. At 15 (p-value 0.08) and 24 (p-value 0.09) months old *Helicobacter* was higher in colon compared to duodenum

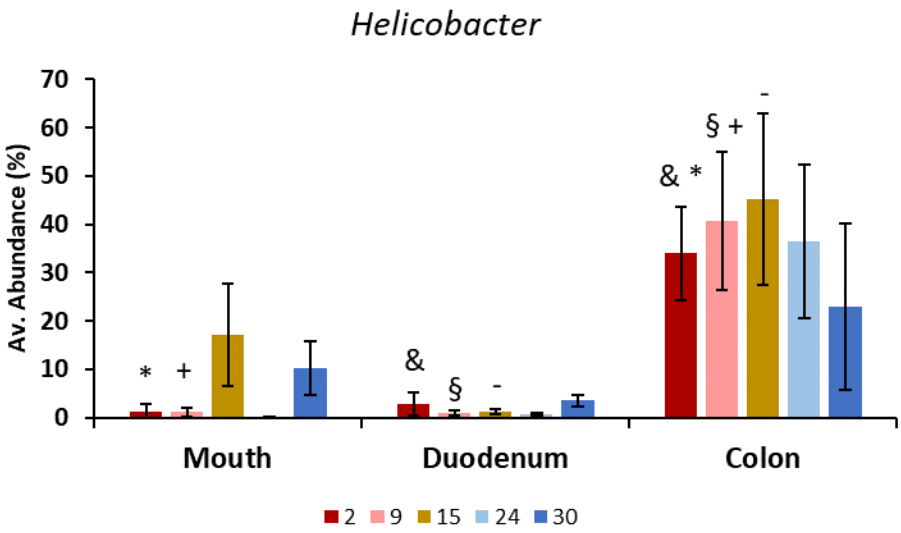

Figure 20 *Average abundances of Helicobacter by site.*

Ages are represented by number of months in different colors. Same symbols above the graphs represent statistical significance between those groups.

We observed some age related changes in genera that were present in low abundances in the colon. The similarities between the samples calculated by the SIMPER analysis (Table 7), showed a specific pattern. In the younger groups at 2 and 9 months of age, duodenal samples had the highest similarity between replicates followed by the colon and the mouth. At 15 months the pattern started to change and the mouth replicates presented the highest similarity (34%) and colon and duodenum had both 32%. The average similarity of the mouth at 24 months of age dropped considerably to 4.7% and was the lowest similarity. While at 30 months of age in the mouth, duodenum and colon the similarities ranged between 20% and 26% being lower compared to the other groups.

Table 7 *SIMPER analysis percentages showing similarities between samples in the same group and groups of the same age.*

			Dissimilarity	
		Similarity	**Duodenum**	**Colon**
2 months	**Mouth**	31.3 %	92.5 %	96.9 %
	Duodenum	39.7 %	-	87.1 %
	Colon	34.8 %	-	-
9 months	**Mouth**	29.4 %	89.8 %	95.9 %
	Duodenum	46.1 %	-	82.6 %
	Colon	41.7 %	-	-
15 months	**Mouth**	34.8 %	70.8 %	75.4 %
	Duodenum	32.4 %	-	79.9 %
	Colon	32.3 %	-	-
24 months	**Mouth**	4.76 %	77.2 %	91.8 %
	Duodenum	28.4 %	-	84 %
	Colon	35.6 %	-	-
30 months	**Mouth**	20.1 %	70.6 %	81.5 %
	Duodenum	22 %	-	81.7 %
	Colon	26.1 %	-	-

Some of the OTUs could be assigned to a species using the NCBI blast tool. Several species were responsible for the separation between the groups. Most of the differences observed were driven by the abundances of *Helicobacter hepaticus*, *Streptococcus danieliae* and *Propionibacterium acnes* (Figure 23).

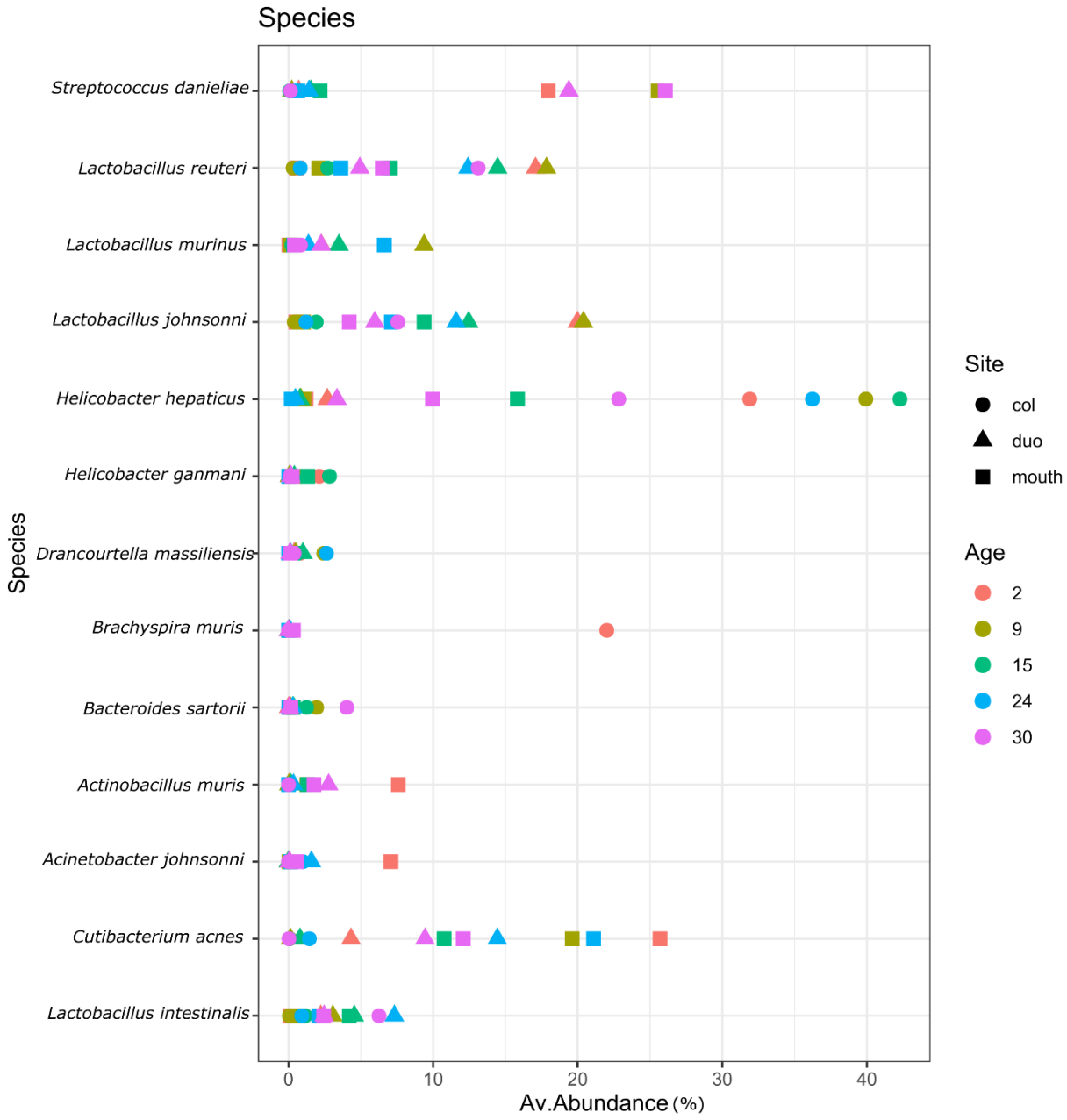

Figure 21 *Average abundances at species level by age and site.*

The average abundance is represented in percentage.

Helicobacter hepaticus was the most abundant species identified in the samples. It was predominant in the colon but showed an increase in the oral samples at age 15 (av.

15.8%) and 30 (av. 9.9%). We observed statistical differences in the *Helicobacter hepaticus* species abundance between the colon and the duodenum at the ages of 9 months (av. 39.9%, p-value 0.01), 15 months (av. 42.2 %, p-value 0.00) and 24 months (av. 32.2% p-value 0.02). We observed also a significant trend in the comparison between the colon of 2 months old mice (av. 31.8%, p-value 0.06) and the duodenum at the same age (av. 2.6%). However, at 30 months old, we did not observe the same behavior (p-value 0.21).

Two species of *Lactobacillus*, *L. johnsonni* and *L. reuteri* showed higher abundances in the duodenal samples in the early ages of 2 and 9 months old. *L. johnsonni* was statistically different in the duodenum of ages 2 and 30 (p-value 0.01) and 9 and 30 (p-value 0.01). *L. reuteri* was statistically different in the duodenum when comparing the ages 9 and 30 and in the colon of the mice at the age 30 months when compared with the young mice of 2 months (p-value 0.02) and 9 months old (p-value 0.02). The abundances of these species decreased with age in the duodenum and were lower in the other segments of the GIT.

Cutibacterium acnes, a common colonizer of the skin microbiota had higher abundances in the mouth samples, probably related to the grooming habits of the mouse. In the oral cavity, the ages 15 (av. 19.7%) and 30 (av. 12.9%) months had lower abundances of this species. In the duodenum they were detected in higher abundances at the older ages of 24 (av. 14.4%) and 30 (av. 9.4%) months of age. Some of the identified species in Figure 6 had abundances lower than 10% and statistical differences were not always observed.

4.5 Discussion

In this study, we identified the global and specific changes of the active microbial community from different segments in the murine GIT at different ages of the lifespan. Our findings indicate that the aging process per se, without dietary and lifestyle changes, influence the oral, duodenal and colonic composition of the active microbial communities.

Several health problems have been linked to aging. Even though aging is not a disease, it eases inflammation that might trigger different chronic diseases [17]. An increment of the biological age is related to frailty and changes in the composition of the microbiome such as dysbiosis and loss of the normal gut microbiota function [18]. Changes in the microbiota composition could result in the low grade inflammation called “inflamm-aging” [17]. Some of the compounds responsible for this inflammatory effect are lipopolysaccharides. Lipopolysaccharides are present in the wall of gram-negative bacteria and due to the increase of the intestinal permeability, they reach the systemic circulation and could accelerate inflamm-aging [19].

Murine models are broadly used to study mammalian biology in health and disease. Mice allow us to understand the fundamental processes involved in aging and the relationship between aging and disease [20]. Often, models that research age-related diseases lack an aging component. Murine models allow adding an aging component due to the shorter lifespan compared to humans [20]. Lately, the microbiome has been highlighted as an important piece in health maintenance and disease progression [21]. Further analysis of how the active microbial communities change during aging could eventually lead to new therapies to postpone age-related diseases[10].

We examined the overall composition of the microbiome using beta-diversity analysis and confirmed previously reported observations that the microbiota composition changes correlate with the age of the host [9]. Changes were observed in each GIT niche that we analyzed. We used two calculations of alpha-diversity. The evenness that identifies changes in microbial dominance or abundance equitability and the richness which calculates the introduction or loss of whole species in the GI microbial community [22]. The diversity indexes showed no statistical significance between the age groups, maybe due to the inter-individual variability. We observed lower diversity measurements in the colon from mice at 30 months of age. In humans, the loss of diversity is one important change in the elderly population and comes together with lower counts of bacteroides, bifidobacteria and lactobacilli [10].

The results from the SIMPER analysis indicated that there is higher variability between samples in older mice compared to younger mice. In previous research, the analysis of the microbiome from the fecal microbiota of the elderly people showed temporal stability and was characterized by extreme variability [23]. In our samples, the increase of this variability starts at 15 months of age and continues in the older ages. The differences within the sampling sites were mostly found in the young groups of mice, while at older ages the genera differences between niches were less frequent. The changes in the microbiome related to aging start in the mouth. We observed that the oral and duodenal niches became more alike as the host ages.

The most significant changes started at 15 months of age. At this age, mice start developing the first senescence changes. This is the start of the upper ages and correlates to humans from 38 - 47 years old [24]. At this age, in our samples, the bacterial

composition of the duodenum and the colon became more similar. Bacterial translocation has been observed from oral environments to gut microbiota [25]. Long-term ingestion of oral pathogens as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* could promote intestinal dysbiosis in susceptible hosts [26]. This bacterial translocation could also happen from duodenum to colon. However, to our knowledge bacterial translocation from the colon to duodenum and vice versa has not been studied.

In this study, we observed that aging was linked with a change in the abundance of the genus *Lactobacillus*, where its average abundances were higher in the duodenum of the youngest groups when compared to the colon. The oldest mice increased the abundance of *Lactobacillus* in the colon and decrease it in the duodenum. Species from this genus among others, have demonstrated to downregulate the pro-inflammatory response at the gut epithelium [27]. Previous research reported lower abundances of *Lactobacillus* species colonizing older mice compared to the young counterpart [28]. Supplementing with probiotic strains of *Lactobacillus* have improved the production of metabolites in aged mice [29].

At the murine age of 24 to 30 months the survival rate is very low and could be comparable to human centenarians [20]. Therefore, the presence of beneficial bacteria could be the reason that the surviving mice were resilient to death.

Pathobionts are bacteria, such as members of the *Helicobacter* genus, present in low abundances in the heathy gut [30]. Several situations, such as a weak immune system or inflammation, that are common in elderly individuals, can result in an overgrowth of these type of bacteria [27]. Therefore, it is common that they cause infection in older hosts.

Helicobacter hepaticus is a common colonizer of the murine low intestine [31]. It does not induce disease in a health status. However, in immune deficiencies, like the lack of IL-10 or its receptor, it can cause intestinal inflammation [32]. In older individuals, an impairment of the immune system is common. Mouse colonized naturally with *H. hepaticus*, had higher sensitivity to tuberculosis [33]. Indicating that the overgrowth of certain pathobionts could also cause an increased incidence of other pathologies [33].

In conclusion, aging is reflected in the active microbial community of the mouth, duodenum and colon. The age-related microbial fluctuations were observed from the phylum to the species level. Further research is required to develop potential personalized strategies to preserve the health status in the aging population by targeting oral, duodenal and colonic microbiome.

4.6 References

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CHAPTER V

5 Discussion

The development of new sequencing technologies and bioinformatic analysis have expanded our knowledge of the microbiome [1]. As culturomics made a comeback and several approaches broaden our understanding of the niche-associated microbiota and functionality of microbes in the human body (Figure 24) [2].

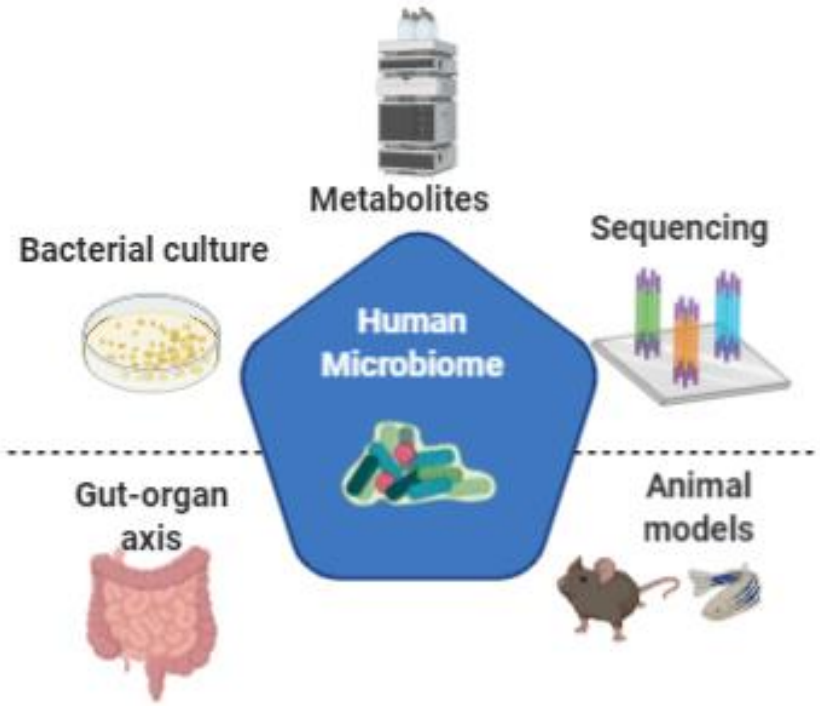

Figure 22 *Schematic representation of the current research approaches for the analysis of the microbiome.*

The microbial ecology of the different body niches is linked to health and disease by the different body organs [3]. The microbiota plays a critical role in health from the beginning in life to the late adulthood [4]. Until this point, there has been a great progress related to

the knowledge of the composition and functionality of the healthy microbiome. However, it is still not clear how throughout the different stages of life, the microbiome switches from sterility in the womb into a disease-prone microbial community [5]. Several non-communicable diseases appear more frequently during late adulthood [6]. As mammals are complex organisms, there is a strong connection between the gut microbes and the different body organs [7]. Recently, the influence of the microbiota in liver diseases opened up a new link between the gut bacteria, the bile acids and the diverse metabolites [8]. Deciphering the changes of the microbiome under different conditions will allow us to design interventions to modulate the microbial communities of the GIT [9–11]. Diet is one of the key factors that modulates the microbiota [12]. However, small changes as the use of supplements have shown to influence the composition of the microbiome [13].

5.1 The importance of oral microbiome research -challenges and approaches

Previous research has implied a close relationship between alterations of the oral microbiota and diseases [14]. The human microbiome project has a list of protocols to sample the oral micro-habitats [15]. These procedures are easier to do in humans as the area surface is bigger with different microbial ecologies in the mouth. Different sampling procedures result in a different microbial community. However, in mice these protocols are difficult or impossible to replicate due to the smaller surface. Experiments in past publications sampled the oral area from mice using dissected maxilla and mandible, others used oral swabs from teeth and gingiva and some used pieces of tongue, buccal mucosa, saliva [16–18] and there was no consensus in how to do it.

In chapter 2, we evaluated two different procedures of sampling the oral cavity from mice in two different ages. Our findings indicated that the different type of sampling induce

differences in the composition of the microbiota. As we analyzed samples from two different ages we observed that aging influenced the composition of the oral microbiome.

These results highlighted that mice also have a niche- dependent oral microbiome and sampling procedures can affect the results of the microbial composition. Therefore, it is crucial that the differences induced by sampling methodologies are taken in consideration when comparing different publications to improve the reproducibility of the results [19].

To our knowledge only a preceding publication from 1989 assessed different types of oral sampling in a murine model using the strain BALB/c. However, to identify the bacterial communities, they used methodologies available at that time, such as direct immunofluorescent technique for bacterial detection and bacterial cultures [20]. The results of this publication are consistent with ours as the swab samples showed to be representative to mucosa, teeth and tongue. One limitation of this publication was the lack of NGS approaches at the time.

The importance of the oral microbiome lays in its clinical potential, as it has already been shown to be a promising diagnostic tool in identifying systemic diseases [21, 22]. In addition to comparability between studies, methodological standardization is as well linked to an increased reproducibility of results [23].

5.2 Aging and the microbiota

The microbial ecology of the gut is never static [24]. Life and environmental factors influence the composition of the microbial communities during the whole lifespan [4]. The microbial ecosystems adapt to the external and internal stimuluses. Dietary patterns, physical activity, hygiene, genetics, diseases, antibiotics, etc… are external factors that modulate the microbiome [25]. Some of these external modulation over time can result in

a negative outcome, like a dysbiosis [26]. Previous evidence showed that shifts in the gut microbiota can also affect the intestinal permeability [27]. An increment in the intestinal permeability is linked to higher inflammation. Several factors can produce a proinflammatory gut environment which also lead to an increased intestinal permeability and this rises the susceptibility of pathobionts to invade the different niches. Inflammation *per se* can also shift gut microbial composition and as previously described aged individuals frequently present low grade inflammation [27]. This makes clear that here is a bidirectional effect of inflammation and the microbiome and it is closely related to aging [28].

Some of the functional and molecular alterations that decrease with age can be detected relatively early in middle-age [45]. Other age related changes may not be present until very late in life in a large percentage of the population [46]. Therefore, it is important to research individuals/animals at different time points because age-associated changes at molecular level can occur also in middle-age individuals. Murine models are a great tool to allow us to understand how the microbial communities become established within their hosts, how their members maintain stable ecological niches and how the dynamics between microbes and host relate to health and disease.

In humans, centenarians are the model of healthy aging as they delayed or avoided chronic diseases. In centenarians from Italy and China, Escherichia/Shigella was increased in both cohorts [29]. An increment of the abundance of Proteobacteria in the gut during aging is commonly found in typical elderly and centenarians. This is in line to our findings as we observed more species belonging to Proteobacteria in the colon from

older mice (Chapter 3 and 4). Faecalibacterium had lower abundances in the long-living people compared to the younger group in both the Chinese and Italian cohorts [29].

In a study assessing the fecal microbiome from mice at different ages and stages of frailty, *Alistipes* were more abundant within the old and middle mouse groups [30]. In our samples (Chapter 4), we observed higher average abundance of *Alistipes* in the colon of older mice however it did not reach significance.

Previous research in mice and humans identified lower counts of *Lactobacillus* in the elderly [31, 32]. In mice the results are contradictory as Languille *et al*. found no significant decrease in *Lactobacillus* related to age in a murine model [30]. In our samples (Chapter 4), we observed that the active microbial portion of *Lactobacillus* increased in the colon with age and decreased in the duodenum of the older mice.

By the time the adult reaches an old age, the microbiome is less diverse and beneficial bacteria are present in lower abundances [4]. Opportunistic bacterial populations are present in higher abundances and due to the lower immune system there can be higher infection rates [33]. In chapter 2 and 4, we observed that older mouse had higher abundances of bacterial species belonging to the *Helicobacter* genus. These findings demonstrated that opportunistic bacteria can overgrow in intestinal environments of hosts from older ages.

In previous and actual research, diseases that prevail with natural human aging are commonly induced in young or developing mice. These models help researchers to understand the biology of disease, but it does not reflect what occur through the natural path of aging when a decrement in immune and physiological functions is common. Our

projects regarding the development of the microbiome from mice at different ages in chapter 2 and 4 could identify the changes in the oral, duodenal and colonic microbial composition due to the aging process without inducing any disease. Understanding the changes in the composition and functionality through aging can be a first step into understanding how the risk of different diseases increase through the lifespan of the host [34].

One limitation of research related to the aging of the microbiome, is a lack of consensus in the methodological approaches of the murine microbiome studies. There is also no agreement of which genetic background of the mice is necessary to study aging [34]. Due to its widespread use in biomedical research the most common strain used for aging studies is C57BL/6 [35, 36]. While no murine strain is completely adequate for study aging, one of the limitations is that the genetic backgrounds change the response to disease, diets and other external and internal factors [37].

The comparison between studies using different strains can be challenging as the lifespan and disease progression is different. Still not much information is available about how the genetic material in the murine models could influence the microbial composition and this can be an influencing factor that we are not taking in considerations.

In conclusion there are several gaps in the knowledge of age-associated diseases due to the lack of adequately designed preclinical research on gastrointestinal health in the area of biological aging. Proper guidelines and consensus on the most adequate animal models, age of animals and sampling approach used in such studies could help us understand the microbiome mechanisms of different age-related diseases and develop diagnostic tools using the oral microbiota.

5.3 The oral (GIT) microbiome changes through aging

The present thesis includes two studies where the oral microbial communities were evaluated (chapter 2 and 4). One related to the active microbial community of different niches and the other, as previously described assessing different types of sampling approaches.

The mouth is an immunological barrier between the host and the environment. It is the first contact between the exterior and the gastrointestinal tract. The oral cavity has many different functions from breathing, to food digestion and chewing. These specific traits influence the assembly of the microbial communities and form a bidirectional relationship between the host and its microbes [38]. Higher concentration of oxygen increases the abundance of aerobes and facultative anaerobes. The production of saliva impacts the oral microbiome, as it mobilizes the bacteria from the mouth and promotes the digestion by liberating nutrients from the food matrix that bacteria can utilize. Saliva also promotes bacterial aggregation by releasing mucins and producing antimicrobial compounds [39].

In old adults, xerostomia or dry mouth are related to lower flow and production of saliva affects 30% of the population after 65 years old. This is influenced by the medication used and preexisting conditions related to aging. Low salivary production and flow also difficult masticating and swallowing [34]. These factors could change the microbial composition and decrease the immunological barrier while allowing some pathogenic bacteria to start GIT colonization from the mouth [39]. Microbes present in the oral microbiome are continuously ingested and colonize the distal GIT. In line with research from Iwauchi *et al*., who found more oral bacteria from plaque and tongue coating transitioned and colonized the gut of elderly than younger adults [40].

The components of the healthy oral microbiome in adults and elderly individuals have not been so deeply studied as the colon. Still there is not enough knowledge about the contribution of the oral microbiota to diseases.

In humans, Firmicutes is the predominant phylum of dental plaque in young adults and older groups are mostly colonized by Proteobacteria [40]. Members of the genus *Actinomyces* species, have been found in significantly higher proportions in the oral biofilm of subjects over 60 years of age. The bacterial profiles of orally healthy elderly people are believed to be more diverse than those of young and middle aged adults [41]. Several authors have also observed similar correlations between *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* with age. A. *actinomycetemcomitans* decreased with aging, while *P. gingivalis* increased [41]. Both of this species are known to produce oral diseases.

It is already known that the transmission of pathogenic microbes from the oral cavity to other sites of the intestinal tract can promote disease progression in susceptible hosts [42]. This previous research could explain the progression of *Helicobacter hepaticus* in chapter 4 and how the colonization from the oral, duodenal and colonic niches developed in the older aged mice.

In both sets of oral samples from this research (chapter 2 and 4), we saw that aging influenced the microbial ecology of the mouth. A study targeting elderly population indicated that frailty is related with oral microbiota composition and showed a distinction between individuals living in a nursing home and healthy subjects [43]. In murine models, aging is not a broadly studied subject. However, in a mouse model of elderly mice, the drug rampamicin prevented aging of the oral cavity by regenerating the periodontal bone,

attenuating gingival and periodontal bone inflammation, and shifting the oral microbiome to a composition that had no statistical differences to young mice [44].

Some of the limitations of using murine models for oral microbiome is that the oral physiology and functionality from the mouth and intestine of mice is different. In some cases, diseases frequent to humans are not found in rodents. However, there are techniques which induce these diseases. Some examples of these techniques for oral diseases are gingival sulcus, oral gavage with periodontal pathogens and dextran sulfate sodium treatment (DSS). DDS for instance, induce innate immune damage to the tissues which results in oral mucosal inflammation and alveolar bone loss. Even though, it can mimic the disease it takes away the natural progression of the disease. This can be a limitation also for our research as it means that not all the age-related diseases occur spontaneously in murine models [34].

To our knowledge, there is still not a complete analysis from the oral microbiome of healthy mouse through the lifespan. As most of this research has been focused in oral diseases as periodontal disease, caries, alveolar bone loss, between others. In our studies, we did not only focus on aged vs young mice, but we also studied the microbial composition changes of middle aged mice. This is an advantage as we can see a broader picture of the microbial changes through the murine lifespan.

This evidence showed that the oral microbiome can be a target to prevent oral and systemic diseases. Understanding the composition and functionality of this niche will allow to develop new approaches for medical diagnosis and treatment.

5.4 Microbiome from the small vs large intestine

The gastrointestinal tract is composed by different organs that house diverse microbiomes with different functions. Each region has specific functions and genetic expressions that regulates the digestive and endocrine processes. As described in the introduction, the small intestine is the entry point of bile acids and pancreatic juices. There is as well a constant flow of gastric juice coming from the stomach and it has a quicker passage rate than the colon [45].

The different properties of each segment of the intestine create specific conditions that allow a specific mixture of microorganisms to thrive. Changes in the gut microbiota of the small intestine could have some implications on the host. Previous studies indicated that microbial genes related to carbohydrate and lipid metabolism change on base of the duodenal microbiome of lean and obese individuals [46]. Therefore, the microbiota of the duodenum could be affecting the bioavailability of dietary nutrients to the host [47].

The analysis of the human gut microbiome has mainly focused on fecal samples and previous publications have shown that it does not depict a whole picture of the microbiome from the different intestinal niches [48, 49]. Sampling different segments of the human upper and lower GIT is difficult, as invasive methods are required [19]. Therefore, the information of a heathy human microbiome of the small intestine and different segments from the large intestine is limited. In case of biopsies from patients, there is the potential contamination with microbes from other niches upon retrieval [19]. Future perspectives are the development of new sampling technologies as bio-sampling capsules which will gather luminal content through the GIT [50]. Due to the limitation of getting biopsies, still

there is not enough knowledge of the microbial communities colonizing the small and large intestinal tissue niches in health and disease contexts through the lifespan [19].

However, in one analysis of duodenal samples from healthy human biopsies was predominantly colonized by *Acinetobacter*, *Bacteroides*, and *Prevotella* and the dominant genera in the mucosa were *Prevotella*, *Stenotrophomonas*, and *Streptococcus*, the last two are aerobic. While the genera *Pseudomonas*, *Sphingomonas* and *Fusobacterium* were more prevalent in the duodenum [51]. The rectal biopsies and fecal samples were predominantly composed by anaerobic colonizers such as *Prevotella*, *Bacteroides*, and *Faecalibacterium* [51].

One of the advantages of murine models is the facility to get tissue from different intestinal sites. A previous analysis of the microbiome from different gastrointestinal segments of mice revealed higher abundances of Bacilli in the stomach and small intestine than that in the large intestine and feces. Clostridia was as well higher in the large intestine and feces than that in the small intestine and stomach [52]. At the genus level, large intestine and feces of mice had statistically higher percentages of *Bacteroides*, *Prevotella*, *Alistipes* ($P<0.05$) [52].

In line with what we observed in chapter 3 and 4, Gu *et al*. found higher abundances of *Lactobacillus* in the small intestine than that in the large intestine and feces. Gu *et al*., also observed large inter-mouse variations, which difficult the observation of significant differences [52]. A network-based analyses showed that samples from cecum, colon and feces were more closely associated with one another from than that from same small intestine sites [52]. This could be comparable to the similarities found in the colonic and

duodenal samples from chapter 4. However, the similarities fluctuated by age and we could not identify a specific behavior.

In chapter 4, we observed significant differences in the active microbial communities from the duodenum and the colon of mice. The differences in both sites started from phylum level. However, in older ages finding differences between the microbiome of the duodenum and the colon was less frequent at every taxonomical level.

Some limitations we encountered were that for the duodenum and colon tissue samples it was not possible to retrieve data from bacteria using shotgun metagenomics. The 16S rRNA gene is a more sensitive analysis that sequences can be recovered from low abundance settings.

The microbiome by itself has been considered an organ. However, it is clear that the microbiome changes are related to different external factors, disease and the lifespan. Still we need to understand that the holobionth is a complex environment. Therefore, research should focus on the understanding as a whole system and not as an independent organ or independent relations with individual body organs. It is important to identify specific strains that are present in old aged individuals with good health, to develop better strategies to increment beneficial bacteria and prevent the outgrowth of pathogenic ones. Understanding which are the components of a healthy microbiota during the different stages of the human and murine lifespan will also allow us to use supplements to modulate the microbiome towards health and prevent chronic diseases that are common in old age.

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CHAPTER VI
SUMMARY

6 Summary

Aging is characterized by several physiological changes. During the lifespan, the biological systems from the body of humans and other animals remain dynamic. These dynamisms include changes in the associated microbial communities that colonize the gut. Throughout the early stages of life, the microbiome develops into a complex ecosystem with thousands of species. Variations related to diet, environmental changes, medications affect the diversity and composition of the microbiota through the lifespan. Some old individuals with higher incidence of chronic diseases have a loss of the stability of the microbiome and an imbalance occurs between the different colonizers of the gut, also named dysbiosis. One of the most distinctive changes occurring with age is the prevalent low grade inflammation, which is named inflamm-aging. This not only changes the microbial composition of the GIT but also affects the permeability. Murine models are well established and help us to understand the complex dynamics between the host and the microbial communities inhabiting the gastrointestinal tract. These models allow us to analyze microbial communities from tissue and mucosa, from all sections of the gut, which is limited in humans. In murine models, mice considered young adults at 2 months of age are the most commonly used. Changes related to aging occur at middle age, which for laboratory mice is 15 months. At 24 months old, C57BL/B6 mice used for this thesis can be considered very old, as the survival rate drops. The general objective of this thesis was to analyze the changes in the bacterial communities in response to aging using a murine model.

Methods standardization is an important topic in microbiome research. In chapter 2 it was compared the efficiency of two sample methods, cotton swab and tissue biopsy, in characterizing the mouth microbiota. In recent years, the mouth microbiome is being seen as a diagnostic tool for not only oral diseases but also systemic diseases. As physiological changes occur with aging, the microbiome from the mouth is affected and there is an increase of pathogens present in the oral surfaces. In murine models, cotton swab is a common tool used for sampling the microbiome of the oral cavity. In our study, we observed similar microbial community structure using both methodologies. However, the species *Streptococcus danieliae*, *Moraxella osloensis*, and some unclassified members of Streptococcus were affected by the different sampling procedures. In this trial, we

included mice at two different ages, 2 months old being considered young and 15 months old considered middle aged mice. We observed changes in the genera *Actinobacillus*, *Neisseria*, *Staphylococcus*, and *Streptococcus* related to the age of the animal and the sampling type. These results showed the importance of sampling standardization in microbiome research and that age has a strong effect on the microbial ecology of the oral cavity.

In chapter 3, it was studied the bacterial communities from duodenum and colon of mice at 2, 15, 24 and 30 months of age in combination with the results of the expression levels of antimicrobial peptides in small intestine and markers of intestinal barrier function. Besides, in this chapter were also assessed the indices of liver damage, inflammation and expression levels of lipopolysaccharide binding protein (Lbp) as well as of toll-like receptors (Tlr) 1-9 in liver tissues. At 24 and 30 months of age there was an increase in inflammation, they developed fibrosis and the levels of endotoxin in plasma were higher. Regarding changes in the microbiome, the duodenum had more changes than the colon related to age. *Allobacullum*, *Bifidobacterium*, *Olsenella*, *Corynebacterium* were the genera that differed statistically in the duodenum through the murine lifespan. Fewer changes were observed in the colon, as *Allobaculum* was the only genus that showed differences between young and old mice.

Additionaly, it was analyzed the impact of aging in the active microbial communities of mouth, duodenum and colon at 2, 9, 15, 24 and 30 months of age (chapter 4). Changes were observed at every age and different taxonomical levels, with a greater shift at 15 months of age. This is related to the age of the mice, as at middle age systemic changes related to the aging process start to occur. At old ages, there was an increment of the pathobiontic species *Helicobacter hepaticus* and *Helicobacter ganmani* in the duodenum and colon.

In conclusion, the analysis from the dynamic changes of the healthy microbiota during the different stages of murine lifespan helps towards unraveling the keys to maintain health and the progression age-related chronic diseases. The oral, duodenal and colonic microbial communities are important pieces of information that can be related to the health status of the host. Research that focuses on assessing the changes in the different

niches and not only in the feces, gives a broader overview of the microbial community of the host.

CHAPTER VII
ZUSAMMENFASSUNG

7 Zusammenfassung

Das Altern ist durch verschiedene physiologische Veränderungen gekennzeichnet. Während der Lebensdauer von Menschen und anderen Tieren bleiben die biologischen Systeme des Körpers dynamisch. Diese Dynamiken umfassen auch Veränderungen in den assoziierten mikrobiellen Gemeinschaften, die den Darm besiedeln. In den frühen Lebensphasen entwickelt sich das Mikrobiom zu einem komplexen Ökosystem mit Tausenden von Arten. Variationen in Bezug auf Ernährung, Umweltveränderungen und Medikamenten beeinflussen die Vielfalt und Zusammensetzung der Mikrobiota während des gesamten Lebens. Bei einigen alten Personen mit einer höheren Inzidenz chronischer Krankheiten ist ein Verlust der Stabilität des Mikrobioms zu beobachten und es tritt ein Ungleichgewicht zwischen den verschiedenen Darmbesiedlern auf, was auch als Dysbiose bezeichnet wird. Eine der auffälligsten Veränderungen ist eine im Alter auftretende niedriggradige Entzündung, die als inflamm-aging bezeichnet wird. Diese verändert nicht nur die mikrobielle Zusammensetzung des GIT, sondern beeinflusst auch dessen Permeabilität. Mausmodelle sind gut etabliert und helfen uns, die komplexe Dynamik zwischen dem Wirt und den im Magen-Darm-Trakt lebenden mikrobiellen Gemeinschaften zu verstehen. Diese Modelle erlauben es uns, die mikrobiellen Gemeinschaften in Gewebe und Mucosa in allen Abschnitten des Darms zu analysieren, was im Menschen limitiert ist.

In Mausmodellen werden am häufigsten Mäuse im Alter von 2 Monaten verwendet, welche als junge Erwachsene eingestuft werden. Veränderungen im Zusammenhang mit dem älter werden treten bei Labormäusen im mittleren Alter (15 Monate) auf. Mit 24 Monaten können C57BL / B6-Mäuse, die für diese Arbeit verwendet wurden, aufgrund der sinkenden Überlebensrate als sehr alt angesehen werden. Das Ziel dieser Arbeit ist es, die Veränderungen in den bakteriellen Gemeinschaften als Reaktion auf das Altern unter Verwendung eines Mausmodells zu analysieren.

Ein wichtiges Thema in der Mikrobiomforschung ist die Standardisierung von Methoden. In Kapitel 2 wurde die Effizienz von den zwei Probenmethoden, Wattestäbchen und Gewebebiopsie, bei der Charakterisierung der Mundmikrobiota verglichen. In den letzten Jahren wurde das Mundmikrobiom als diagnostisches Instrument nicht nur für orale Erkrankungen, sondern auch für systemische Erkrankungen eingesetzt. Mit

zunehmendem Alter treten physiologische Veränderungen auf, welche das Mikrobiom im Mund beeinflusst und eine Zunahme von Krankheitserregern auf den Mundoberflächen beobachten lässt. In Mausmodellen sind Wattestäbchen ein übliches Werkzeug zur Probeentnahme des Mikrobioms der Mundhöhle. In unserer Studie beobachteten wir mit beiden Methoden eine ähnliche Struktur der mikrobiellen Gemeinschaft. Jedoch wurden die Anteile der Arten *Streptococcus danieliae*, *Moraxella osloensis* und einige nicht klassifizierte Mitglieder von *Streptococcus* von den verschiedenen Probeentnahmeverfahren beeinflusst.

In dieser Studie wurden Mäuse zwei verschiedener Altersstufen untersucht, wobei die 2 Monate alten Mäuse als jung und die 15 Monate alten als im mittleren Alter eingestuft wurden. Wir beobachteten Veränderungen in den Gattungen *Actinobacillus*, *Neisseria*, *Staphylococcus* und *Streptococcus* in Bezug auf das Alter des Tieres und dem Probentyp. Diese Ergebnisse zeigten, dass das Alter einen starken Einfluss auf die mikrobielle Ökologie der Mundhöhle hat und wie wichtig eine standardisierte Probenentnahme in der Mikrobiomforschung ist.

In Kapitel 3 wurden die bakteriellen Gemeinschaften aus Zwölffingerdarm und Dickdarm von Mäusen im Alter von 2, 15, 24 und 30 Monaten in Kombination mit den Ergebnissen der Expressionsniveaus von antimikrobiellen Peptiden im Dünndarm und Markern der Darmbarrierefunktion untersucht. Außerdem wurden in diesem Kapitel die Indizes für Leberschäden, Entzündungen und Expressionsniveaus von Lipopolysaccharid-Bindungsprotein (Lbp) sowie von Toll-like-Rezeptoren (Tlr) 1-9 in Lebergeweben bewertet. Im Alter von 24 und 30 Monaten kam es bei den Mäusen zu einem Anstieg der Entzündungswerte, sie entwickelten eine Fibrose und die Endotoxinspiegel im Plasma waren höher. In Bezug auf Veränderungen im Mikrobiom kam es im Zwölffingerdarm zu mehr altersbedingten Veränderungen als im Dickdarm. *Allobacullum*, *Bifidobacterium*, *Olsenella*, *Corynebacterium* waren die Gattungen, die sich im Zwölffingerdarm über die Lebensdauer der Maus statistisch unterschieden. Im Dickdarm wurden weniger Veränderungen beobachtet, *Allobaculum* war die einzige Gattung, die Unterschiede zwischen jungen und alten Mäusen zeigte.

Zusätzlich wurde der Einfluss des Alterns in den aktiven mikrobiellen Gemeinschaften von Mund, Zwölffingerdarm und Dickdarm im Alter von 2, 9, 15, 24 und 30 Monaten analysiert (Kapitel 4). Veränderungen wurden in jedem Alter und auf verschiedenen taxonomischen Ebenen beobachtet, im Alter von 15 Monaten kam es zu der größten Veränderung. Dies hängt mit dem Alter der Mäuse zusammen, da im mittleren Alter systemische Veränderungen im Zusammenhang mit dem Alterungsprozess auftreten. Im hohen Alter gab es eine Zunahme der pathobiontischen Arten *Helicobacter hepaticus* und Helicobacter ganmani im Zwölffingerdarm und Dickdarm.

Zusammenfassend lässt sich sagen, dass die Analyse der dynamischen Veränderungen der gesunden Mikrobiota in den verschiedenen Lebensstadien der Maus zur Entschlüsselung der Gesundheit und des Fortschreitens altersbedingter chronischer Krankheiten beiträgt. Die oralen, duodenalen und kolonalen mikrobiellen Gemeinschaften sind wichtige Informationen, die auf den Gesundheitszustand des Wirts schließen lassen. Forschungen, die sich auf die Bewertung der Veränderungen in den verschiedenen Nischen und nicht nur im Kot konzentrieren, geben einen breiteren Überblick über die mikrobielle Gemeinschaft des Wirts.

CHAPTER VIII
APPENTIX

8 Appentix

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Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic *"The effect of aging in the murine gut microbiome"* is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath. I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, Germany 9th September 2020

Place, Date

Signature

Angélica María Hernández Arriaga

Annex 4

Instructions on the importance and criminal legal consequences of the declaration in lieu of an oath

according to Sec. 18(3) sentence 6 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

The University of Hohenheim requires a declaration in lieu of oath on the independence of the scientific work done in order to ensure that the doctoral candidates have done the scientific work independently.

Because the legislators place a particular importance on declarations in lieu of oath and these declarations can have serious consequences, the legislators have placed criminal penalties on false declarations in lieu of oath.

If a person willfully (that means knowingly) submits a false declaration, the punishment can be imprisonment for up to three years or a fine.

If a person negligently submits a false declaration (that is, it is submitted even though the person should have realized that the declaration was not correct), then the punishment can be imprisonment for up to one year or a fine.

The criminal provisions can be found in Sec. 156 of the Criminal Code (StGB, false declaration in lieu of oath) and in Sec. 161 StGB (negligent false oath, negligent false declaration in lieu of oath).

Sec. 156 StGB: False Declaration in Lieu of Oath

Persons who make a false declaration in lieu of oath to an institution responsible for accepting such declarations or persons who make false statements on such a declaration are subject to imprisonment of up to three years or a fine.

Sec. 161 StGB: Negligent False Oath, Negligent False Declaration in Lieu of Oath

161(1): If an action described in Secs. 154 and 156 are done negligently, the punishment is imprisonment of up to one year or a fine.

161(2): There is impunity if the perpetrator corrects the false declaration in a timely manner. The provisions in Sec. 158(2 and 3) apply mutatis mutandis.

I acknowledge the instructions on declarations in lieu of oath.

Stuttgart, Germany 9th September 2020

Place, Date

Signature

Angélica María Hernández Arriaga

Annex 5

Declaration of agreement of the digital version of the dissertation and consent to it being checked with plagiarism software

according to Sec. 18(3) sentence 4 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

I, Hernández Arriaga, Angélica María, born in Irapuato, Guanajuato, Mexico, hereby declare that the unencrypted text document I submitted with the application to initiate the doctoral examination process for the dissertation with the topic

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