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“Impact of genetics and diet on the murine gut mycobiome “

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"Das Leben

Kommt auf alle Fälle

Aus einer Zelle,

doch manchmal endet's auch in einer solchen –

bei Strolchen." (Heinz Ehrhardt)

Für Christian

Content

Abstract

The mycobiome, including the diversity and dynamics of fungi, is distributed across and within the human body with the highest abundance in the gut. Pathogenic fungi like *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococccus* may cause invasive and chronic infections. Modern high-fat diets like the so-called Western Diet are a leading cause for obesity and increase the risk for autoimmune and inflammatory diseases. This has been linked to changes in the overall gut microbiome. Research recently aims to unravel genetic associations to these disease phenotypes as well as possible fungal interactions with host genetics and also environmental factors such as diet. This study focused on fungal ITS2 gene region NGS using the Illumina Miseq sequencing platform to characterize the gut mycobiome in mice. The establishment of an NGS protocol for fungal DNA from murine cecum content samples was the first part of this work. A total of 1.154 of mice derived from a 4-way autoimmune-prone intercross line were fed either a Western, calorie-restricted or control diet. Characterization of the gut mycobiome of 477 of these mice via ITS2 NGS was performed. The phyla Ascomycota and Basidiomycota were most abundant in the gut of AIL mice with Ascomycota constituting over 96 % of all taxa in the calorie-restricted mice and roughly 92 % in the Western Diet mice. At genus level, *Penicillium* was most abundant found in all mice (53.3 %), besides *Aspergillus* (8.4 %), unknown Ascomycota (7.8 %) and *Candida* (7.7 %). No significant difference in fungal alpha diversity was found for the three dietary groups, while differences were observed for the beta diversity. For the Western Diet group, *Wallemia sebi*, *Penicillium decumbens*, *Aspergillus rubrum*, *Kluyveromyces marxianus* and *Nannizzia gypsea* were identified as indicator species while *Phoma herbarum*, *Aspergillus nidulans* and *Neoascochyta paspali* were indicator species in calorie-restricted mice. The highest phenotypic variation in fungal lineages was explained by cage (mean = 26 %), while host genetics explained on average 9.1 % and diet 1.2 %. Regarding genetics, a total of 52 QTL for 43 taxonomic lineages were mapped and single genes that are associated with fungal taxa on chromosomal loci were identified, such as *Nox1*, *Vtn* and *Kctd1*. Future knock-out and gene expression studies could shed light on their disease contributions. Through human dietary intervention studies, one could gain further insights into the effects of diet on specific fungal taxa and on gut biosis, respectively.

Zusammenfassung

Das Mykobiom, bestehend aus der Vielfalt und Dynamik von Pilzen, ist auf und im menschlichen Körper mit der höchsten Häufigkeit im menschlichen Darm verteilt. Pathogene Pilze wie *Candida, Aspergillus, Pneumocystis* und *Cryptococccus* können lebensbedrohliche und chronische Infektionen verursachen. Moderne fettreiche Ernährungsformen wie die sogenannte *Western Diet* sind eine der Hauptursachen für Fettleibigkeit und erhöhen das Risiko für autoimmune und entzündliche Erkrankungen. Dies wurde bereits mit Veränderungen des Darmmikrobioms in Verbindung gebracht. Zur Zeit sind Untersuchungen zum Einfluss der Genetik und der Umwelt (z.B. Diät) und deren Interaktion mit dem Mikrobiom in das wissenschaftliche Interesse gerückt. Diese hier vorliegende Arbeit konzentrierte sich auf die NGS der Pilz ITS2 Genregion unter Verwendung der Illumina Miseq-Sequenzierungsplattform zur Charakterisierung des Darmmykobioms in einem Mäusemodell. Die Etablierung eines NGS-Protokolls für Pilz-DNA aus Blinddarminhalt von Mäusen war der erste Teil dieser Arbeit. Insgesamt 1.154 Mäuse, die aus einer Auszuchtlinie stammten, wurden entweder mit einer Westen Diet, einer kalorienreduzierten Diät oder einer Kontrolldiät gefüttert. Nachfolgend wurde die Zusammensetzung des Darmmykobioms von 477 dieser Mäuse mittels ITS2 NGS charakterisiert. Die Stämme Ascomycota und Basidiomycota waren im Darm von AIL-Mäusen am häufigsten nachweisbar, wobei Ascomycota über 96 % aller Taxa der Mäuse ausmachte, die mit kalorienreduzierter Diät gefüttert wurden, und ungefähr 92% derer, die mit westlicher Diät gefüttert wurden. Auf Gattungsniveau war *Penicillium* mit 53,3% am häufigsten neben *Aspergillus* mit 8,4%, unbekannten Ascomycota mit 7,8% und *Candida* mit 7,7%. Für die drei Ernährungsgruppen wurde kein signifikanter Unterschied in der alpha-Diversität der Pilze festgestellt, für die beta-Diversität wurden jedoch Unterschiede beobachtet. Für die Mäusegruppe der westlichen Diät wurden *Wallemia sebi*, *Penicillium decumbens*, *Aspergillus rubrum, Kluyveromyces marxianus* und *Nannizzia gypsea* als Indikatorarten identifiziert, während *Phoma herbarum*, *Aspergillus nidulans* und *Neoascochyta paspali* als Indikatorarten bei kalorienreduzierten Mäusen identifiziert wurden. Die höchste phänotypische Variation des intestinalen Mykobioms wurde durch den Käfigeffekt mit einem mittleren Prozentsatz von 26 % erklärt, während die Wirtsgenetik im Durchschnitt 9,06 % erklärte und Ernährung 1,2 %. 52 QTL für 43 taxonomische Linien konnten assoziiert und Gene, die mit verschiedenen Pilztaxa auf

Chromosomenorten interagierten, identifiziert werden, so wie z.B. *Nox1, Vtn* und *Kctd1*. Zukünftige Knock-out- und Genexpressionsstudien könnten Aufschluss über ihren Beitrag zur Entstehung von Krankheiten geben. Durch Ernährungsinterventionsstudien im Menschen könnte man weitere Einblicke in die Auswirkungen der Ernährung auf bestimmte Pilzarten bzw. auf die Darmflora gewinnen.

List of abbreviations

ABCA	ATP-binding cassette sub-family A member
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AIDS	acquired immunodeficiency syndrome
AIL	advanced intercross line (of mice)
ANA	antinuclear antibodies
BAL	bronchoalveolar lavage
BLAST	basic local alignment search tool
BMI	body mass index
bp	base pairs
capscale	constrained analysis of principal coordinates
CD	Crohn's disease
CDK	cyclin-dependent kinase
CGD	chronic granulomatous disease
cM	centimorgan
cm	centimeter
CNS	central nervous system
CO^2	carbon dioxide
Cq	quantification cycle (qPCR)
CRP	c-reactive protein
dbRDA	distance-based redundancy analysis
ddH2O	double-distilled water
dl	deciliter
DNA	desoxyribonucleic acid
dNTP	deoxynukleosidtriphosphate
DO	diversity outbred
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	et alii (and others)
EV	Epidermodysplasia Verruciformis
FFC	functional fungal community

FITC	fluorescein isothiocyanate
FLI	Friend leukemia integration transcription factor
FU	arbitrary fluorescence unit
g	gram; gravity
GAG	glycosaminoglycan
GB	gigabyte
GM	galactomannan
GPI	glycosylphosphatidylinositol
GT	gastrointestinal tract
GWAS	genome-wide association study
h	hour
Hif	hypoxia-inducible factor
HIV	human immunodeficiency viruses
HLA	human leukocyte antigens
HMP	Human Microbiome Project
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
HT	high-throughput
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ID	identity
ITS	internal transcribed spacer region
kbp	kilobase pairs
KCTD	potassium channel tetramerization domain containing protein
kg	kilogram
l	liter
LD	loading dye; linkage disequilibrium
LEfSe	linear discriminant analysis effect size
LOD	logarithm of odds
LSU	large subunit
maf	minor allele frequency
MAGEA	MAGE family member protein

MAPK	mitogen-activated protein kinase
Mbp	megabase pairs
min	minute
ml	milliliter
mm	millimeter
MM	master mix (PCR)
mm^3	cubic millimeter
mol	mole
n	nano
NADPH	nicotinamide adenine dinucleotide phosphate
NC	negative control
NGS	next-generation sequencing
NO	nitric oxide
OTU	operational taxonomic unit
p	pico
PBS	phosphate-buffered saline
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PF	passing filter
pH	potentia hydrogenii
PRR	pattern recognition receptor
PV	phenotypic variation
qPCR	quantitative PCR, real-time PCR
QTL	quantitative trait loci
RA	rheumatoid arthritis
RDP	ribosomal database project
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature

SBS	sequencing by synthesis
SD	standard
sec	second
SLE	systemic lupus erythematous
SNP	single nucleotide polymorphism
SPRI	solid-phase reversible immobilization
SSU	small subunit
TNF	tumor necrosis factor
TRIS	tris(hydroxymethyl)aminomethane
UTR	untranslated region
UV	ultraviolet
V	volt
WGS	whole genome sequencing
µg	microgram
µl	microliter
%	percentage
°C	degree Celsius

List of figures and tables

1 Introduction

Microbes are distributed almost everywhere in and on the human body. While the bacterial microorganisms within the microbiome have been shown to not only modify human physiology like its immune development, immune functions, energy acquisition, vitamin-cofactor availability as well as xenobiotic metabolism, and newly shown also neurological development and behaviour (Seed 2014; Pflughoeft and Versalovic 2012; Tremlett et al. 2017; Cryan et al. 2020), fungal contribution and their functions within the microbiome and interactions with the host have been less extensively studied. The so-called mycobiome includes the diversity and dynamics of fungi distributed across and within the human body. Concerning the associations of fungi with infectious and autoimmune diseases as well as their key roles in maintaining the microbial biosis in different parts of the body, little is understood (Seed 2014; Walsh TJ 1996; Qin et al. 2010). In addition, the influence of host genetics on the fungal diversity and composition is poorly investigated. Recently, studies on human genetic disorders and genetic polymorphisms aim to unravel the potential interaction of complex diseases and genetics with the mycobial composition (Underhill and Iliev 2014).

1.1 The fungal cell wall

By now, taxonomists tend to believe that fungi are more closely linked to animals than plants. They live heterotrophic, do not have plastids and contain a chitinous cell wall (1993, Baldauf-Palmer). In 2012 Adl et al. defined common fungal characteristics as the presence of β-glucan and chitin in their cell wall (figure 1), a unicellular or mycelial growth, the presence of an amino adipic pathway for the biosynthesis of lysine and the presence of flattened mitochondric cristae (Adl et al. 2012). The fungal cell wall is a complex and very robust shield. Some fungi have the most robust cell walls found in nature. As a dynamic structure essential for cell shape, viability, morphogenesis and pathology, it is known to be regulated by the environment. The fungal cell wall is made of components that are mostly not represented in humans resulting in recognition of conserved elements by the human immune system. These therefore determine fungal virulence while displaying an ideal drug target. The extent of its importance was for instance shown by de Groot et al. in 2001, who described one fifth of the yeast genome to be devoted only to the biosynthesis of its cell wall. In general, the fungal cell wall

has a robust core of fibrous gel-like carbohydrate polymers. Branched β-(1,3) and β-(1,6) glucans with variable attached sugars or proteins and chitin form layers around the cell while chitin acts as an exoskeleton and the cell wall still remains flexible (Gow et al. 2017).

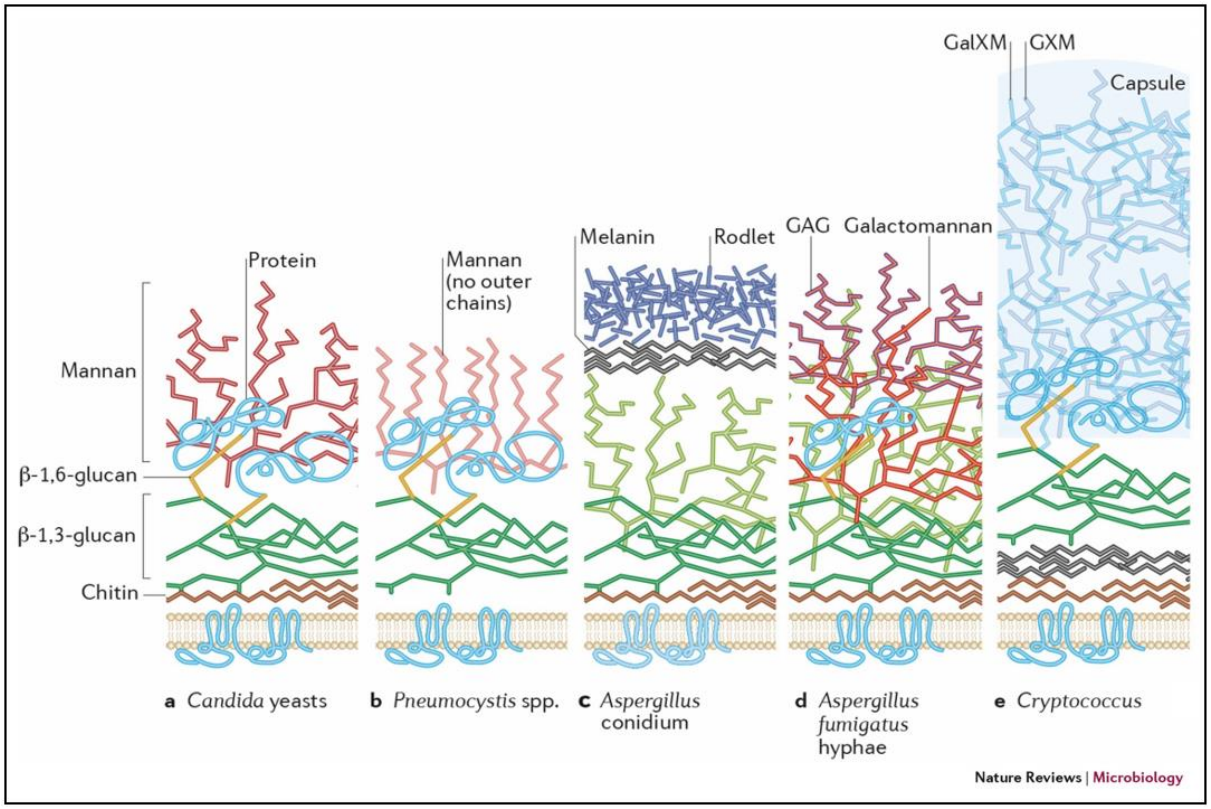


Figure 1: Schematic structure of common fungal cell walls (Gow and Latge et al. 2017, modified). Shown are cell wall structures of important fungal pathogens with their major components and their hypothetical interconnections. The common core of branched β-(1,3) glucan, β-(1,6) glucan and chitin is found in almost all fungal cell walls with differences in attached components like (a) mannosylated proteins attached via Glycosylphosphatidylinositol (GPI) anchors (*C. albicans*), (c) modified mannan chains directly attached to the cell wall polysaccharide core of *Aspergillus* spp. with an outer layer of hydrophobins and melanin or (d) hyphae that contain Galactomannan (GM) and Galactosaminoglucan (GAG) or (e) a capsule that is attached to α-(1,3) glucan in the underlying wall (*Cryptococcus* spp.).

1.2 Fungal taxonomy

Since Whittaker classified the fungal kingdom in 1969 with the definition of core clades (Eumycota), the so called “true fungi” (Whittaker 1969), the old taxonomic classification

of Linnaeus, who described the binomial nomenclature in 1758 and the fungi as belonging to the plant kingdom, has been reviewed many times. Today, modern taxonomic classification of fungi (figure 2) includes nine lineages of true fungi which are divided into three groups (Naranjo-Ortiz and Gabaldón 2019): (**i**) Zoosporic fungi (Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota) that derived from a single cell organism with posterior flagella (secondary loss over time) and include many intracellular parasites, plant pathogens and others that show a diverse morphology, (**ii**) Zygomycetous fungi (Zoopagomycota, Mucuromycota, Glomeromycota) that reproduce sexually, show hyphal growth and that were able to emerge on terrestrial ground. Within the Zygomycetous fungi there are many parasitic classes but also plant symbionts and mycorrhizae. Some representants of the class Mucoromycota can cause rare infections in humans that are strongly invasive (Kwon-Chung 2012) while others are used for fermentation in food industry (Conti et al. 2001). The most well described group of fungi refers to the (**iii**) Dikarya which include the largest fungal phylum Ascomycota (sac fungi) and the second species rich fungal phylum Basidiomycota (yeasts and molds).

The Dikarya are often described as subkingdom of the fungal kingdom which includes more than 97% of all described fungi. Their sexual lifecycle alternates between a haploid phase wherein the gametes are produced, and which ends with nuclear fusion at the time of zygote formation in the diploid phase, which is then followed by meiosis. The dikaryotic condition, wherein each parent forms a dikaryon through cytoplasmatic fusion of two monokaryotic hyphae and which happens before nuclear fusion, is one characteristic feature of the Dikarya after which the group ins named (Stajich et al. 2009; Hibbett et al. 2018). Due to the formation of zygotes and independent meiosis Dikarya show increased recombination and diversity. Dikarya mostly include species with hyphae or unicellular yeasts and commonly produce regular septa with central pores that have regulatory effects on the cytoplasm and organelles. Their membrane sterol is ergosterol and some of the Dikarya even form multicellular reproductive or vegetative structures (Naranjo-Ortiz and Gabaldón 2019; Stajich et al. 2009). Recently, studies could show how adaptable dikaryons are due to the ability of some proportions of the two nuclei to vary in a colony and thereby adjusting to changes in the environment (James et al. 2008).

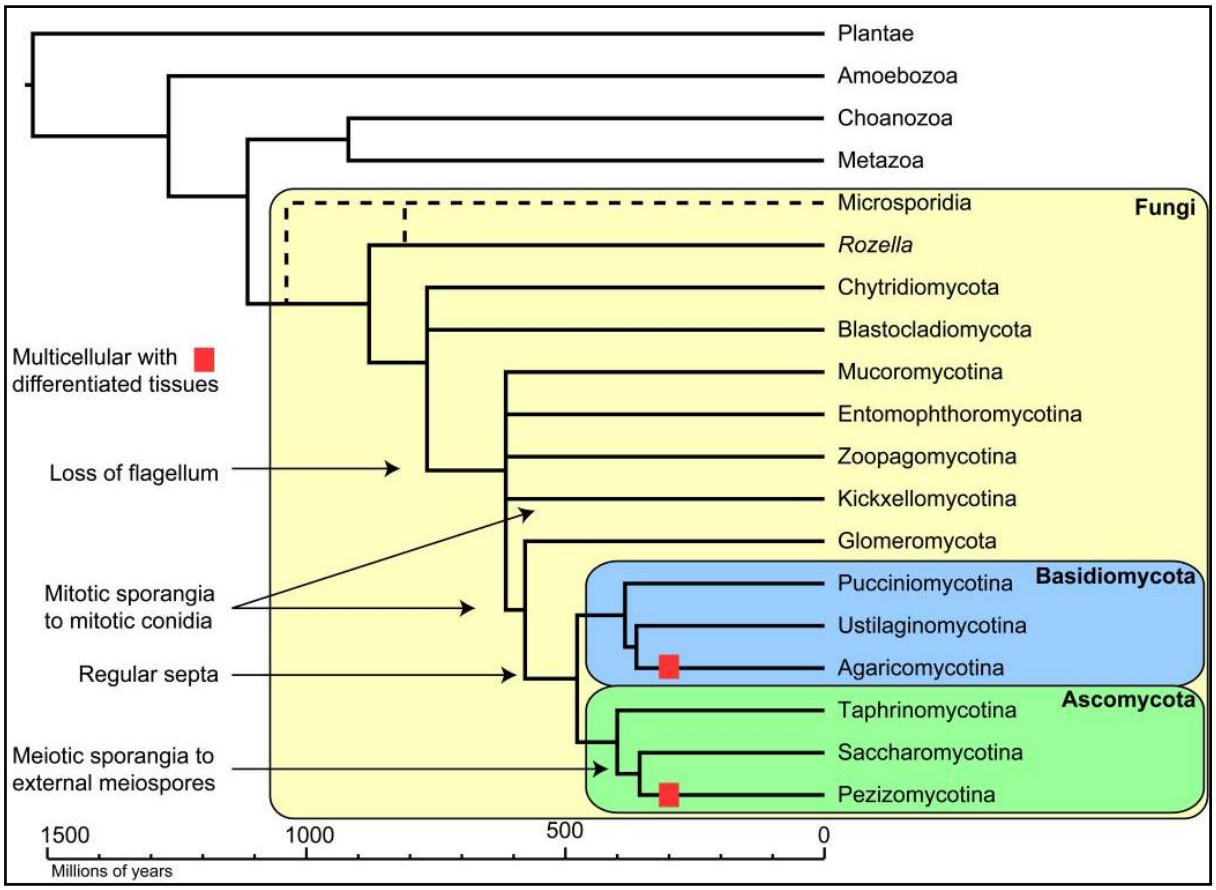


Figure 2: Simplified modern fungal taxonomy (James et al. 2006). Fungi are described as separate kingdom which branches together with the plant kingdom from the Eukarya. The secondary loss of the flagellum within the Zoopagomycotina reaches until the two big phyla of true fungi Ascomycota and Basidiomycota, which display the main interesting fungi involved in a variety of environmental pathways and also involved in research on fungal diseases in humans.

1.2.1 Ascomycota

The Ascomycota are the largest fungal phylum including two third of all described fungal species (Schoch et al. 2009). These sac fungi form an ascus with eight spores and morphologically present as simple yeast forms or highly complex fruiting bodies. Species of the class Saccharomycetes like *Candida* are used in experimental studies because they are easily manipulated in the laboratory, and together with species of the genera *Penicillium, Neurospora,* and *Schizosaccharomyces* this group has been study subject in Nobel prize research (Naranjo-Ortiz and Gabaldón 2019; Stajich et al. 2009). Three subphyla are classified so far including the physiologically divers

Taphrinomycotina which have very compact and reduced genomes and the Pezyzomycotina displaying the most diverse subphylum with 13 classes and 67 orders, also including the lichens, which are associations between fungi and algae or cyanobacteria.

The most important subphylum Saccharomycotina describes yeasts that are able to switch to filamentous forms, that can easily adapt to the environment and that are known to be associated with several animal niches like the gut and the mucosa (Stajich et al. 2009; Naranjo-Ortiz and Gabaldón 2019). Representants of this subphylum have gene rich genomes that have undergone evolutionary gene loss and reduction of nucleotide sequences like introns. This has led to conditions where only one gene locus can have hundreds of ribosomal RNA (rRNA) gene tandem copies which has enabled the sequencing and identification of approximately 10% of all Saccharomycotina today (Dujon et al. 2004; Proux-Wéra et al. 2013; Dujon and Louis 2017). Saccharomycotina contain important species like the industrial yeast *Saccharomyces cerevisiae* or parasitic *Candida* species and others which are responsible for many severe systemic human mycoses and skin diseases (Heitman 2006).

1.2.2 Basidiomycota

With over 30,000 described species the Basidiomycota are the second species rich phylum of the Dikarya and account for 34% of described fungi. Given the name, they form reproductive structures called Basidia which are specialized club-like cells that produce sexual spores and in which meiosis takes place (Adl et al. 2019; Hibbett et al. 2007). The Basidiomycota contain rusts, smuts and mushrooms with several growth forms like yeast, hyphae or dimorphic and they comprise many yeasts and molds. They overall present the most complex fungi concerning cell cycle and multicellularity. Classification divides them into three subphyla including the parasitic fungi Pucciniomycotina, the Ustilaginomycotina and the Agaricomycotina which all bear model systems for research in genetics, development and sexual reproduction (Stajich et al. 2009; Steinberg and Perez-Martin 2008; Kües 2000). While researchers are certain that the Pucciniomycotina share some ancestral traits with the Ascomycota, a big part of their diversity is hidden through the lack of phenotypes and characteristics of many classes (Tedersoo et al. 2017). Although the subphylum Ustilaginomycotina

mostly presents plant and rarely animal pathogens, its genus *Malassezia* is commonly found as colonizer in normal mammalian skin. But it can also be involved in diseased skin conditions and dandruff (Velegraki et al. 2015; Stajich et al. 2009).

Within the clade Agaricomycotina fungal growth as yeast, hypha or both is found as well as self-fertile species. Divers multicellular fruiting bodies define the mushrooms in this clade which are formed to increase basidia surface area and display an important source of food. Besides that, they are also involved in the process of wood decay but, most importantly, they can be human pathogens like the well-known *Cryptococcus* genera or *Tremella*, that belong to the class of the Tremellomycetes (Stajich et al. 2009; Hibbett et al. 2007; Adl et al. 2019). *Cryptococcus* appears as encapsulated yeast. The species *C. neoformans* and *C. gattii* are known to cause the leading invasive fungal infection cryptococcosis and the widely distributed cryptococcal meningitis in immunocompromised humans (Nielsen et al. 2005; Perfect 2012; Park et al. 2009). One important class, which is described as sister clade of the Agaricomycotina but stands isolated within the Basidiomycota, are the Wallemiomycotina and its genus *Wallemia*. The organisms belonging to this genus are the most xerotolerant, xerophilic and halophilic species worldwide (Zalar et al. 2005; Zajc and Gunde-Cimerman 2018).

1.3 The human mycobiome

Fungi belong, with an estimated species number of five million, to one of the largest and most diverse kingdoms of living organisms. Within the last decade whole-genome sequencing of fungal species has led to the formation of fungal genome-trees which are built from DNA or protein sequences consisting of a small number of highly conserved genes. Over 400 whole-genome sequences for fungal species are already publicly available and the field of genetic research on fungi is greatly expanding due to the rise in fungal infections worldwide but also because of their great potential in food and pharma industry (Choi and Kim 2017; Stajich et al. 2009). Based on the information on fungi that have been gathered from researchers worldwide, fungi have become a more interesting subject to a variety of experimental and genetic studies based on the mycobiome that colonizes the human body.

Recently, research focuses on interactions of the mycobiome with the host and its genes in crosstalk with environmental factors such as body sites (niches), diet and other influences (Seed 2014; Vorobyev et al. 2019). Since several years, the number of papers on fungal research has largely increased. In brief, several studies focused on the mycobiome in health and disease showing for example that the oral mycobiome of patients infected with the human immunodeficiency virus (HIV) is different from that of uninfected controls (Hager and Ghannoum 2018), *Candida* and *Saccharomyces* species seem to play a role in hepatitis B infections (Chen et al. 2011) or an abnormally high abundance of *Candida tropicalis* is linked to inflammatory bowel disease (IBD) (Stamatiades et al. 2018; Trojanowska et al. 2010). With these studies, researchers worldwide aim to find new treatment possibilities based on the mycobiome, so that changes in diversities of the commensals but also of the pathogenic fungi could possibly predict the outcome or severity of a disease. In 2016 Kalan et al. showed in a longitudinal study on 100 diabetic-foot ulcers, for instance, that even chronic cutaneous wound nonhealing, which causes significant morbidity and mortality in diabetic, obese and elderly people, is not only influenced by bacterial but also an increased interkingdom biofilm formation between bacteria and for example yeast species. Fungal communities become more heterogeneous over time and their diversity increases with antibiotic administration. Using fungal internal transcribed spacer region (ITS) sequencing the authors showed also that pathogenic fungi, which could not be identified through culture (culture-based methods only captured 5% of fungi residing in the chronic wounds), reside in chronic wounds and can even predict the healing (Kalan et al. 2016).

Figure 3 displays the different fungal niches in and on the human body, showing that for example *Candida* and *Aspergillus* lineages are almost ubiquitously present in humans including also the skin and the gastrointestinal tract (GT) (Ghannoum 2016). They occur as commensals but when microbial community balance is disrupted in for example immunocompromised people or due to other environmental influences, these potentially pathogenic fungi can induce fungal infections. Alterations in the mycobiome composition in the gut have been linked to IBDs like Crohn´s disease (CD), while *Malassezia* species for example can lead to superficial skin diseases (Ott et al. 2008; Zhang et al. 2012). Hence, the influence of environmental factors such as genetic susceptibility, hygiene or nutrition on fungal composition can define the mycobiome.

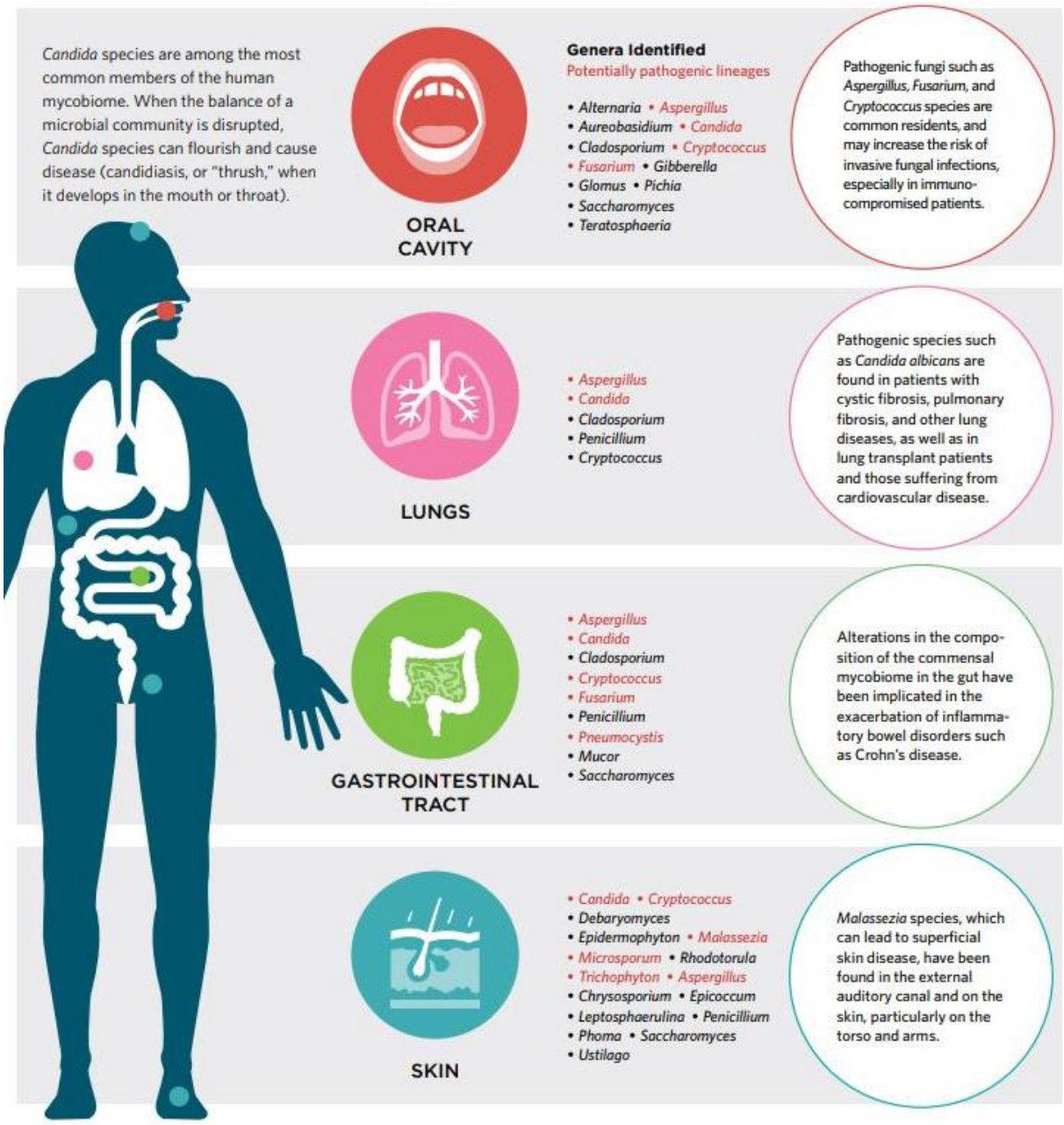


Figure 3: Overview on the human mycobiome, its niches and diseases (Ghannoum 2016, modified. In: The Scientist, The Mycobiome (Feature)). Different fungal niches on and in the human body and the influence of different environmental factors on fungal communities.

1.3.1 Niches of the mycobiome

As mentioned above, fungal communities are present in and on different human body sites like the vaginal canal, the skin, the gut, the oral cavity or the lung (Seed 2014). In 2010 Ghannoum et al. showed that *Candida* species were the most frequent fungi in

the oral cavity of healthy donors, followed by *Cladosporium* and Saccharomycetales but also *Aspergillus* and *Fusarium* spp. representing pathogenic microbes. Two separately performed studies on the oral mycobiome using ITS region sequencing gave different abundances of core fungi present in each of the samples (Ghannoum et al. 2010; Dupuy et al. 2014). While in 2010 Ghannoum et al. found via principal coordinate analysis (PCoA) that *Candida* and *Cladosporium* were the most abundant fungi in 75% and 65% of the donors, Dupuy et al. identified in 2014 *Malassezia* and *Epicoccum* as most abundant genera which made up 38% and 33% of the total oral mycobiome, respectively. These differences show that not only are there variations in the curation of the used databases as well as in the individuals belonging to the general sample cohort, but also may differences in sample collection and handling as well as different analysis tools in each study lead to a big variance in the resulting data (Seed 2014).

Since the oral mycobiome plays a key role in the entry of microbes into the enteric and respiratory tract in altering systemic innate and adaptive immunity (Seed 2014), it is not surprising that the lung mycobiome seems to mostly arise from the oral cavity (Seed 2014; Charlson et al. 2012) and is altered during conditions (Pragman et al. 2012). A recent study focused on the characterization of the lung mycobiome, which could be shown to be dominated by *Aspergillus* species in healthy people and *Candida* species in donors who suffered from different diseases (Nguyen et al. 2015a). Concerning the vaginal microbiome, its bacteria have been well studied (Ma et al. 2012), while the vaginal mycobiome has only recently been focused on. A study showed that 58% of the vaginal constituents out of 251 samples were Ascomycota of which 37% were Saccharomycetales, *Candida* being the most abundant species (Drell et al. 2013).

But also our largest organ, the human skin, with its various niches for fungal growth, is colonized by a variety of microorganisms like bacteria, viruses, parasites and fungi, whom some may occasionally cause skin diseases when the skin barrier function is disturbed (Leung et al. 2016; Sugita et al. 2002; Tajima et al. 2008; Park et al. 2012; Harada et al. 2015; Baroni et al. 2004; Murphy et al. 2018). The abundance of fungi on healthy human skin varies with the region the individuals live in. Leung et al. (2016) gave a taxonomic overview on the skin mycobiome of Chinese individuals describing common skin commensals like *Aspergillus*, *Penicillium*, *Candida* and *Cryptococcus*,

but also pointing out, that especially concerning the skin as an organ with a variety of niches, fungal diversities are not equal within different anatomic sites. The forearm and palm sites for example were mainly dominated by the common skin fungus *Malassezia* (57% of fungal community within each sample), which as a lipophilic genus is even more abundant in forehead samples. In 2012 Zhang et al. showed the distribution of *Malassezia* on the human skin, followed by Findley et al. in 2013, who analyzed and characterized the human skin mycobiome and showed the dependency of fungal abundances on the different body sites.

1.4 The gut mycobiome

The total human microbiome is with an estimated number of roughly one trillion organisms most abundant in our intestines and also shows the highest diversity there (figure 4) (Qin et al. 2010). Densities of total microbiota in the gut can reach up to 10^{12} per ml and masses up to one kg (Goodrich et al. 2017). Not only are the combined microbiota essential for the intestinal epithelium and also for the immunity of the mucosa, but also for metabolic functions (Cerf-Bensussan and Gaboriau-Routhiau 2010). Hence, mucosal border and its microbial environment are a complex of bacteria, fungi, parasites and viruses that reside in an ecosystem and is constantly monitored through pattern recognition receptors (PRRs), which condition the mucosal immune cells as well as epithelial cells and promote the innate immune system (Jacobs 2015). The lack of microbiota in germ-free mice leads to a development of immature lymph nodes and spleens and other abnormalities in postnatal development like impaired organ functions or lipid cycling (Smith et al. 2007) that can be reversed upon introduction of microbiota (Hooper 2004). Therefore, the maintenance of a biosis of different microbiota in the gut is crucial for immune development (Bauer H. et al. 1963).

The mycobiome is established early in life very soon after birth and is influenced by the exposure to different microorganisms. Until recently researchers believed, that the fungal composition can hardly be changed by environmental influences like for example diet because the oral uptake only leads to a transient presence of some fungi, whereas others like *Candida*, *Saccharomyces* or *Trichosporon* can belong to the resident gut flora (Hof 2017).

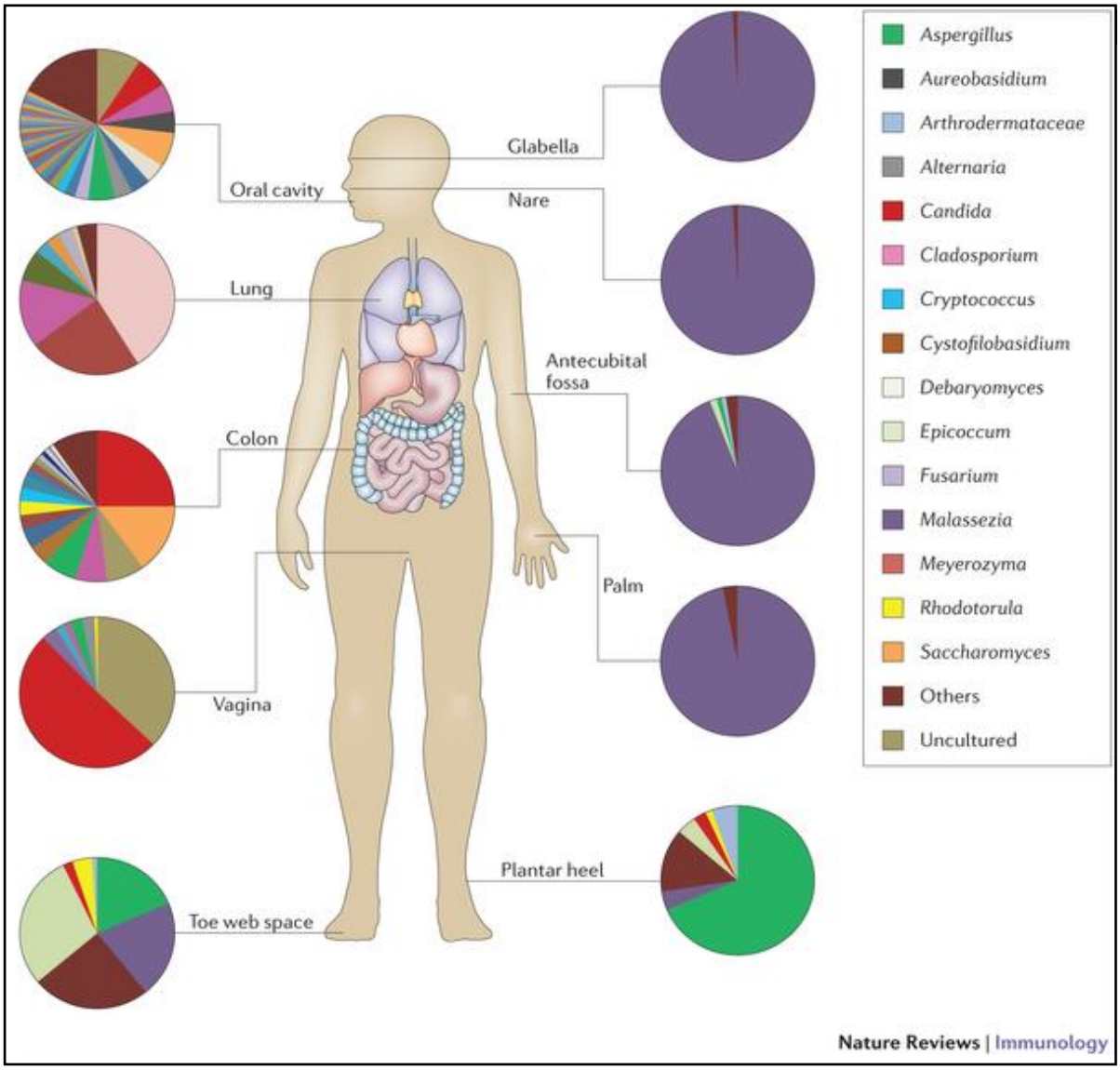


Figure 4: Fungal diversities and commensals on different human body sites. (Underhill and Iliev 2014). Fungal diversities differ between human body sites with the oral cavity and the colon bearing the highest. The healthy intestinal mycobiome is dominated by commensal *Candida* and *Saccharomyces* species.

Fungal presence in the GT is limited to only 0.1% of the total gut microbiota, which long time hindered the investigation of the fungal contributions in disease (Li et al. 2018; Qin et al. 2010; Arumugam et al. 2011). Mouse studies showed that the highest concentration of fungi is found in the distal colon (Iliev et al. 2012). Together with other microbiota, the underestimated fungi maintain intestinal homeostasis and systemic immunity by for example production of beneficial metabolites like fatty acids or carotenoids or by metabolizing toxic compounds and, in addition, also by directed

binding and export of pathogenic bacteria through mannans on their surface (Hof 2017; Hernández-Santos and Klein 2017). The gut's ecosystem is maintained by multiple interactions between microbial members like bacteria, fungi and viruses (Filyk and Osborne 2016). Now, it is known that diet, antibiotic treatments and infections influence the microbial diversity in the gut (Dale and Lied 2020; Nishida et al. 2018; Jandhyala et al. 2015). For example could the gut flora composition of overweight humans be shifted towards the microbial composition of healthy non-obese controls by transition to a low-fat diet (Scarpellini et al. 2010; Turnbaugh et al. 2008).

Although already challenged, some believed the healthy infant to be born sterile (Aagaard et al. 2014) and that its first exposure to microbes, both bacteria and fungi, happens during the passage of the infant through the vaginal canal during labour (Ma et al. 2012). It has been shown that the intestines of a high percentage of preterm infants is colonized with *Candida* spp. within the first six weeks of life and that their general risk for invasive fungal diseases is significantly increased, which can be explained by several factors such as prematurity of the intestinal and systemic immunity, broad antibiotic exposure during and after birth and delayed feeding due to caesarean section (Kaufman et al. 2006). In 2011 La Tuga et al. showed that seven out of eleven stool samples from preterm infants had fungal DNA with the most abundant order Sacharomycetales, showing different *Candida*, *Cladosporium*, *Cryptococcus* and *Sacharomyces cerevisiae* species. In addition, Malasseziales, Eurotiales and others were found (LaTuga et al. 2011).

1.4.1 Composition of the gut mycobiome

Of the worldwide estimated 5 million different fungal species, roughly 300 species are potentially pathogenic to humans and can cause diseases in humans (Blackwell 2011; Taylor et al. 2001). Although these are relatively few fungi, they have been found to be responsible for many infections ranging from superficial skin and nail infections to invasive infections of the lungs, blood or brain (Brown et al. 2012). Until 2015 more than 267 distinct fungal taxa from the human gut have been reported and they can mostly be distinguished between resident and non-resident fungi with resident or autochthonous fungi being able to grow at 37°C for a colonization of the gut (Suhr and Hallen-Adams 2015). As mentioned in section 1.2 the fungal kingdom includes the subkingdom Dikarya with two large phyla: (**i**) the spore forming Ascomycota that

represent yeasts like *Candida* as well as molds like *Penicillium*, *Cladosporium* or *Aspergillus* and (**ii**) the hyphae forming Basidiomycota, which include the opportunistic pathogen *Malassezia* as well as *Trichosporon* and the pathogenic yeast *Cryptococcus.* These fungi (figure 3) have been shown to play a role within the human gut mycobiome (Nash et al. 2017; Bennett and Turgeon 2016; Stajich et al. 2009).

For the yeast genera *Candida* including species like *C. albicans, C. tropicalis, C. arapsilosis* or *C. glabrata* the mammalian digestive tract seems to be the primary niche in healthy individuals (Moran et al. 2012). But these resident fungi normally are not significantly present in soil, air or food (Saleh et al. 2011). The genus *Malassezia*, as described above, has been reported to play a role in skin diseases and has been shown to dominate the skin mycobiome, but already since 1969 one believes that it might play a role in the gut mycobiome too (Cohen et al. 1969; Findley et al. 2013; Gupta et al. 2004). These fungi are dependent on their host, because they lost the ability to synthesize their own lipids, what makes them great colonizers of the skin and probably also of the gut (Gupta et al. 2004). Other potential colonizers of the healthy gut are the molds *Cladosporium* (including yeasts of the Dipodascaceae like *Galactomyces,* (figure 4), *Aspergillus* and *Penicillium*, which are filamentous fungi belonging to the Ascomycota and which have been reported in up to 22% of gut fungal studies (Geiser et al. 2006; Hallen-Adams and Suhr 2017; Cohen et al. 1969). Unlike *Aspergillus*, which is of environmental origin (soil, plants, air) and which can survive human physiological temperature (Mortensen et al. 2010; O'Gorman and Fuller 2008), *Penicillium* is a food borne species which is used in food production (cheese) and for the development of several β-lactam antibiotics (Decontardi et al. 2018; Banjara et al. 2015), but of which some species also produce common human allergens (Visagie et al. 2014). Another species, that originates in food, is *Saccharomyces cerevisiae* which is besides *Candida albicans* one of the most commonly detected fungi in faecal samples (Angebault et al. 2013).

S*accharomyces boulardii* is described as having probiotic properties, which refers to microorganisms that provide health benefits due to effects that can positively modify the gut microbiome by stimulating the growth and activity of certain bacteria or fungi (Gibson and Roberfroid 1995; Hutkins et al. 2016). Its beneficial effect in antibiotic-mediated or bacteria-mediated diarrhoea has been reported repetitively (Terciolo et al. 2019; Czerucka et al. 2007; Pothoulakis et al. 1993; Pothoulakis 2009; Sen and

Mansell 2020), but *S. boulardii* only persists in the human GT for five days after administration stops in healthy humans (Moré and Swidsinski 2015). Tung et al. showed in 2009 that by stimulation of the anti - *Clostridioides difficile* toxin IgA production and other anti-inflammatory pathways, *S. boulardii* shows the potency together with parts of the intestinal microbiome to modify intestinal functions (Qamar et al. 2001; Pothoulakis 2009). Additionally, administration of either chitin or β-glucan, both constituents of the fungal cell wall (see section 1.1 and figure 1), or living *S. boulardii* cultures to mice prevented them from developing obesity phenotypes that were induced by high-fat diet (Everard et al. 2014; Neyrinck et al. 2012).

Metabolites produced by *Penicillium* genera have been reported to exhibit anti-inflammatory as well as insulin-sensitizing activities (Lee et al. 2013) and *Aspergillus terreus* administrated as dietary supplement along with a high-fat diet could reduce hepatic steatosis compared to rats fed only a high-fat diet (Jang et al. 2015). But besides these well described fungi most of the species richness of the gut mycobiome is formed by rarely-detected fungi like edible mushrooms (Suhr et al. 2016), plant pathogens (Gouba et al. 2014), xerophiles like *Wallemia* (Chen et al. 2011) and wood decay fungi (Hamad et al. 2012; Hallen-Adams et al. 2015) of which is believed that they do not have an influence on the gut ecology.

1.4.2 The gut mycobiome in diseases

Fungal infections with *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus* kill more than one million people annually and in general, fungal infections show a high worldwide mortality which is higher than that of for instance tuberculosis or malaria. Additionally, the rapid development of resistances to antifungal drugs displays an immense problem for health and research (Brown et al. 2012; Denning and Bromley 2015; Janbon et al. 2019). Collected research on the role of fungi in diseases has proven their contribution in driving disease pathogenesis (Underhill and Iliev 2014; Huseyin et al. 2017a; Sokol et al. 2017). The most common fungus that is found in the human intestines is *Candida albicans*, which shows an elevated prevalence in different intestinal diseases like IBD or irritable bowel syndrome (IBS), where overall changes in the gut mycobiome mediate visceral hypersensitivity in patients (Sokol et al. 2017; Longstreth et al. 2006; Botschuijver et al. 2017). Sokol et al. further saw that the alpha diversity of fungi, meaning their species richness, from faecal samples of more than

200 IBD patients was decreased. And while *C. albicans* abundance was elevated, the prevalence of *S. cerevisiae*, which is a known probiotic, was reduced (Sokol et al. 2017). Colonization with *C. albicans* in antibiotic-treated mice can also worsen other diseases like for example allergic airway diseases (Noverr et al. 2004).

Another study from 2015 on *C. albicans* showed that specific bacterial species can suppress its growth in the murine gut. Fan et al. were able to show in this study that mice that are colonized with the gram-negative obligatory anaerobic bacterium *Bacteroides thetaiotaomicron* are more resistant to *C. albicans* colonization in the gut than mice without. The expressed hypoxia-inducible factor 1-α (Hif-1α) by *B. thetaiotaomicron* apparently leads to a production of specific antifungal peptides which increase the killing of *C. albicans* (Fan et al. 2015). Other researchers showed that mice that were chronically fed alcohol had a generally increased fungal burden and a great expansion of *C. albicans* in the intestines compared to mice with an alcohol-free diet (Yang et al. 2017). Microbial dysbiosis has already been reported in neurological diseases like multiple sclerosis or Alzheimer's disease, and especially for the gut bacteria, an influence in rheumatoid arthritis, diabetes, cancer but also skin diseases like psoriasis or systemic lupus erythematous could be shown (Golombos et al. 2018; Forbes et al. 2016; Wang et al. 2018). These results might also be linked to an effect of the gut mycobiome on the central nervous system (CNS) (Enaud et al. 2018).

A competitive association between bacteria and fungi in the gut seems to influence for example the blood-brain-barrier permeability, which was shown to be increased in germ-free mice due to a reduction in tight junction protein expression, and which could be decreased through microbial colonization of their digestive tract (Braniste et al. 2014). The relationship of the total gut microbiome and the CNS is called gut microbiota-brain-axis (Dinan and Cryan 2017) and recent studies show that microbiota including fungi play a key role in many CNS functions (Rhee et al. 2009; Kennedy et al. 2017; Enaud et al. 2018). The anxiety-like behavior of germ-free mice could also be reversed after colonization of the gut with a commensal microbiome (Buffington et al. 2016) and probiotic supplements showed to positively affect anxiety and depression symptoms (Pirbaglou et al. 2016). Fungi can synthesize neurotransmitters that are involved in brain activation and, conversely, neuromediators also impact the gut mycobiome in increasing fungi virulence (Reyes-García et al. 2012; Mayr et al. 2005). For *C. albicans* it has been shown that the fungus is able to produce histamine which

plays a role in appetite regulation, sleep-wake rhythm an cognitive activity (Voropaeva 2002).

But the most widely researched intestinal disease in dependence of the total gut microbiome is probably IBD. Inflammatory intestinal diseases like IBDs including CD and ulcerative colitis are thought to be mediated by the total gut microbiome including fungi (Gu et al. 2019; Lam et al. 2019; Pothoulakis 2009). Both diseases can be associated with a variety of pathogenic factors such as environmental changes, genetic susceptibility, a dysregulation and dysbiosis of the gut microbiota and the general immune response (Souza and Fiocchi 2016). Already in 2008 Ott et al. found that the faecal fungal community in IBD differs from that in healthy controls. Furthermore, mouse models have shown that for example the dectin-1 PRR, which is present on innate immune cell surfaces that interacts with the major polysaccharide motif on fungal cell walls β-glucan, influences mouse IBD symptoms (Iliev et al. 2012; Brown and Gordon 2003). It was demonstrated how a mutation in the widely expressed major fungal recognition C-type lectin receptor encoding gene *dectin-1* was associated with ulcerative colitis. Dectin-1-deficient mice lost more weight, showed alterations in histology as well as in the cytokine production towards a proinflammatory milieu compared to the wild-type mice, which was mediated by *Candida tropicalis*. An antifungal treatment of the dectin-1-deficient mice with fluconazole decreased disease severity (Iliev et al. 2012).

What also could be shown is a generally increased total fungal load of *Candida* and *Malassezia* species in the faeces and mucosa of CD patients while the fungal diversity is lower in those of ulcerative colitis patients. Apparently a caspase recruiting protein polymorphism favours the colonization of the gut with *Malassezia* which enhances inflammation (Lam et al. 2019). But, not only alterations in the gut mycobiome could be shown in IBD, also inter-kingdom alterations take place. This means the bacterial-fungal network is dramatically disturbed in IBD patients compared to that in healthy controls (Sokol et al. 2017). Kapitan et al. specified a negative correlation and disruption of the bacterial microbiome as a prerequisite for fungal overgrowth (Kapitan et al. 2019).

1.4.3 The influence of diet

The primary route for fungi to enter the GT is through ingestion, which starts after birth and after the contact with yeast species like *Candida*, that first colonize the infant during the passage through the vaginal canal (Penders et al. 2006; Bliss et al. 2008). Besides the host's genotype and genetic predisposition, host physiology (sex, age and present comorbidities), general lifestyle (hygiene and occupation) or the exposure to antibiotics, diet is one of the major factors influencing the total microbiome in the gut and thereby the immune system (Paterson et al. 2017; Cui et al. 2013; Strati et al. 2016). Dietary intervention studies on the diversity of the gut microbiome highly depend on sample size, individual habits and environmental factors as well as the composition of the diet. Several studies showed that the total gut microbiome can be altered through diet (David et al. 2014; Heisel et al. 2017).

The intake of dietary fat together with changes in the gut bacterial composition could already be associated with obesity in mice, which is also a growing health problem in developed and industrialized countries (Heisel et al. 2017). In 2017 Heisel et al. described different relative abundances of bacteria and fungi in the gut with disrupted interactions and a dramatic reduction of co-abundances between intra-and interkingdom microbial pairs. They further found that a high-fat diet decreased bacterial alpha diversity. In general, diet high in fat (which is a major constituent of Western diet), cholesterol and sugar and low in dietary fiber influences not only the risk for obesity but also for related diseases like diabetes and atherosclerosis, and it can lead to an overall inflammatory response by changing also the mycobiome (Turnbaugh et al. 2008; Tilg and Moschen 2014; Caesar et al. 2010; Cani et al. 2008). And this is not surprising, as to fungal cells can be up to 100-fold larger than typical bacterial cells. They compromise for a big mass of biomaterial and can therefore contribute to unique metabolic functions (Huffnagle and Noverr 2013).

Many fungi become transient colonizers in the gut upon food digestion (Forbes et al. 2018). In 2014 David et al. found that gut bacteria respond to nutrient availability, while gut fungi mostly derived from food. The same fungal species were found in both faecal samples and food that was consumed by the participants. Standard mouse chow also differs in fungal composition from high-fat diet, that shows a higher *C. albicans* and a lower *S. cerevisiae* abundance. Its intake leads to different mycobial composition in

the mouse gut (Heisel et al. 2017). Food-borne *S. cerevisiae,* that is also found in fermented beverages, can survive challenges of the human GT and thus can become a commensal (Rizzetto et al. 2014). In 2016 Strati et al. performed metagenomics and detected sequences that belonged to edible fungal genera. Hence, they described the dietary fungi intake as potential confounding effect on the shaping of the gut mycobiome. But some fungi like *C. albicans* also have the ability to switch between different morphologies including cellular, pseudohyphae and hyphae forms and therefore display successful microorganisms that colonize the mucosa by expressing genes involved in environmental resistances (Sudbery 2011; Vautier et al. 2015). Recently, it could be shown that diet itself can shift the microbiome in the mouse gut including both bacteria and fungi even before the onset of disease and thereby overcomes genetic susceptibility for a certain disease (Vorobyev et al. 2019).

But already since 2013, when Hoffmann et al. performed deep sequencing of the ITS region from healthy human faecal samples, fungal research has gained importance. They found that in 98% of all samples *Saccharomyces* were present and that *Candida* and *Cladosporium* were the second and third most prevalent fungi. But they couldn't define, which of these fungi were resident or derived for example from dietary sources intake. Nonetheless, in this study the researchers could already show that *Candida* and *Saccharomyces* were associated with a diet rich in carbohydrates, whereas donors with diets rich in amino acids had a lower abundance of *Candida* in the stool. The *Candida* abundance could therefore be positively correlated with carbohydrate consumption and negatively correlated with that of total saturated fatty acids, while short chain fatty acids drove for instance *Aspergillus* abundance (Hoffmann et al. 2013). By adding coconut oil to the daily diet Gunsalus et al. showed in 2015 that the increased amount of *C. albicans* and resulting infections could be reduced in mice fed with a coconut oil-rich diet compared to mice fed with a diet rich in beef tallow and soybean oil. Likewise, Hallen-Adams and Suhr (2015) sequenced fungal DNA from vegetarian and conventional diet consuming persons and showed that fungi were proportionally more common in vegetarians with *Fusarium, Penicillium, Aspergillus* and *Malassezia* being detected more often than in the conventional diet consuming subjects. Compared to a study of David (David et al. 2014) one sees a discrepancy in the results mostly due to the inclusion of more or less dairy products in the participants' dietary regime. Another pitfall of this study was that fungal communities in the gut seem

to be less stable than bacterial communities (Lozupone et al. 2012; Hallen-Adams et al. 2015). One of the most common fungi that persisted over time, when samples of the same individual were taken longitudinally, was *Candida tropicalis*, while other species showed a high variation and could not be determined as resident (Hallen-Adams et al. 2015).

Moreover, fungi produce numerous metabolites that can help maintain human homeostasis. Some can serve as signalling molecules with anti-inflammatory and antibacterial effects. Recent studies demonstrated the impact of shifts in the gut fungal composition on human diseases like obesity, hepatitis, cirrhosis, autism or IBD (Mogilnicka and Ufnal 2019; Mar Rodríguez et al. 2015; Bajaj et al. 2018; Strati et al. 2017). These prior findings suggest a great impact of diet on the gut mycobiome. But what remained unknown from these studies were the potential interactions of the mycobiome with its host genes. Microbiome next-generation sequencing (NGS) studies in mice have shown that their gut and skin microbiome is defined by host genetics and can even modulate the variation of metabolic traits (Vorobyev et al. 2019; Srinivas et al. 2013; Belheouane et al. 2017). Thus, the diversity of the total microbiome is influenced by environmental and host genetic factors that have been associated with several diseases (McKnite et al. 2012) (figure 5).

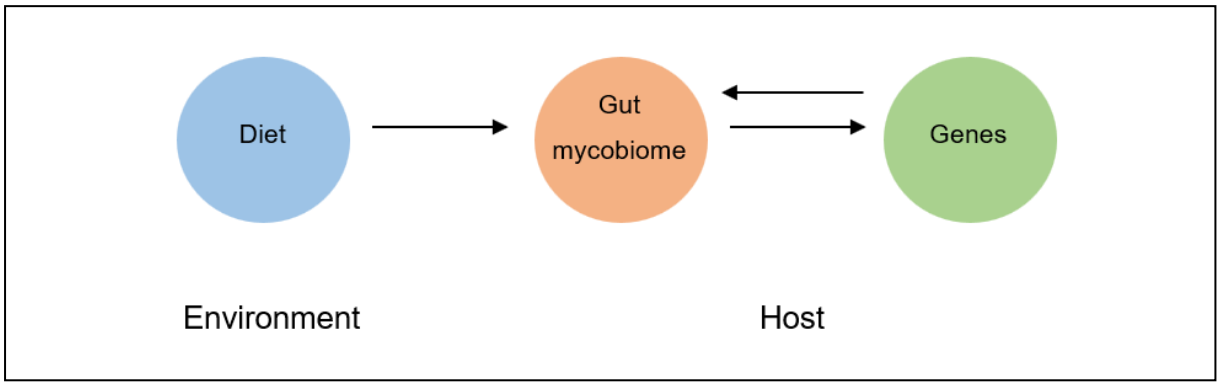


Figure 5: Interaction between the gut mycobiome, genes and environment. Hypothesis by (McKnite et al. 2012): The environment influences the hosts immune system and gut mycobiome via diet, while changes in the mycobiome might influence hosts genetics and the other way around.

1.4.4 Complex traits and quantitative trait loci

As described above, several factors influence the mycobial composition in the gut, those being either genetic or environmental. Complex traits, or quantitative traits, can be complex diseases or phenotypes that are a result of an interplay of genetic variations and environmental influences (Grisel and Crabbe 1995). What defines a complex trait is that they are not inherited following the Mendelian inheritance laws and they are not driven by a single gene locus, for example like eye color or blood type, but by multiple (Boyle et al. 2017; Grisel and Crabbe 1995). Since 2002 Genome-wide association studies (GWAS) successfully study associations between single nucleotide polymorphisms (SNPs) and their corresponding gene loci, the quantitative trait loci (QTL) (Visscher et al. 2017). Studies focus on QTL identification of complex traits like common human diseases or complex phenotypes like human height or weight (Abiola et al. 2003; Ozaki et al. 2002; Visscher et al. 2017). A lot of variants identified through GWAS only contribute minorly to disease susceptibility and different phenotypes due to their apparently small effect size. Hence, they only account for a small percentage of the predicted heritability. Others show large effects and many rare alleles seem to essentially contribute to disease risk (Gibson 2012). GWAS identified gene loci which carry these genetic variants often as noncoding regions of the genome and which are likely to affect gene regulation (Welter et al. 2014). Complex diseases like alcoholism, schizophrenia or autism are described to be polygenic and influenced by many genes located in different areas of the DNA (Goldman 1993; Grisel and Crabbe 1995). In 2009 Frazer et al. described that most variants can only explain a small portion of familial clustering. Thus, the missing heritability of some complex traits still needs to be explained (Manolio et al. 2009).

To understand the linkage between genetic variation and disease in complex traits researchers try to identify the approximate location of the influencing genes within the genome, the so called QTL (Grisel and Crabbe 1995). Due to variations of even one gene in its alleles between individuals in a population and the fact that one person can carry two different versions of that allele from its mother and father, respectively, the identification of QTL can be challenging (Grisel and Crabbe 1995). Most of the QTL studies on complex traits are performed in mice, which is relatively comparable to humans due to large common genome regions (Copeland et al. 1993). Humans show

many physiological, anatomical and metabolic similarities with mice like many comparable protein functions, but there are considerable differences in for example size, shape and longevity due to alterations in gene expression (Boguski 2002; Suzuki et al. 2004; Tautz 2000). More detailed information on QTL will be given later in this introduction (see section 1.5.4).

Complex traits have been a subject to genetic studies since over 20 years with Fisher describing his “infinitesimal model” already in 1919, when he supposed that, if a specific trait is affected by many genes due to a random sampling of their alleles, each gene will produce a continuous and normally distributed phenotype (Fisher 1919). Since 2006, exome sequencing through GWAS has provided details for the understanding of complex traits with the most important loci seeming to only have small effect sizes (Visscher et al. 2017; Manolio et al. 2009). Also, the more genes are involved, the less is their contribution to the heritability of a certain trait (Barton et al. 2017). Boyle et al. described in 2017 that the association signals for complex traits are spread across the genome and include many genes that are not directly connected to the disease. Therefore, they suggested the presence of core disease-regulating genes whose functions are affected by interconnected gene regulatory networks. They furthermore claimed that genes outside the core pathways explain most of the heritability of a complex trait (Boyle et al. 2017).

Common SNPs with low effect sizes have been described by other researchers to account for the majority of heritability (Yang et al. 2010; Shi et al. 2016). For many diseases hits identified through GWAS helped to highlight specific molecular processes like for example the role of autophagy in CD (Jostins et al. 2012). But especially the accumulations of small effects on key genes as well as regulatory pathways accounting for disease risk can drive complex diseases (Furlong 2013; Chakravarti and Turner 2016). Disease-associated SNPs showed an enrichment in active chromatin, so their genetic contribution to the complex trait is heavily concentrated in transcribed regions or relevant tissues (Boyle et al. 2017; Trynka et al. 2013; Finucane et al. 2015; Kundaje et al. 2015). Hence, many complex diseases seem to be driven by a large number of genes with no direct disease relevance. But their effect on a smaller number of core genes and, in addition, virtually any variant

with regulatory effects in a given tissue can show effects on all diseases that are modulated through that tissue (Boyle et al. 2017).

Within complex diseases a quantitative trait normally crosses a certain threshold in order to be expressed (Abiola et al. 2003). From their ITS2 gene and 18S rRNA sequencing of 317 healthy human stool samples, that were collected longitudinally for the Human Microbiome Project (HMP) cohort (Turnbaugh et al. 2007), Nash et al. drew the conclusion that a core gut mycobiome consisting of 15 most abundant genera might exist (Nash et al. 2017). Several studies on mycobial communities using high throughput NGS have shown that there are fewer operational taxonomic units (OTUs) assigned fungal sequences than bacterial abundance (Hallen-Adams and Suhr 2017; Nash et al. 2017). Hallen-Adams et al. observed 72 OTUs in 45 human stool samples which were distributed mostly in the two big fungal phyla Ascomycota and Basidiomycota with *Candida tropicalis* and Dipodascaceae representants being the most abundant species. Other studies using human stool samples also identified between 66 and 75 fungal genera of which *Saccharomyces*, *Candida* and *Penicillium* were the most prevalent (Heisel et al. 2015; Huseyin et al. 2017b). Gut-associated fungal identification by Nash et al. gave, upon ITS2 sequencing, 701 fungal OTUs including 247 named genera. Here, the prevalence of *Saccharomyces*, *Candida* and *Malassezia* was the highest among all samples (Nash et al. 2017).

Diseases that are inherited as complex traits are for instance heart diseases, Alzheimer's disease, as mentioned above schizophrenia and autism as well as for example diabetes, rheumatoid arthritis (RA) or also systemic lupus erythematous (SLE) (Abiola et al. 2003; Grove et al. 2019; Yoo 2015; Kathiresan and Srivastava 2012; Sullivan et al. 2003; Jansen et al. 2019; Regan et al. 2012; Field and Tobias 1997; McCarthy and Menzel 2001; Padyukov et al. 2004). The genetic variations go together with environmental factors, which are normally collected as metadata. These are such as race, age and gender, diet and body mass index (BMI), smoking and several other factors, that all have influence on disease susceptibility. And the probability of developing a certain disease can be increased or decreased, depending on the presence of disease-linked susceptibility genes (Nash et al. 2017; Strati et al. 2016; Abiola et al. 2003). This gene-environment interactions have been shown for instance for the human leukocyte antigen (HLA) locus and smoking that together

enhance the risk for RA (Padyukov et al. 2004). Nash et al. suggested in 2017 that due to no statistical significance in the covariants age, gender or tobacco consume other environmental factors like diet or the hosts genetics may play a larger role.

The influence of diet on complex phenotypes could be shown in a recent study where the tribute of diet to the modulation of complex phenotypes like weight or other metabolic traits (48% of phenotypic variation (PV) in final body weight was explained by diet) was characterized, and showed that autoimmune-prone mice that carry the genetic susceptibility to develop SLE were highly influenced by the dietary regime and in part protected from disease development upon consumption of a calorie-restricted diet (Vorobyev et al. 2019). SLE is a multisystemic autoimmune disease which can lead to nephritis or neuropsychiatric disorders (Doria et al. 2006; Anaya et al. 2013). Susceptibility to lupus is determined by an interplay of genetic and environmental or/and hormonal trigger factors like for instance microbial pathogens that induce antinuclear antibody (ANA) production, UV-light, alcohol, cigarette smoke or vitamin D deficiency (Anaya et al. 2013; Mak and Tay 2014; Cooper et al. 1998; Hedrich 2018). ANA can be found in serum even many years before the onset of lupus disease and they are widely used as disease indicator (Arbuckle et al. 2003). GWAS already identified over 40 robust genetic associations of genes that induce the transcription of proteins involved in SLE pathogenesis, but they only account for one to two percent of the disorder (Han et al. 2009; Harley et al. 2008; Rhodes and Vyse 2008; Cervino et al. 2007). That said, the outcome of complex diseases like SLE may not only be determined by the host genetics, but also by microbial changes, including the fungi, that together with environmental influences might majorly contribute to disease susceptibility.

1.5 Background of the mycobiome analysis

1.5.1 Sample collection

Since more than a decade the knowledge on culture-independent, high-throughput (HT) sequencing technology concerning the mycobiome and its function has greatly expanded (Nilsson et al. 2019). A mycobiome analysis always starts with the sample collection yielding a high number of fungal cells. This can be challenging due to the low abundance of fungi compared to the high abundance of bacteria in the enteric tract,

the vagina as well as on the skin (Li et al. 2018). Also molecular methods like polymerase chain reaction (PCR) can be disturbed by contaminations of the samples through the personnel or the animals (Seed 2014). Swabs and scrapings of the skin have been established as very good sample material for fungal analysis. But that also depends on the body site where the sample is taken from, regarding the different types of dry, moist and oily body sites (Findley et al. 2013; Park et al. 2012). The lung mycobiome can be characterized by either deep sputum samples, bronchoalveolar lavage (BAL), where the airways are saline-washed and sampled by vacuum suctioning, or finally by endoscopy (Cabrera-Rubio et al. 2012). While the oral mycobiome can be analyzed from oral washes and cell collection by centrifugation (Ghannoum et al. 2010) and also from buccal swabs from the cheek (Wade 2013), the intestinal mycobiome is most frequently characterized from faeces as non-invasive method (Hoffmann et al. 2013), but also biopsies or surgical samples taken via oral cavity through the rectum are used for identification of mucosal-associated fungi (Araújo-Pérez et al. 2012).

1.5.2 Molecular identification

To characterize fungal species and their diversities within biological samples HT NGS of rRNA regions is performed frequently. This includes the first described 18rRNA region (Dollive et al. 2012) or the ITS region (figure 6), which are gene regions internal to the 18S, 5.8S and 28S rRNA gene sequences (White et al. 2013). Sequencing is done via PCR (Bell 1989) amplification of this targeted regions using specifically designed primers that are directed against the, through most of the fungi, conserved flanking regions of their variable regions. And these regions themselves differ between the different taxa (Martin and Rygiewicz 2005). In order to extract fungal nucleic acids from biological samples physical methods for cell lysing including lysis of the fungal chitin cell wall are necessary. For this purpose, bead-beating methods for difficult to lyse tissues have been developed which are often combined with strong homogenization and addition of lysing proteins (Goldschmidt et al. 2014; Middelberg 1995; Harder 2008; Müller et al. 1998). After sequencing of the target gene region, the raw data has to be processed and bioinformatically analyzed. Until today, there are different reference databases available as well as analysis tools for the assignment of fungal taxa to the sequences obtained from amplicon sequencing (Findley et al. 2013;

White et al. 1990; White et al. 2013; Kõljalg et al. 2005; Pruesse et al. 2007; Mahé et al. 2012; Santamaria et al. 2012; Di Bella et al. 2013; Cole et al. 2003; Schloss et al. 2009; Caporaso et al. 2010; Mitra et al. 2011). Most of the times, the template is amplicon DNA, which is more stable than the active communities-describing RNA. Amplicons can be sequenced on different sequencing platforms such as Illumina HiSeq-MiSeq, Roche 454 and Ion Torrent, with Illumina MiSeq being one of the most widely used sequencing platforms and sequencing chemistries.

Culture-based methods for the identification of fungal species are still important. One the one hand traditional culture methods can be used to isolate and culture fungi of interest, which have a low abundance within complex microbial communities (Seed 2014), but on the other hand they are labour- and time-consuming in establishing the suitable growth conditions. In addition to that, there are a lot of fungal species that are difficult or unable to be cultured (St-Germain and Summerbell 1996). To overcome the pitfalls of culture-dependent fungal identification NGS is performed nowadays (Heisel et al. 2015; Lindahl et al. 2013).

1.5.3 Next-generation ITS region sequencing of fungal DNA

As mentioned previously, many fungi are difficult to culture or even uncultivable (Huffnagle and Noverr 2013). In order to overcome these pitfalls the nuclear rRNA cistron has been used for fungal diagnostics and phylogenetic studies since over 10 years (Begerow et al. 2010). In 2005 the first HT sequencing platform 454 Life Science (Brandford, CT, USA) launched research (Margulies et al. 2005) and only in 2009 the first fungal ecology studies based on the HT sequencing (Buée et al. 2009; Jumpponen and Jones 2009; Opik et al. 2009) were published. In 2010 Ghannoum et al. successfully performed one of the first sequencing-based approaches to characterize the healthy human mycobiome of the oral cavity. In 2012 Schoch et al. proposed that the ITS regions should be used as the primary and universal barcode for fungal identification (Schoch et al. 2012). Besides a mitochondrial gene region (*CO1*) encoding for the cytochrome c oxidase subunit which is used as barcode for animals, researchers found the ITS region gene most suitable for a successful fungal identification due to its capability in identifying a broad range of fungi (Hebert et al. 2003; Schindel and Miller 2005; Schoch et al. 2012).

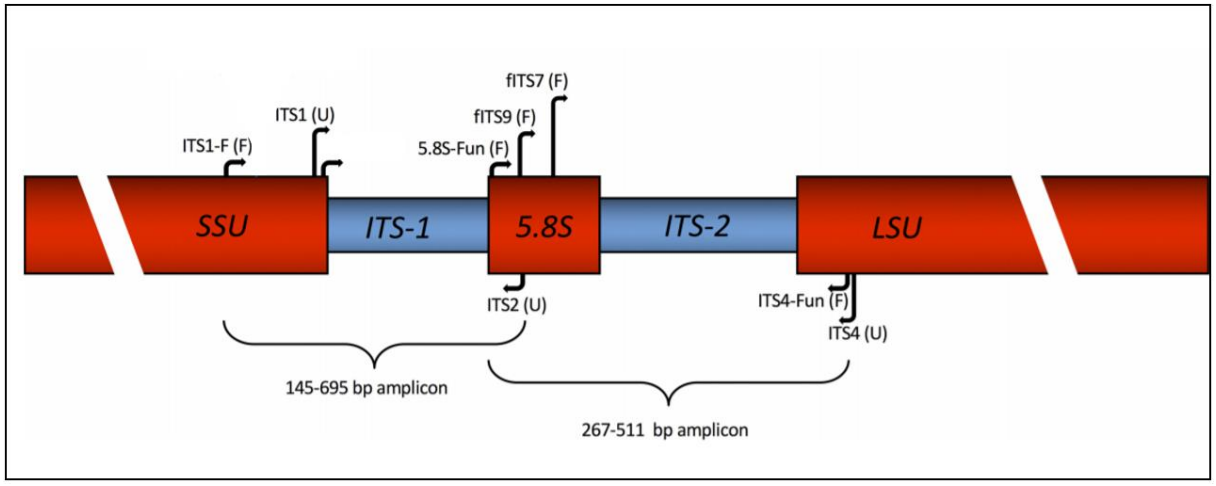


Figure 6: Schematic overview on the fungal ITS region gene and primer map (Taylor et al. 2016, modified). The transcribed but nonstructural spacer regions ITS1 and ITS2 (blue blocks) are located between the 18S small subunit nuclear rRNA gene (SSU), 5.8S (structural RNA gene) and the 28S large subunit rRNA gene (LSU). Primers designed to target these regions are shown and in parentheses, F describes primers selecting against nonfungal taxa and U universal eukaryotic primers. The bp length at the bottom are size estimates of the amplicon lengths. Gene sizes and primer positions are not to scale.

The ITS region genes are hypervariable and at the same time highly conserved throughout many organisms because they do not code for ribosome components (Gweon et al. 2015). The 18S, 5.8S and 28S ribosomal RNA genes (figure 6) are transcribed as one unit by the RNA polymerase I (Schoch et al. 2012). ITS regions are then removed from the rRNA cistron through posttranscriptional splicing before ribosomal assembly (Schoch et al. 2012; Gweon et al. 2015; Lindahl et al. 2013). One big advantage of this region is that the rDNA operon occurs in multiple copies within a genome and can provide up to 100 times more DNA template from starting material than single-copy genes (Herrera et al. 2009). Also even closely related species will differ in their sequence while the intraspecific variation stays low (Lindahl et al. 2013; Gazis et al. 2011; Schoch et al. 2012). ITS genes show more variability in fungi than small subunit (SSU) genes which makes a specific genetic identification possible (Eberhardt 2010).

When deciding on which ITS region to sequence, both ITS1 and ITS2 region sequencing have their advantages and disadvantages but both give comparably

similar results when using a 97% similarity cut-off regarding taxonomic coverage and species identification (Blaalid et al. 2013). The length of ITS2 is generally less variable and does not bear problems like co-amplification of a 5' SSU intron which is common in many Ascomycetes (Lindahl et al. 2013). ITS1 sequencing can lead to a bias towards Basidiomycetes identification, whereas ITS2 amplicons rather favours Ascomycetes identification (Bellemain et al. 2010). In this work, ITS2 region sequencing was used for analysis of the mycobiome.

1.5.4 QTL-mapping and the use of outbred mouse strains

As mentioned above, HT sequencing has provided a lot of efficiency in the discovery and analysis of QTL and the identification of SNPs of large sample cohorts (Suzuki et al. 2004; Gupta et al. 2008). For the detection of QTL a linkage disequilibrium (LD) between marker variant and casual variants is needed due to the non-random association of alleles at different loci in the corresponding population (Slatkin 2008). A QTL can be described as genetic locus on a chromosome with alleles affecting a certain PV in a measurable quantitative trait, and by mapping a specific gene or locus to a chromosomal segment within 10-30 centimorgans (cM) one can specifically determine SNP occurrences and positions (Abiola et al. 2003). In fact, the higher the number of included samples in the analysis, the higher the significance for a given size of a QTL (Abiola et al. 2003). Lander and Kruglyak suggested the use of logarithmic odds ratio (LOD) threshold values to be able to avoid false QTL detection during analysis. But because of linkage, neighboring genomic positions are not independent and will therefore show equivalent test statistics (Lander and Kruglyak 1995; van Ooijen 1999).

Nowadays, in QTL analysis, permutational tests to obtain threshold values which are adjusted for multiple testing are much more precise (Doerge and Churchill 1996). Here, phenotypes are shuffled separately within genotyped and ungenotyped individuals and genome scans are repeatedly done on these shuffled versions of the obtained data to estimate an appropriate LOD threshold for the yield of true QTL. And permutation can also be applied when the examined trait is not normally distributed (Manichaikul et al. 2007; Doerge and Churchill 1996). Hence, besides the LOD score, statistical analyses always give peak positions as well as the estimated confidence interval to compare the mapped position of a QTL with that for another QTL which controls related traits (Abiola

et al. 2003). When the QTL have been mapped to chromosomal segments, QTL-Fine mapping is further done to locate the genes precisely within a range less than one to five cM. To identify candidate genes from an already identified QTL set full genome sequencing and genotyping of the used mouse sample cohort is necessary, and only those genes with a strong effect on the phenotype can be targeted (Abiola et al. 2003). While determing a representative QTL, one should always be able to state evidence supporting the relation between the gene of function and the expression of the investigated complex trait (Abiola et al. 2003). That said, mouse crosses with many recombinant events are most successful because the genetic variation is essential for identification of candidate genes that underlie complex phenotypes (Brekke et al. 2018). In order to follow the given hints of a correlation of the mycobiome and its interactions with environmental factors and genes in humans, the use of mice is an easier approach to study these questions in mammals.

The use in genetic research of for example recombinant inbred strains or/and crosses (Bailey 1971; Threadgill et al. 2002), the most commonly used congenic or/and recombinant congenic strains (Bennett et al. 2002; Stassen et al. 1996) or also heterogeneous stocks (Talbot et al. 1999), consomic strains (Nadeau et al. 2000), near-isogenic lines (Martin et al. 1991), recombinant QTL-ingression strains (Vadasz et al. 2000) or knock-out strains (Bolivar et al. 2001) changed towards the use of outbred mice when trying to understand complex human diseases (Talbot et al. 1999; Solberg Woods 2014). Outbred mice, that are nonisogenic and created to maintain maximum heterozygosity, can display a more comparable model for studies on genetic contributions to complex traits in humans due to the high genetic diversity in human populations (Solberg Woods 2014). More recent animal models like the diversity outbred (DO) mouse line (Churchill et al. 2012) result in larger QTL with hundreds of genes that have to be narrowed down to single loci for the identification of candidate genes. But they can give a tremendously higher quantity of information and a higher possibility of SNPs identification within the mouse genome (Solberg Woods 2014).

The statistical power of study designs using inbred strain backcrosses and intercrosses is high but the mapping resolution is limited and identification of causal variants can take a lot of time depending on data size (Churchill et al. 2012). In this work, an advanced intercross outbred (AIL) mouse line crossed over 20 generations (Vorobyev

et al. 2019) was used to display human genetic diversity for the identification of gene candidates (see also section 2.2).

1.6 Aim of this work

While the bacterial microorganisms within the microbiome have been shown to modify human physiology like its immune development and function, fungal contribution and interactions with the host have been less extensively studied. In addition to that, the influence of host genes on fungal diversity and composition has been poorly investigated until now. The worldwide number of fungal infections is increasing due to a higher prevalence of immunocompromising comorbidities that can subsequently result in the turning of commensals into pathogens and that are driven by environmental factors. In this study the aim was to investigate how host genes and diet influence the intestinal mycobiome in the mouse as model organism for mammals.

The contribution of susceptibility genes together with the influence of diet on the variability of an investigated complex trait has come to focus recently. In modern industrialized countries diet displays a major factor in disease development and severity as to the so-called Western diet contains a high amount of fat, cholesterol and carbohydrates. The gut is one of the main human immune organs, and to investigate the correlation and impact of diet on the gut fungal population, an AIL mouse line was created by crossing a characterized wildtype mouse strain with three autoimmune-prone mouse strains for over 20 generations resulting in an experimental cohort with a high genetic diversity. By feeding a total of 1.154 of these mice either a Western, calorie-restricted or control diet characterization of their gut mycobiome and identification of candidate genes, that contribute to variations in the complex phenotype of these mice and that are linked to the changes of fungal diversity under the influence of diet as a confounding factor, were aimed. These identified genes could further be objective to knock-out studies for the investigation of their disease contribution.

In order to identify these genes, the establishment of an isolation protocol for fungal DNA from murine gut samples as well as the development of ITS2 region NGS from these samples is one part of this work. The influence of the gut fungal population on complex phenotypes is suggesting a pharmacotherapeutic potential concerning the development of drugs that modulate the mycobiota. This may also include probiotic

treatment options or even fecal transplantations. The understanding of the host-genome interactions of the mycobiome could offer biological insights leading to more knowledge on the correlation between fungal communities and the hosts genes as well as on factors that have the potency to shape the mycobiome.

2 Material and methods

2.1 Chemicals, kits and laboratory equipment

The basic chemicals used in this work were purchased from the companies Sigma, Roth, and Merck. The basic laboratory equipment (plastic material and glass bottles) was purchased from the companies Eppendorf, Sarstedt and Schott Duran. All chemicals and kits were used according to the protocols of the manufacturers. The water used for analysis was always deionized and RNAse/DNAse-free.

2.1.1 List of devices used in this work

Device	Manufacturer
OHAUS Precision Standard balances	Ohaus, Nänikon (CH)
EMB 220-1 Digital laboratory balances	Kern&Sohn, Balingen (D)
Magnetic thermo stirrer RCT basic	IKA-Werke Staufen (D)
Microcentrifuge	Sigma Laborzentrifugen, Osterode am Harz (D)
Pipetus	Hirschmann Laborgeräte, Eberstadt (D)
Waterbath Tyep3042 to dissolve buffer crystals	Köttermann Labortechnik, Uetze/ Hänigsen (D)
Mini centrifuge Labnet C-1202	Labnet International, Inc., Woodbridge (USA)
-20° Celsius freezer for storage	Liebherr, Biberach an der Riß (D)
4-8° Celsius fridge for storage	Liebherr, Biberach an der Riß (D)
-80° Celsius freezer for storage	Liebherr, Biberach an der Riß (D)
Heating-ThermoMixer	HLC by DITABIS, Pforzheim (D)
UVC/T-AR DNA/RNA UV-cleaner box	Biosan, Riga (LV)
Precellys tissue homogenizer	Bertin Technologies SAS, Montigny-le-Bretonneux (F)
MiSeq Sequencer	Illumina, San Diego (USA)
Agilent Bioanalyzer	Agilent, Santa Clara (USA)

Power Pack Basic Gel electrophoresis system	Bio-Rad, Hercules (USA)
microwave	Gorenje, Velenje (SVN)
Vilber E-Box CX5.TS for gel imaging	Vilber Lourmat SAS, Collegién (F)
Laptop for data analysis Lenovo Flex 2-14	Lenovo, Quarry Bay (HK)
Magnetic plate for 96-well plates	Thermofisher Scientific, Dreieich (D)
Mastercycler ep Realplex for qPCR	eppendorf, Hamburg (D)
Table centrifuge 5415 R	eppendorf, Hamburg (D)
Nanodrop 2000 spectrophotometer	Thermofisher Scientific, Dreieich (D)
Speed Mill Plus homogenizer	analytik jena, Jena (D)

2.1.2 List of materials used in this work

Material	Manufacturer
Eppendorf Research pipettes (0.5-10µl, 0.1-2.5µl 2-20µl, 10-100µl, 20-200µl; 100-1000µl)	Eppendorf, Hamburg (D)
Eppendorf Research pipette 0.5µl-10µl Multichannel (12)	Eppendorf, Hamburg (D)
Pipette tips for Research (10µl, 200µ, 1000µl)	Sarstedt, Nümbrecht (D)
Biosphere Filtertips 0,1µl-20µl farblos	Sarstedt, Nümbrecht (D)
Biosphere Safe Seal 0.5, 1.5ml, 2.0ml Tube RNAse/DNAse-free	Sarstedt, Nümbrecht (D)
Sterile tubes 15ml and 50ml	Sarstedt, Nümbrecht (D)
PCR Soft tubes 0.2ml transparent, DNA/DNAse/RNAse-free	Biozym Scientific GmbH, Oldendorf (D)
Serological one-time-use pipettes (5ml,10ml, 25ml)	Sarstedt, Nümbrecht (D)
Plate sealing foil for 96-well plates, transparent	Sarstedt, Nümbrecht (D)
Alu plate sealing foil for 96-well plates	Sarstedt, Nümbrecht (D)

Eppendorf Combitips advanced, 5ml, 10ml Biopur	Eppendorf, Hamburg (D)
Qiagen collection tubes 2ml	Qiagen, Venlo (NL)
Glass bottles for buffer storage	Schott Duran, Main (D)
X-TRACTA Tips for gel extraction	Biozym, Hessisch-Oldendorf (D)
96-well PCR Plate half skirt flat	Sarstedt, Nümbrecht (D)

2.1.3 List of Reagents and kits used in this work

Reagent/kit	Manufacturer
DNeasy PowerLyzer PowerSoil Kit	Qiagen, Venlo (NL)
MiSeq V3 reagent kit (600 cycles)	Illumina, San Diego (USA)
MinElute Gel Extraction Kit	Qiagen, Venlo (NL)
dNTP Mix (10 mM)	Thermofisher Scientific, Waltham (USA)
DNA Gel Loading Dye (6x)	Thermofisher Scientific, Waltham (USA)
Gene Ruler 100bp Plus ready-to-use DNA Ladder	Thermofisher Scientific, Waltham (USA)
SYBR Green and SYBR Safe DNA Gel stain	Thermofisher Scientific, Waltham (USA)
Absolute 200 proof Ethanol Biology Grade	Thermofisher Scientific, Waltham (USA)
MiSeq V2 reagent kit (250 cycles)	Illumina, San Diego (USA)
AMPure Beads XP Kit	Beckman&Coulter, Brea (USA)
Agilent High Sensitivity DNA Kit	Agilent, Santa Clara (USA)
PhiX Control v3	Illumina, San Diego (USA)
High Capacity cDNA Reverse Transcription Kit	Thermofisher Scientific, Waltham (USA)
NEBNext Library quantification Kit for Illumina	New England Biolabs, Ipswich (USA)
ITS primers	Metabion, Planegg (D)
Qiagen DNAse/RNAse-free DNAse set	Qiagen, Venlo (NL)
Proteinase K	Qiagen, Venlo (NL)
Phusion Hot Start II DNA Polymerase	Thermofisher Scientific, Waltham (USA)

HD Green gel stain	Intas Science Imaging, Göttingen (D)
GeneRuler 1 kb Plus ready-to-use DNA Ladder	Thermofisher Scientific, Waltham (USA)
Ethanol 70%	Carl Roth, Karlsruhe (D)
Agarose	Biozym, Hessisch-Oldendorf (D)
Ultrapure Agarose	invitrogen, Karlsbad (USA)
Deionized water (H_2O millipore)	Ampuwa Plastipur Fresenius Kabi, Bad Homburg (D)

2.1.4 List of software used in this work

Software	Manufacturer
Microsoft Excel (Office) 2010-2016	Microsoft Corporation, Redmond (USA)
X64 Linux system	https://github.com/torvalds/linux
R	R Core Team, 2013, http://www.R-project.org
Vegan library package	Dixon, 2009, available at https://github.com/vegandevs/vegan
PIPITS	Gweon et al. (2015), https://github.com/hsgweon/pipits
VSEARCH	V2.7, https://github.com/torognes/vsearch
RDP Classifier-2.12	Wang et al. (2007)
Linux ubuntu software	Linux Foundation, San Francisco (USA)
QIIME1	V1.9.1, http://qiime.org/index.html
Conda environment	http://anaconda.com
Python	V2.7.1,https://docs.conda.io/en/latest/miniconda.html
Office365	Microsoft, Redmond (USA)
Vision-Capt 16.16.0.0	Vilber Lourmat SAS, Collegién (F)

2.2 Animals and sample collection

All animal experiments have been conducted prior to this work by certified personnel. The study was set up by Dr. Artem Vorobyev. The MRL/MpJ, NZM2410/J, BxD2/TyJ and Cast/EiJ breeding pairs were obtained from the Jackson laboratories (Maine, USA), and further breeding was performed in the animal facility of the University of Lübeck, Germany. By crossing at equal strain and gender distributions as previously described (Srinivas et al. 2013) an AIL mouse line was generated (figure 7). Through random and sequential intercrossing this experimental population can provide increased mapping resolution due to an increased probability of recombination between loci. For example, only eight additional mating generations using the same population size and QTL effect already reduce a 95 % confidence interval of a QTL five times (Darvasi and Soller 1995). The mice used in this study were intercrossed for 20 generations. After weaning at three to four weeks of age offspring mice were transferred into separate cages. Either female or male mice were put into cages and were randomly assigned to one of the three diets: control mouse chow (Altromin Spezialfutter GmbH, Lage, Germany) ad libitum, calory-restricted diet consisting of 60 % of the control mouse chow and a Western diet rich in cholesterol, butterfat, salt and sugar (ssniff Spezialdiäten GmbH, Soest, Germany) until the age of six month.

A total of 1,154 mice were held under specific-pathogen-free conditions at a twelve-hour light/dark cycle at the animal facility of the University of Lübeck, Germany. After two- and four-months blood samples were taken by facial vein puncture and by cardiac puncture at six months of age after euthanization. The blood samples were analyzed with a HemaVet950 (Drew Scientific Inc, Miami Lakes, FL USA). In addition, stool samples were collected and the weight of the animals was obtained on the second, fourth and sixth month of dietary intervention. At the age of six months, all mice were euthanized using CO2 and cecum content samples were collected into RNA*later*™ Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA), incubated at 4 °C for 24 hours, centrifuged and frozen at -20 °C until further analysis. The mice were scored blindly and all animal experiments were conducted according to the European Community rules for animal care, approved by the respective governmental administration (Ministry for Energy, Agriculture, the Environment and Rural Areas, file number 27-2/13) and performed by certified personnel.

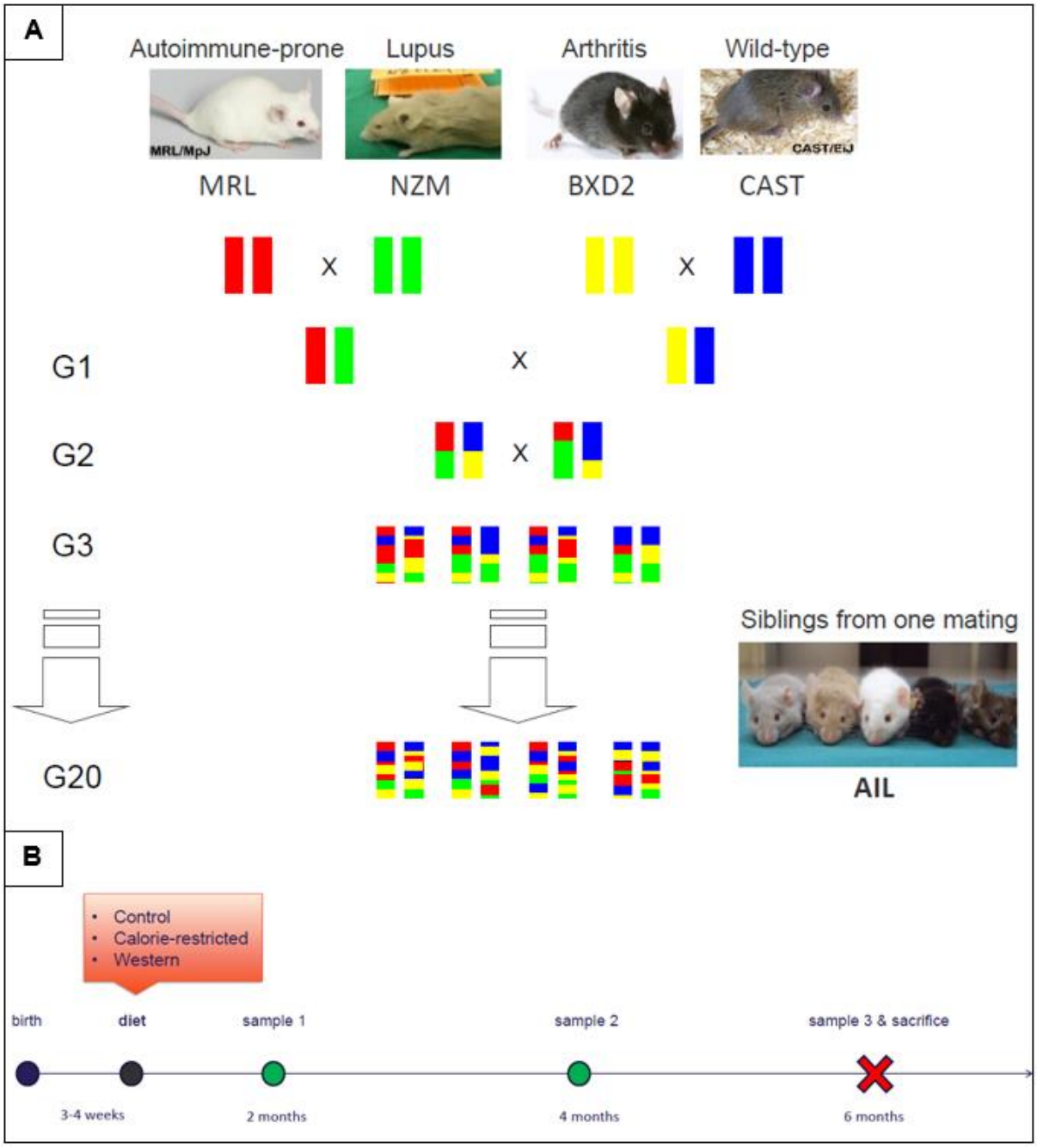


Figure 7: AIL Breeding scheme and experimental setup. A: NZM (Lupus-prone), BDX2 (Arthritis-prone), MRL (Pancreatitis-prone) and CAST wildtype mice were crossed over 20 generations (G) with 50-60 matings per generation allowing for multiple genetic recombinations among the 4 strains. Genomes of different strains are represented by different colors. Siblings from one mating represent high genetic and phenotypic diversity in AIL mice. B: Experimental setup of the feeding and sampling strategy. Mice were divided in groups and fed three different diets (caloric restriction, control and Western) shortly after weaning (3-4 weeks) and samples were taken after 2 months, 4 months and after 6 months during sacrifice. The experiments were conducted beforehand by Dr. Artem Vorobyev together with certified personnel.

2.3 Genotyping of the mice

In order to identify QTL, mice were genotyped prior to this work. Genomic DNA was isolated from the tail tips of a total of 1,154 mice at the final stage after six months. The DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) was used to isolate DNA according to the manufacturer's instructions. Isolated DNA was quantified with the NanoDrop2.0 (Implen, Munich, Germany) and stored at -20 °C until further use. DNA was analyzed by MegaMUGA genotyping array (Neogen Genomics, Lincoln, Netherlands) covering 77,800 markers throughout the mouse genome. Using the plink toolset (http://pngu.mgh.harvard.edu/purcell/plink/), non-informative SNPs were filtered out based on a minor allele frequency (maf) of > 0.05, a missing geno probability of < 0.1 and common homozygous SNPs among the founders resulting in 55,458 SNPs, which were used in downstream analysis.

The Hap.py R tool (https://github.com/Illumina/ hap.py/blob/master/doc/happy.md) was then used for probabilistic reconstruction of the AIL mouse genome in terms of that of the four founder strains. The posterior probability that each mouse was in one of the four possible genotype states was determined by using a hidden Markov model at every adjacent marker interval across a chromosome. This probability was then converted to three-dimensional arrays in R, and an intraindividual relationships representing kinship matrix was calculated using the kinship.probs online function (DOQTL R package, https://www.rdocumentation.org/packages/DOQTL) by a certified bioinformatician. Afterwards, each trait was fitted for sex, diet as a fixed effect and kinship as a random effect to estimate residuals (r) using the hglm R package (Hagerty et al. 2000) . A total of three types of single-locus QTL effects on traits were tested. The host-genotype (G) association with r, where log likelihood ratios of traits for each interval across the genome were calculated and converted to LOD scores, then the G x Diet association with r by calculating LOD scores while comparing likelihood values for G and G x Diet, and finally, LOD scores were also calculated for G x Sex interactions. The confidence interval for a QTL was described by a 1.5 LOD drop.

2.4 Whole genome sequencing of founder strains

Genomic DNA of three of the founder strains (NZM2410/J, MRL/MpJ, BxD2/TyJ) was isolated prior to this work as described in section 2.3. Analysis of the founder strains

was performed in the course of a previous study by Dr. Artem Vorobyev and colleagues (Vorobyev et al. 2019). The genome of the wildtype CAST strain was publicly available. To detect possible DNA degradation, the quality of the obtained genomic DNA was controlled by electrophoresis on a 0.7 % agarose gel at 15 V overnight. Whole genome sequencing (WGS) was performed on a HiSeq X machine, using 150 x 2 paired end sequencing (Quick Biology, Pasadena, CA, USA) resulting in a fastq dataset. The quality of the sequenced reads was evaluated by Fastqc software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads with a phred score less than 30 were filtered. The remaining reads were aligned to a C57BL/6J GRCm38 mouse reference genome and BAM files were obtained for each strain using the BWA-MEM (v0.7.10) software with default parameters (http://bio-bwa.sourceforge.net/bwa.shtml#13). The BAM files were further evaluated for the alignment quality using a reference genome and the Qualimap software (http://qualimap.bioinfo.cipf.es/).

Downstream analysis for SNP and indel detection was performed as previously described (B et al. 2015). Each BAM file was sorted and filtered for possible PCR and optical duplicates using Picard Tools version v1.141 (Li et al. 2009), SNP and indel calling around indels were improved by realigning the reads by using the GATK v3.5 'IndelRealigner' tool. A combination of SAMtools mpileup (v1.5) and BCFtools call (v1.5) was used for the identification of SNPs and indels. Low confidence variants and false positive SNPs and indels due to alignment artifacts were removed and thereby only high-quality and homozygous variants resulted. Additionally, the SNPs and indels common among the four founders were filtered out and SNPs and indels were annotated for their functional class, consequence and known transcripts if available using Ensembl VEP server (https://www.ensembl.org/info/docs/tools/vep/index.html).

2.5 Gut mycobiota analysis

2.5.1 DNA isolation and PCR

Fungal DNA was isolated with the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Venlo, Netherlands) from 600 cecum content samples of genotyped AIL mice from generation 18-20 with addition of Proteinase K (Qiagen, Venlo, Netherlands) to ensure proper lysis of the fungal cell wall. After 2 hours shaking at 900 rpm at 50 °C cecum content

was homogenized with a Precellys 24 tissue homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France) and isolation of the DNA was performed following the manufacturer's protocol. During each isolation, one negative isolation control (NC, empty buffer used as sample input) was included. The nuclear ribosomal (ITS) 2 region was amplified using a dual indexing approach as previously described (Ghannoum et al. 2010; Kozich et al. 2013). The ITS-specific sequencing primers ITS4 and fITS7 were linked to a unique eight-base multiplex identifier designated as XXXXXXXX (Ihrmark et al. 2012), a 10-nucleotide pad sequence for the prevention of hairpin formation (underlined), a 2-nucleotide linker sequence and ITS2-specific primer sequence with the reverse primer being degenerated at one position (forward 5′-AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTATGGTAATTGGTCCTC CGCTTATTGATATGC-3′, reverse 5′-CAAGCAGAAGACGGCATACGAGATXXXXXXXXAGTCAGTCAGCCGTGA[AG]TCA TCGAATCTTTG-3′). Figure 8 shows the primer construct. All PCR amplifications were conducted in a 25µl volume using the Phusion Polymerase (Thermofisher, Waltham, Massachusetts, USA). The cycling conditions were as follows: initial denaturation for 30 s at 98 °C; 35 cycles of 9 s at 98 °C, 30 s at 50 °C, and 30 s at 72 °C, final extension for 10 min at 72 °C. Template-free reactions were performed with all forward and reverse primer combinations used on the day of PCR performance to make sure the primers were not contaminated. The full primer sequences used in this work are shown in table 1.

Primers

Illumina adapter----------INDEX----------f/r pad-----linker---gene-specific primer
AATGATACGGCGACCACCGAGATCTACAC---XXXXXXXX---TATGGTAATT---GG---TCCTCCGCTTATTGATATGC

Figure 8: Construct of customized sequencing primers. The ITS-specific sequencing primers contained the Illumina adapter which was linked to a unique eight-base multiplex identifier (XXXXXXXX), a 10-nucleotide pad sequence, a 2-nucleotide linker sequence and the ITS2-specific primer sequence.

	Primer name	ID	Sequence
A	ITSF.SB501	1	AATGATACGGCGACCACCGAGATCTACACCTACTATATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB502	2	AATGATACGGCGACCACCGAGATCTACACCGTTACTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB503	3	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB504	4	AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB505	5	AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB506	6	AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB507	7	AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB508	8	AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB509	9	AATGATACGGCGACCACCGAGATCTACACCTGAAGTCTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB510	10	AATGATACGGCGACCACCGAGATCTACACACGATCGTTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB511	11	AATGATACGGCGACCACCGAGATCTACACATATGGCCTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB512	12	AATGATACGGCGACCACCGAGATCTACACTTCGATGGTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB513	13	AATGATACGGCGACCACCGAGATCTACACTTGGTACGTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB514	14	AATGATACGGCGACCACCGAGATCTACACCGTTGGATTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB515	15	AATGATACGGCGACCACCGAGATCTACACCGTTAAGCTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB516	16	AATGATACGGCGACCACCGAGATCTACACACAGCTCATATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB517	17	AATGATACGGCGACCACCGAGATCTACACGACAAGTGTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB518	18	AATGATACGGCGACCACCGAGATCTACACGCATTAGCTATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSF.SB519	19	AATGATACGGCGACCACCGAGATCTACACTGTGGACTTATGGTAATTGGTCCTCCGCTTATTGATATGC
B	ITSR.SA701	A	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA702	B	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA703	C	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA704	D	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA705	E	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA706	F	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA707	G	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA708	H	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA709	I	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA710	J	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA711	K	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA712	L	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA713	M	CAAGCAGAAGACGGCATACGAGATTACGTACGAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA714	N	CAAGCAGAAGACGGCATACGAGATGATCACGTAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA715	O	CAAGCAGAAGACGGCATACGAGATGTGACAGAAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA716	P	CAAGCAGAAGACGGCATACGAGATAACCGGAAAGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSR.SA717	Q	CAAGCAGAAGACGGCATACGAGATCAACTGGTAGTCAGTCAGCCGTGARTCATCGAATCTTTG
C	ITSRead1		TATGGTAATTGGTCCTCCGCTTATTGATATGC
	ITSRead 2		AGTCAGTCAGCCGTGARTCATCGAATCTTTG
	ITSIndex		CAAAGATTCGATGA[CT]TCACGGCTGACTGACT

Table 1: Full sequences of primers used in this work. Primers are listed as forward primers (A), reverse primers (B) and Illumina sequencing primers (C). The full sequences are shown. The IDs were used in the preparation process for an easier handling. Blue: Illumina sequencing adapter and linker. Yellow: Unique Barcode Identifier.

2.5.2 ITS2 gene sequencing

2.5.2.1 Library preparation

The PCR products were quantified on a 1.5 % agarose gel (Biozym, Hessisch-Oldendorf, Germany) that was run at 120V for 5 minutes followed by 110 V for 1 hour. Gel pictures were taken on a Vilber E-Box CX5.TS (Vilber Lourmat SAS, Collegién, France) and image analysis was done using the image analysis software Vision-Capt version 16.16.0.0 (Vilber Lourmat SAS, Collegién, France). After quantification and quality control 477 fungal PCR products were equimolarly mixed into subpools and those were further run on an ultrapure agarose gel (Thermofisher, Waltham, Massachusetts, USA) and the bands around 470 bp, representing the amplicon product, were extracted with the MinElute Gel Extraction Kit (Qiagen, Venlo, Netherlands). Then, the concentration of each subpool was determined with the NEBNext Library quantification Kit for Illumina (New England Biolabs, Frankfurt am Main, Germany) on an Eppendorf Mastercycler ep Realplex (Eppendorf, Hamburg, Germany) according to the manufacturer's instructions.

The quantified subpools were combined into two equimolar libraries, each library containing between 220 and 257 samples for sequencing. The libraries were then further purified using AMPure Beads XP Kit (Beckman&Coulter, Brea, CA, USA) and again quantified with the NEBNext Library quantification Kit. Prior to sequencing, the average amplicon size of the library was determined by the Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). Each library was then sequenced on a MiSeq (Illumina, San Diego, CA, USA) using the MiSeq v3 600 cycles sequencing chemistry (Illumina, San Diego, CA, USA) at a concentration of 17.5 pM together with 10 % of a PhiX Control v3 library (Illumina, San Diego, CA, USA). From those samples, which did not reach a statistical threshold in sequencing, a third library was pooled and sequencing was repeated.

2.5.2.2 Sequencing by synthesis

NGS has enabled HT sequencing and is therefore nowadays commonly used for large-scale projects to produce millions of reads from one sample. It shows several more advantages over the "old" gold standard, the Sanger method, which still requires an electrophoresis to separate the DNA products and which is lacking complexity. Besides

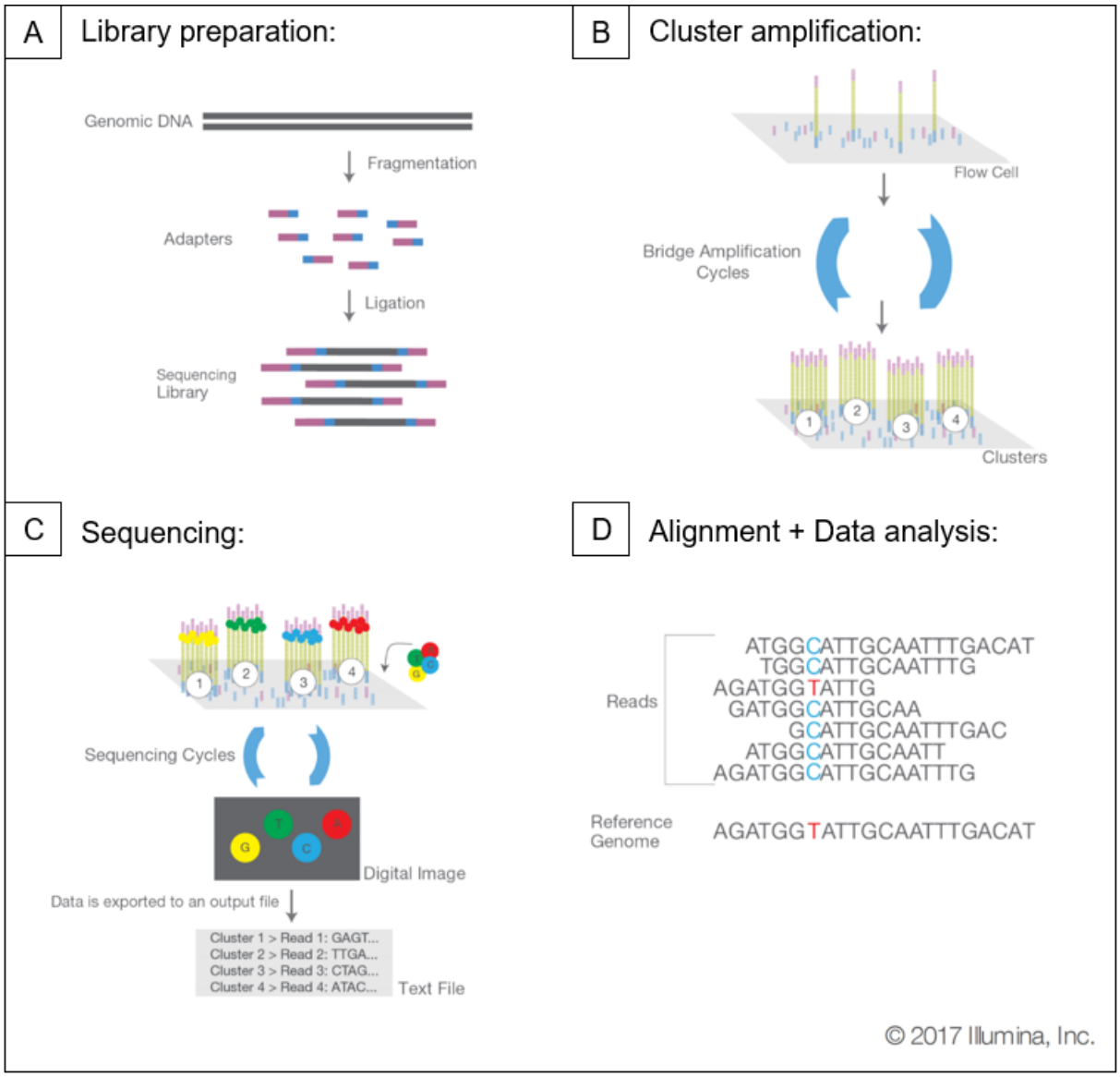


Figure 9: Illumina MiSeq NGS principle and workflow (https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf, modified). Illumina NGS workflow including library preparation, cluster amplification, sequencing and alignment with data analysis. A: the NGS library is prepared from DNA fragments that contain specialized adaptors at both fragment ends. B: The fragments are hybridized to the flow cell surface, a glass slide coated with a loan of oligonucleotides, and are bridge amplified into clonal clusters. C: Fluorescently labeled nucleotides are added and single bases are incorporated and imaged through cluster emission. Bases are identified through their emission wavelength and its intensity. The sequencing cycle is repeated “n” times (300 cycles each for forward and reverse read) to create a read length of “n” bases. Forward and reverse reads are output in separate files and can later be connected by nucleotide overlaps. D: The joined reads are then aligned to a reference sequence to identify differences in the nucleotide sequences.

other new sequencing approaches, the sequencing by synthesis (SBS) technology is widely used (figure 9). SBS is a base-by-base sequencing by using a DNA polymerase which extends a to the template hybridized primer by a single nucleotide. Each nucleotide is identified and monitored. The difference to Sanger sequencing is that individual bases are detected simultaneously on a high-density array, the flow cell, where over tens of millions of DNA sample spots can be arrayed and sequenced (Guo et al. 2010; Bubnoff 2008; Sanger et al. 1977).

2.6 Data processing and statistical analysis

All statistical analysis was done with the help of Dr. Yask Gupta. The raw fungal data was generated as gzipped FASTQ format files on the Illumina MiSeq sequencing platform and consisted of ~20 million reads. The reads were analyzed on a x64 Linux system (https://github.com/torvalds/linux) using the automated open-source bioinformatic pipeline PIPITS (Gweon et al. 2015), which is shown schematically in figure 10. Sequences were classified using the RDP Classifier-2.12 (Cole et al. 2003) against the UNITE fungal data set (Kõljalg et al. 2005). The paired-end reads were merged and quality filtered using the “PIPITS_PREP” module using the fastx-toolkit for merging paired reads while filtering out reads below $q < 20$. Subsequentially, the ITS gene region reads were extracted with the “PIPITS_FUNITS” module. The UNITE UCHIME dataset was used for reference-based chimera removal and UCHIME (Nilsson et al. 2015) for de novo chimera removal by the vsearch (Rognes et al. 2016) algorithm version 2.8 with $E = 0.5$.

Reads were classified from phylum to genus level using the RDP classifier with the UNITE database as reference at a confidence of 0.80 and 1000 iterations (Cole et al. 2003; Kõljalg et al. 2005) and OTU clustering at a 97 % threshold was performed with default vsearch parameters. The RDP classifier was also used to taxonomically classify the FASTA sequences for each OTU. And after singleton removal the OTU abundance table was extracted and further ecological analysis was then done in QIIME (Caporaso et al. 2010). The samples were rarefied (subsampled) to 5,000 reads and statistical analysis was done using R (RCore Team, 2013, http://www.R-project.org) including the vegan library package (Dixon, 2009, available at https://github.com/vegandevs/vegan).

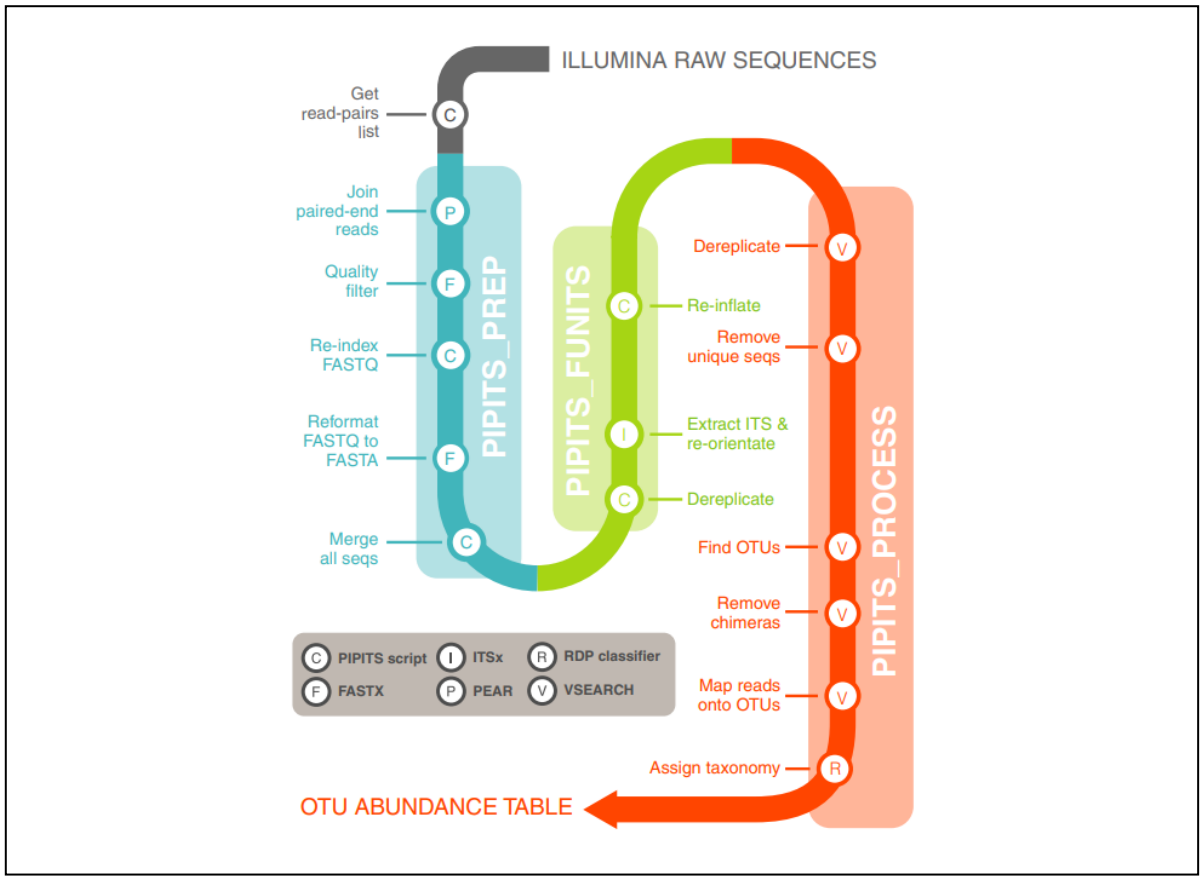


Figure 10: Schematic overview on the PIPITS analysis workflow (Gweon et al. 2015). PIPITS_PREP (blue) joins paired-end reads, quality filters the reads upon a certain threshold and reformates data into FASTA format. PIPITS_FUNITS (green) dereplicates sequences and extracts the ITS gene region. PIPITS_PROCESS (red) processes the extracted sequences while removing unique sequences and creating OTU abundance tables by mapping the reads onto OTUs and assigning the reads to taxonomy.

The alpha diversity, meaning the species richness of the samples, was obtained using the Shannon index (mean) and significance was assessed using the Mann-Whitney U test and/or Kruskal-Wallis test. For the beta diversity the non-Eucledian Bray Curtis metrics as well as the Jaccard distance were used for computing the dissimilarity between the samples. Further, PcoA was performed (Pearson 1901; Jolliffe and Cadima 2016). The significance of the beta diversity among the three different groups (stages) was calculated utilizing the distance-based redundancy analysis (dbRDA, https://rdrr.io/cran/vegan/man/capscale.html#heading-7) with removal of the effect of diet. Using the adonis function in R (https://CRAN.R-project.org/package=Cite) significance was assessed and differentially abundant fungal taxa could be identified using LEfSe, a tool developed by the Huttenhower group and implemented in QIIME

to find biomarkers between two or more groups based on relative abundances (Segata et al. 2011). Due to a comparably low diversity of fungal species the minimum cluster size was kept at five. And after calculation of the functional fungal community (FFC) eigenOTUs were correlated to different traits like disease stage, sex or diet to further identify FFCs associated with a certain trait.

2.7 QTL-mapping

Chromosomal regions of the mouse genome, that were already mapped with distinct complex traits within a confidence interval of 2-3 Mb, should further be resolved to single or multiple genes using the WGS of the founder strains. The analysis was performed by Dr. Yask Gupta. For each QTL the founder allele effect was estimated. Differences in one or two alleles from other founder strains due to single diallelic polymorphism, that can lead to trait variations, were manually inspected and strain-specific SNPs and indels were kept in an investigated QTL. In addition, identified SNPs and indels were favored based on their consequences (Ensembl VEP). This led to filtering of the genes that were not polymorphic among the founders and that were causing variation in the trait. The remaining genes in the QTL were investigated for association with traits by curated databases such as GWAS Catalog (Buniello et al. 2019) and GeneCARD (Stelzer et al. 2016) and thorough literature search by two independent investigators. Once having identified several traits associated with genes, SNPs and indels associated with the genes were investigated. Polymorphisms in the 5'UTR (untranslated region), 3'UTR (regulator of gene expression) and missense mutation were most important.

To analyze the relationship of the hosts genetics with the gut mycobiota, residuals of taxonomical abundances using a linear mixed model were identified. Here, generation was considered as fixed effect and cage as random effect. Then, these residuals were used as traits to investigate associations of the gut mycobiota with the host genetics only (Additive QTL), host genetics interacting with diet (Intdiet) and host genetics interacting with sex (Intsex). In every model, diet and sex were considered as additive covariate and kinship was used to account for relation individually among all mice.

3 Results

3.1 Protocol establishment and sequencing of fungal ITS2 region

3.1.1 Isolation of fungal DNA

As already mentioned in 1.5 molecular identification of the mycobiome from different sample types starts, aside from sample collection, with the isolation of nucleic acid from the sample, in this case the fungal DNA. For that, a variety of DNA isolation kits are produced nowadays including enhanced lysis of the cells via heat incubations, additional use of lysing enzymes like proteinase K as well as specific bead-beating methods including strong homogenization. In order to address the question, how to most efficiently and fast isolate fungal DNA from our murine cecum content samples and further process them with NGS for a subsequent abundance analysis of fungal taxa, an isolation protocol with a commercially available DNA isolation kit should be established in the first part of this work.

The Qiagen Dneasy Powerlyzer Powersoil Kit (Qiagen, Venlo, NL) was chosen due its ability to extract DNA even from tough soil microbes using the bead-beating method (figure 11). After testing the isolation of fungal DNA with this kit on random murine stool samples as well as on cecum samples (tissue pieces), cecum content as representative sample type for the identification of resident fungal species found in the murine gut was chosen for this work. It could be lysed and homogenized well through our applications and yielded a sufficient amount of DNA of minimum 30 ng/µl for downstream analysis. Because of the low amounts of fungal DNA in the gut compared to bacterial DNA and the even lower amount of fungal RNA, this work only focused on the fungal DNA, representing the standing fungal gut communities. The isolation kit included bead tubes and a lysis buffer, different buffers for lysis enhancement, inhibitor removal and washing, and specific DNA spin columns that included a silica DNA binding membrane. All solutions are shown in figure 11.

In order to sufficiently lyse the fungal chitin cell wall and remove proteins from the DNA lysate, additional 20 µl of the enzyme Proteinase K (Qiagen, Venlo, NL, not included in the kit) were added to the sample in the bead tube together with bead lysing buffer and the lysing enhancer solution. Proteinase K is a serine protease that derives from the mold *Tritirachium album Limber*. It has peptide bond hydrolysing properties with a

low specificity and a strong proteolytic activity even if strong detergents are present (Butler et al. 1991; Ebeling et al. 1974). The concentration used in this protocol was 400 µg/ml which is comparably high (the recommended concentration is between 50 and 300 µg/ml, depending on the manufacturer) but yielded the best DNA results in our analysis. The minimum two-hour incubation at minimum 800 rpm shaking at 50 °C made sure that the majority of fungal cells was properly lysed. The amount of Proteinase K was determined by test isolations using different amounts of protein and comparing the DNA yield.

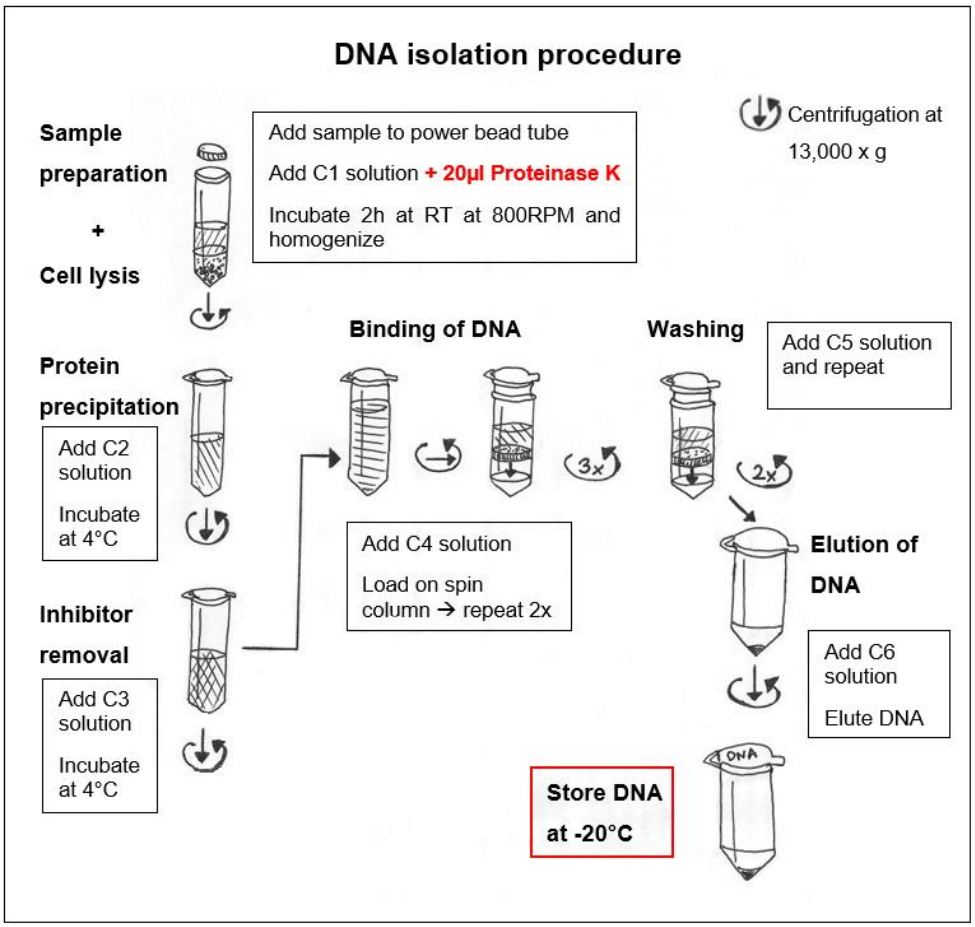


Figure 11: DNA isolation procedure with the Qiagen Dneasy PowerLyzer PowerSoil Kit. Samples are mixed with Powerbead lysing solution, mixed with C1 lysis enhancer solution and added to a Powebead tube. Additional 20µl of Proteinase K (Qiagen, Venlo, NL) are added, followed by a 2 h incubation at 50°C and 800 rpm. Then samples are incubated 5 min at 4° with solution C2 and inhibitor removal solution C3. DNA is then mixed with solution C4 and loaded onto a MB spin column which leads to a binding of DNA to the column membrane. After washing with solution C5 and removal of residing ethanol, the DNA is eluted with solution C6 and stored on ice or at -20°C until further analysis.

Between each step of adding specific solutions to the sample in the collection tube centrifugation was always carried out at room temperature (RT) at minimum of 13,000 x g speed and the flow-through was discarded. A decrease in centrifugation velocity or/and centrifugation at lower temperatures resulted in a poorer DNA yield after isolation. One critical step along the isolation procedure was the proper cleaning of all plastic ware, pipettes and other instruments as well as working spaces prior to the start of the isolation. RNAse away solution was used vigorously along with 70 % ethanol spray cleaning of all devices prior to each isolation. Gloves were disinfected between each centrifugation step of the protocol and changed regularly throughout the procedure and masks were worn to avoid contaminations through the personnel.

After stabilization and washing, the DNA was eluted with the elution buffer in a volume of 100 µl. The reduction of the elution volume by half did not yield significantly higher DNA concentrations but the incubation of the elution buffer on the membrane for several minutes prior to centrifugation did and was therefore applied. Immediately after eluting the DNA, it was stored on ice and total DNA concentrations were measured in a photometer where the DNA was checked on purity by measuring the absorbance at 260 nm and 280 nm and only pure DNA was used for PCR. A 260/280 ratio of ~1.8 indicated a pure DNA. A significantly lower ratio (≤1.5) indicated the presence of proteins, phenols, or other contaminants that absorb at 280 nm wavelength. For samples with a lower DNA concentration than 30ng/µl or significantly low 260/280 ratios the isolation procedure was repeated.

3.1.2 PCR design

The PCR reaction was carried out in a total reaction volume of 25 µl (table 2) in order to avoid repeated isolation in case the PCR sample would finish or not suffice for all further applications during the NGS process (subpooling). Primers were chosen and designed following the dual indexing approach as described in section 1.5. The barcoding strategy was chosen in the most convenient and time saving way. A total of 17 samples (16 samples plus one NC) were processed in the PCR using the same forward primer and 17 different reverse primers (full primer sequences in table 1, NC always reverse “Q”). Record was kept on each combination of barcodes for the identification of the samples later on. The most suitable annealing temperature for the primers was chosen as 50 °C with the other cycles being performed standardized and

most suitable for the used enzyme (Phusion polymerase, Thermofisher, Waltham, USA).

Reagent	**Volume**
5x HF Phusion Buffer	5 µl
dNTP mix (10mM)	0.5 µl
ITS primer forward (2µM)	4 µl
ITS primer reverse (2µM)	4 µl
Phusion polymerase	0.25 µl
H_2O deionized/RNAse/Dnase-free	9.25 µl
Template DNA (diluted to 30ng/µl)	2 µl
Total reaction volume	**25µl**

Table 2: PCR reaction mix protocol. The PCR Master mix (MM) included the 5 x HF Phusion Buffer, dNTP mix, water, the forward primer and the Phusion polymerase. The amount of water could be reduced by 2 µl in case of low DNA concentration in the sample, the template volume was increased to 4 µl, respectively.

The Phusion polymerase was chosen as reaction enzyme due its high proofreading capacity and fidelity. Less errors are reported to happen with this enzyme compared to others which are used for PCR reactions (McInerney et al. 2014; Li et al. 2006). Quality and purity of the PCR reaction was validated by running a negative reaction control including deionized water, instead of template DNA, that was kept under the sterile UV-hood during the whole investigation. The enzyme was kept at -20 °C just until the use and was immediately put back to the freezer after usage to avoid damaging of the enzyme. The UV-hood was used to provide an RNAse/DNAse-free and completely disinfected area for the preparation of the PCR reaction. No nucleic acid was taken under the hood to avoid any possible contamination of the PCR reagents. The optimal cycle number of the PCR reaction was tested in separated runs to yield a high amplification rate of the ITS2 region gene within the sample while keeping amplification of artefacts low. The full PCR program is shown in table 3.

Step	Temperature	Time	
Initial denaturation	98°C	30 sec	
Denaturation	98°C	9 sec	
Primer Annealing	50°C	30 sec	35x
Elongation	72°C	30 sec	
Final extension	72°C	10 min	
Hold	4°C	∞	

Table 3: PCR settings. Initial denaturation was followed by 35 cycles of denaturation, primer annealing and elongation with subsequent final elongation for 10 min. Samples were then cooled down to 4 °C and held infinitely.

3.1.3 Gel electrophoresis

To confirm a successful PCR and the DNA integrity agarose Gel electrophoresis was done on a 1.5 % agarose gel including fungal DNA amplicon products and the NC, PCR negative control (water) as well as primer controls (see also section 2.7, figure 12). While establishing this step, miss-shaped DNA bands were observed which could not be correctly and precisely quantified (figure 12A). In order to overcome this problem and to obtain straight bands in the gel that could be quantified using an optical quantification program, different agarose concentrations ranging between 0.5 and 2 % as well as different gel chambers, combs and power supplies were tested. Finally, the nucleic acid gel stain was changed from SYBR green gel stain (Thermofisher Scientific, Waltham, USA) to the HD green gel stain (intas Science Imaging, Göttingen, D) which resulted in straight and non-deformed bands (figure 12B). Additionally, the voltage of the power supplies was increased up to 120 V for the first five minutes of the electrophoresis run and then lowered down to 110 V for the following hour. This led to a faster and deeper sinking of the PCR products into the pockets of the gel at the beginning of electrophoresis. All PCR products were loaded into the gel pockets with a 12-channel 0.1 – 10 µl multipipette (eppendorf, Hamburg, D) to decrease the loading time. This avoided diffusion of the PCR products into the gel buffer during the loading process. Gel pictures were taken by exciting the fluorescently dyed DNA fragments in the gel with UV-light. The gel pictures were further subject to quantification.

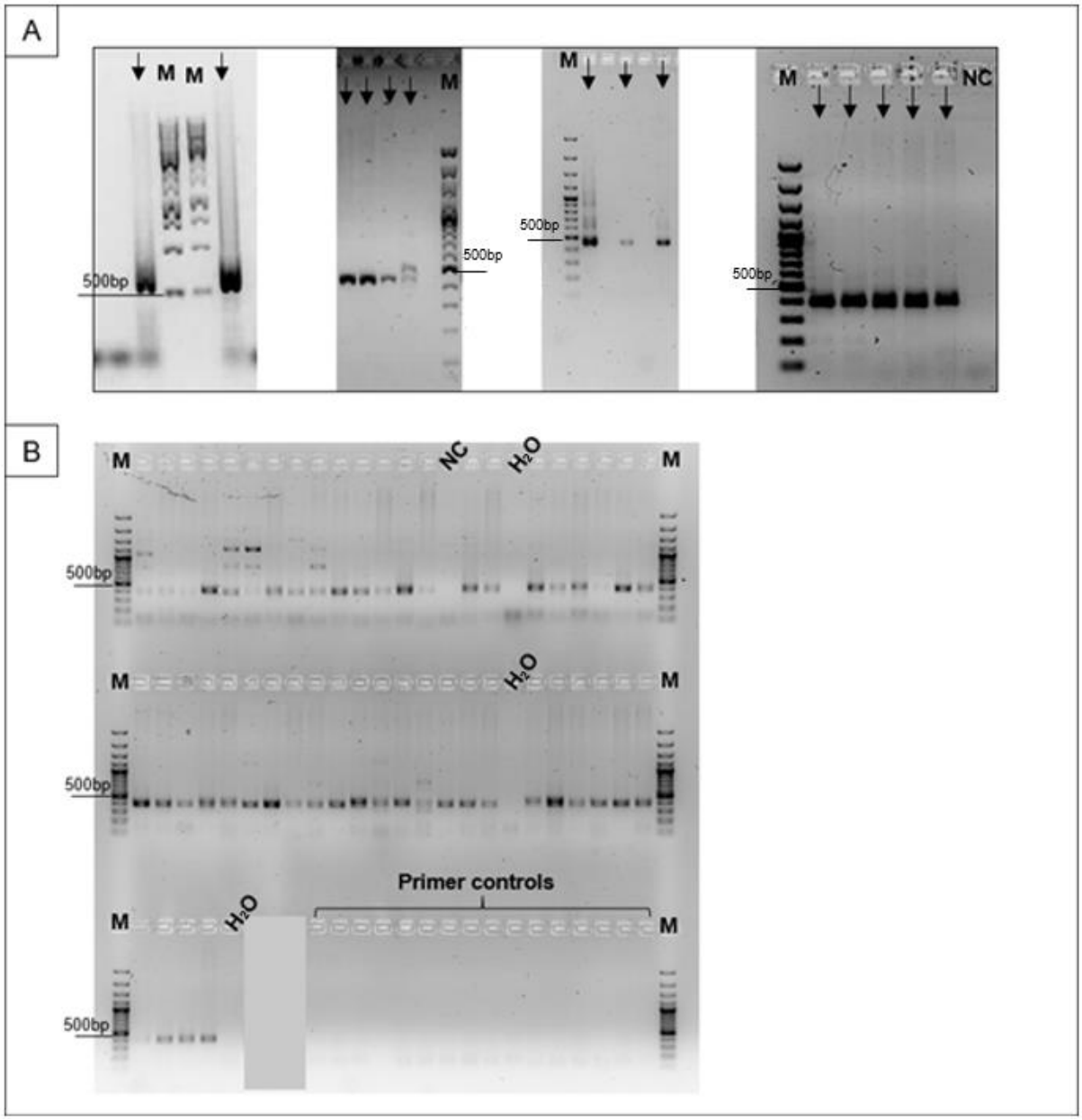


Figure 12: Gel electrophoresis with amplicons from fungal DNA. 1.5 % agarose gels were run 5 min at 120 V followed by 1 h at 110 V and 5 µl of PCR product/Loading dye (LD)-mix (5.5 µl sample + 1 µl LD) were loaded into the pockets. M is the DNA ladder (1 kbp or 100 bp plus gene ruler, reference band size highlighted at 500 bp). Fungal ITS2 constructs ran between 400 and 500 bp A: Example gel pictures of ITS2 PCR products (arrows). From left to right: the first two pictures show misshaped DNA bands observed in gels dyed with SBR Green gel stain. The third and fourth picture show straight bands dyed with HD Green gel stain. B: PCR products from subpool 3-5 (visible bands other than the ladder), NC and negative PCR control (H_2O) and primer controls.

3.1.4 Quantification and subpooling

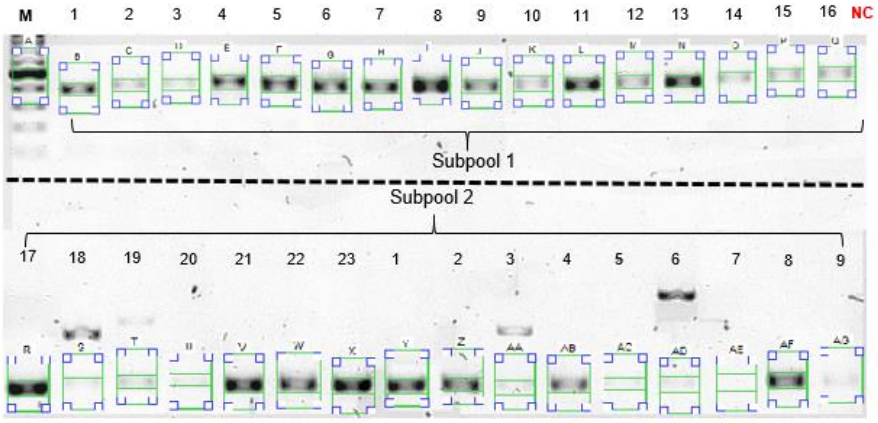
The quantification of the PCR products with the Vision-Capt software resulted in a total sum of 477 PCR products (library 1 = 220, library 2 = 257) that were further used for subpooling and library preparation (figure 14). Products that didn't reach the necessary concentration of minimum 5.2 ng/µl were repeated in PCR with doubled template amount (4 µl) and were re-quantified in Gel electrophoresis and Vision-Capt. If the concentration was still below the threshold, samples were excluded from the further analysis. Therefore, not all subpools contained 17 samples in the end. Figure 13 shows a successful example quantification of fungal amplicon DNA within subpool 1 and 2. The marker band signal intensity and volume were used as reference (100 %) with known ng gel input within the loaded 5 µl, so that the PCR product concentrations could be calculated as percentages of the reference band. Equimolar subpools consisting of 100 ng of each included PCR product amplified with the same forward primer and different reverse primers were pooled on ice and stored at -20 ° degrees until gel extraction.

Due to the presence of several differently sized DNA fragments in the majority of PCR products, the subpools had to be run on an ultrapure agarose gel for subsequent extraction from the gel to include only the fragments that were within the bp range of the desired ITS2 region construct (between 400 and 500 bp). For the gel extraction, subpools were mixed with 2.5 times their volume of colorless LD for library extraction (contained 50 mM TRIS (pH 8), 40 mM EDTA and 40 % Sucrose) and were cut out from the gel (figure 14). Gel extraction pipette tips were used for cutting, which fitted exactly to the band sizes in the gel and were changed after each subpool when also the UV-light was turned off to protect DNA from degrading during excision.

3.1.4.1 Purification of the subpools

To extract and purify the DNA from the gel the Qiagen MinElute kit (Qiagen, Venlo, NL) was used. This kit yields highly concentrated DNA with minimal loss from agarose gels and recovers fragments from 70 bp up to 4 kbp of length. The principle is based on the binding of the DNA at a uniquely adapted silica membrane within a spin column which can bind up to 5 µg of DNA through high salt concentrations and a pH of ≤ 7.5 while contaminants pass through and are washed away.

A



B

			% volume	ng in gel	concentration [ng/µl]	
Sample ID	Forward-Primer	Reverse-Primer	100,00	80,0	16	µl to take for 100ng DNA
DNA 1	IST-1	MID A	61,582	49,2656	9,85312	10,15
DNA 2	IST-1	MID B	44,261	35,4088	7,08176	14,12
DNA 3	IST-1	MID C	39,079	31,2632	6,25264	15,99
DNA 4	IST-1	MID D	71,249	56,9992	11,39984	8,77
DNA 5	IST-1	MID E	93,746	74,9968	14,99936	6,67
DNA 6	IST-1	MID F	80,445	64,356	12,8712	7,77
DNA 7	IST-1	MID G	71,226	56,9808	11,39616	8,77
DNA 8	IST-1	MID H	117,412	93,9296	18,78592	5,32
DNA 9	IST-1	MID I	55,864	44,6912	8,93824	11,19
DNA 10	IST-1	MID J	49,335	39,468	7,8936	12,67
DNA 11	IST-1	MID K	90,891	72,7128	14,54256	6,88
DNA 12	IST-1	MID L	35,899	28,7192	5,74384	17,41
DNA 13	IST-1	MID M	90,068	72,0544	14,41088	6,94
DNA 14	IST-1	MID N	49,129	39,3032	7,86064	12,72
DNA 15	IST-1	MID O	62,488	49,9904	9,99808	10,00
DNA 16	IST-1	MID P	64,393	51,5144	10,30288	9,71
NC	IST-1	MID Q		0	0	10,00

Figure 13: DNA quantification process using gel imaging. A: PCR products of subpool 1 and 2 (partly) are compared to the ladder signal (M, lane A) in volume and intensity of UV detection using the Vision-Capt software. Numbers indicate DNA sample IDs. The regions of interest are manually selected according to their peak signals (green blocks around the bands in lanes B-Z and AA-AG) and background correction is applied. NC (red) is not visible in this image. Only the bands appearing at around 500 bp are included. B: Percentages of the reference volume and DNA concentration per µl PCR product are calculated for subpool 1. Sample Amounts are then adjusted to 100 ng of DNA to assure high quality of downstream analysis. From each NC 10 µl were pooled into each subpool, no matter the calculated concentration (NC = 0, no visible band, in this example), to subtract possibly appearing reads after sequencing.

Gel pieces of a maximum weight of 400 mg were used for one membrane column. The gel was dissolved for 10 min at 50 °C while the pH of the solution was monitored

through the added pH indicator within the binding buffer the whole time and extraction was proceeded according to the manufacturer's protocol. DNA was eluted from the membrane with 10 µl of 10 mM Tris-chloride elution buffer (pH 8.5).

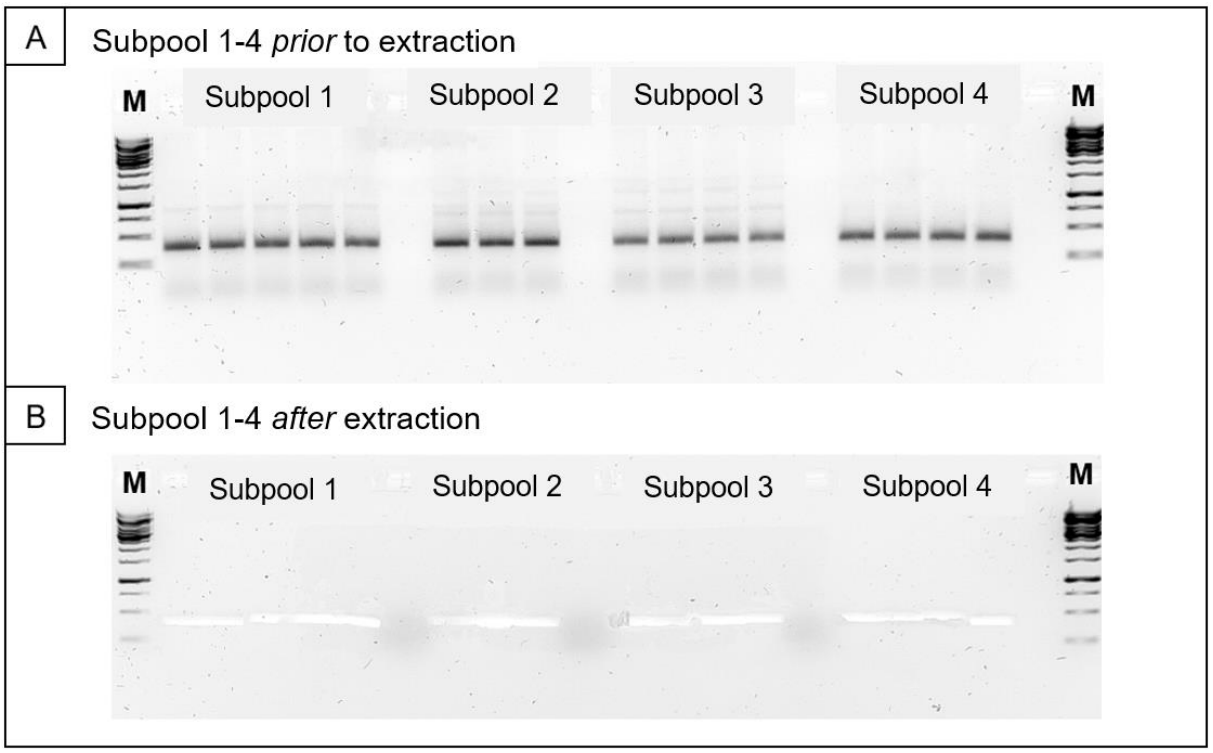


Figure 14: Gel extraction of the subpools. The figure shows subpool 1-4 prior (A) and after (B) extraction under UV-light. Subpools were run 5 min at 120 V followed by 1 h at 110 V in a 1.5 % ultrapure agarose gel. 10 µl of subpool were loaded into each pocket and extracted each with one extraction tip into clean eppendorf tubes, only around the size of approximately 470 bp. M is the 1 kbp gene ruler DNA ladder.

3.1.5 Library construction

3.1.5.1 Quantification of the subpools and library pooling

For equimolar pooling of the subpools into DNA libraries the subpools further had to be specifically quantified. For accurate quantification the NEBNext Library Quant kit for Illumina (New England Biolabs, Ipswich, USA) was used. It is a real-time PCR (quantitative PCR, qPCR) kit which detects only Illumina adaptors including DNA fragments through fluorescent labelling and measurement of fluorescence intensity

after each cycle. The fluorescence signal thereby is proportional to the DNA amplicon concentration in the sample at that moment (Higuchi et al. 1992). The initial fluorescence signal is non distinguishable from that of the background and therefore, the initial number of template DNA molecules proportionally corresponds to the point at which the fluorescence intensity passes the detectable threshold (Kubista et al. 2006), the quantification cycle (Cq). Cq values are then calculated according to the standard curve (Heid et al. 1996; Bustin et al. 2005).

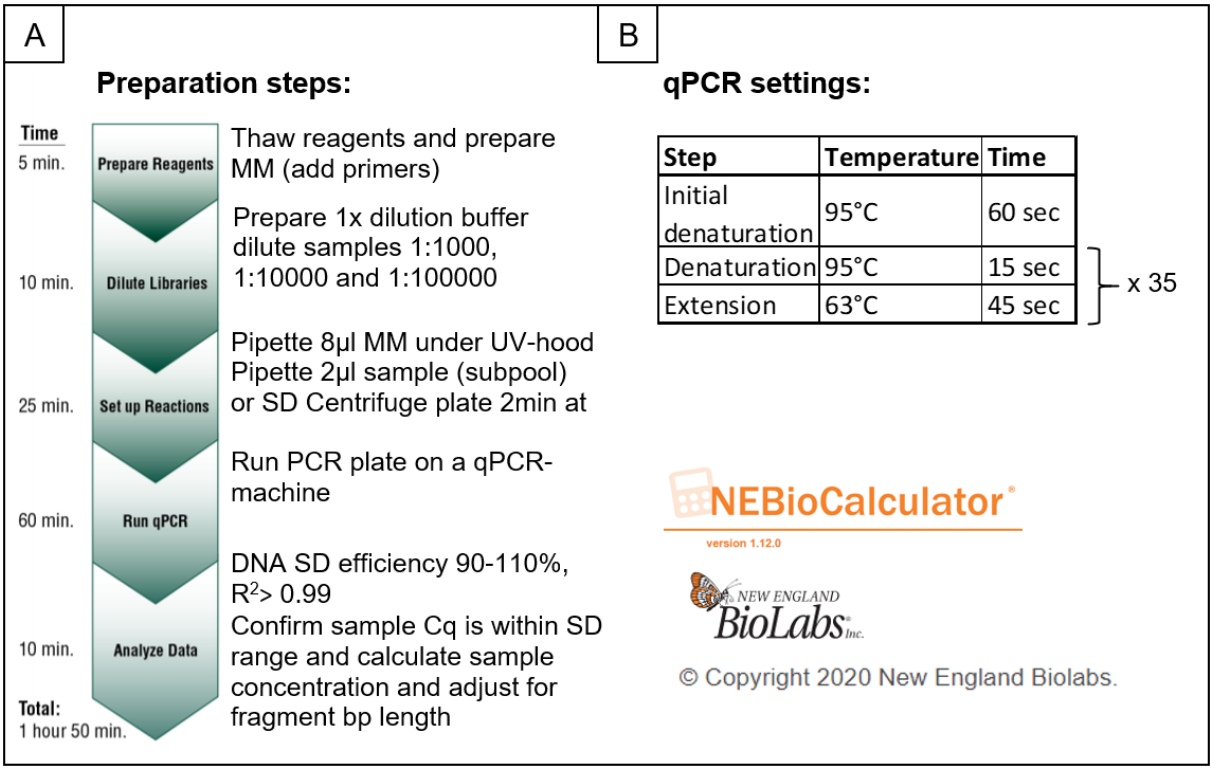


Figure 15: qPCR workflow and PCR settings (www.neb.com; NEB #E7630S/L instruction manual 2020, changed). A: Workflow of the qPCR. B: qPCR settings. Concentrations of amplicon DNA were calculated according to the NebNext Biocalculator and adjusted to an estimated fragment length of 470 bp.

Figure 15 shows the workflow of the qPCR kit with qPCR settings. The qPCR was performed on a 96-well plate. It was important to vortex and shortly spin down all reagents prior to pipetting to minimize bias. The four standards (SD; 0.01pM, 0.1pM, 1pM and 10pM), a negative control (here: NCT), containing only sample dilution buffer and PCR reagents, and subpool dilutions of 1:10000 and 1:100000 were included as

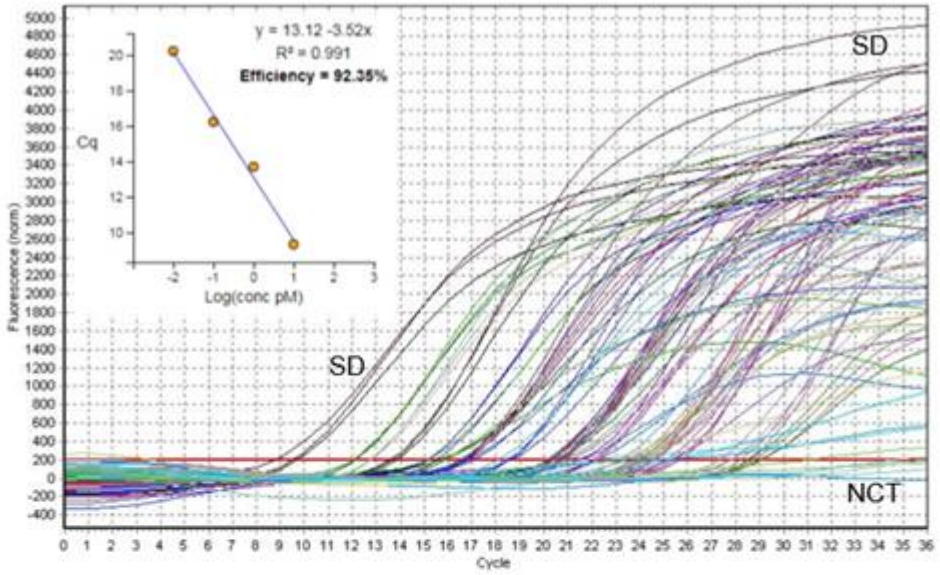
triplets on the plate. First qPCR runs gave positive results for the NCT after 10-20 cycles which could be resolved by pipetting the qPCR MM reagents and dilution buffer under the UV-hood to minimize contaminations.

Results of the qPCR from subpools 1-13 (part of library 1) with efficiency of the SDs between 90 and 110 % and $R^2 > 0.99$ are shown in figure 16A. R^2 is the coefficient of correlation that derives from the SD curve of the SD serial dilution (Heid et al. 1996). Subpool concentrations calculated from Cq values ranged from 2.65 ng/µl to 14.72 ng/µl and 19 subpools with a total number of 220 samples were included in library 1 (figure 16B). Calculated amplicon concentrations of the subpools 1.2-19.2 (library 2) ranged from 1.55 ng/µl to 20.8 ng/µl and qPCR SD efficiency was always between 90 and 110 % (data not shown). qPCR was repeated if the values were outside the recommended range. 18 subpools with a total number of 257 samples were included in library 2 (figure 16C). In total 15 NC were included in sequencing.

3.1.5.2 Purification of the libraries

Prior to quantification, 40 µl of the libraries were purified with the Agencourt AMPure XP system which uses the solid phase reversible immobilization technology (SPRI). This principle is based on the immobilization of DNA through carboxyl coated magnetic particles that was described as purification method for nucleic acids in 1994 (Hawkins et al. 1994) and has been used widely in research ever since. Under conditions of high polyethylene glycol (PEG) and salt concentration the DNA binds to the surface of the magnetic particles and is thereby immobilized on this solid phase. The DNA-bead complex can then be vigorously washed before elution in water or TRIS-buffer (figure 17A). With this method it is possible to recover amplicons larger than 150 bp and efficiently remove unincorporated dNTPs, primer dimers, salts and other contaminants. For the elution of the DNA from the beads they had to be dried at RT for 2-5 min depending on the size of the bead cluster. At this critical step the surface of the beads had to be monitored very intensely because the balance point at which all ethanol had evaporated but the beads were not completely dry was only visible in the change of the appearance of the bead surface. Lower DNA concentrations were seen when beads dried out completely during purification or when ethanol residues were still present prior to elution. All libraries were eluted in 15 µl of solution C6 from the DNA isolation kit (described in figure 11, see also section 3.1.1).

A



B

Subpool	No. of samples	NEB kit conc. [nM]	Subpool [ng/µl]	sample [ng/µl]	µL to take	Total ng in library
1	17	16,58	5,06	0,30	7,66	38,75
2	7	23,21	7,08	1,01	2,25	15,96
3	7	8,68	2,65	0,38	6,03	15,96
4	10	18,2	5,55	0,56	4,11	22,79
5	9	11,36	3,46	0,38	5,92	20,51
6	9	9,44	2,88	0,32	7,13	20,51
7	8	13,42	4,09	0,51	4,46	18,23
8	11	16,58	5,06	0,46	4,96	25,07
9	15	11,21	3,42	0,23	10,00	34,19
10	16	17,11	5,22	0,33	6,99	36,47
11 rep	15	22,59	6,89	0,46	4,96	34,19
12 rep	13	32,68	9,97	0,77	2,97	29,63
13 rep	9	10,63	3,24	0,36	6,33	20,51
14	9	36,52	11,14	1,24	1,84	20,51
15 rep	16	48,26	14,72	0,92	2,48	36,47
16	8	31,67	9,66	1,21	1,89	18,23
17	13	42,34	12,91	0,99	2,29	29,63
18	15	45,79	13,97	0,93	2,45	34,19
19 rep	13	39,27	11,98	0,92	2,47	29,63
Total	220	Volume [µl]	87,18	min [ng]	2,28	
		min [ng/µl]	0,23	ng library	501,46	

C

Subpool	No. of samples	NEB kit conc. [nM]	Subpool [ng/µl]	sample [ng/µl]	µL to take	Total ng in library
1-2 rep	14	68,21	20,80	1,49	1,16	24,15
2-2 rep	14	71,73	21,88	1,56	1,10	24,15
3-2	17	11,68	3,56	0,21	8,23	29,32
4-2	12	8,94	2,73	0,23	7,59	20,70
5-2	12	31,09	9,48	0,79	2,18	20,70
6-2	16	28,74	8,77	0,55	3,15	27,60
7-2	15	12,08	3,68	0,25	7,02	25,87
8-2	17	9,66	2,95	0,17	9,95	29,32
9-2	13	7,77	2,37	0,18	9,46	22,42
10-2	15	22,31	6,80	0,45	3,80	25,87
11-2	12	8,58	2,62	0,22	7,91	20,70
12-2	9	5,09	1,55	0,17	10,00	15,52
13-2	12	10,66	3,25	0,27	6,37	20,70
14-2	13	30,05	9,17	0,71	2,45	22,42
15-2	15	104,7	31,93	2,13	0,81	25,87
16-2	11	74,27	22,65	2,06	0,84	18,97
17-2	14	39,91	12,17	0,87	1,98	24,15
18-2	12	19,76	6,03	0,50	3,43	20,70
19-2 rep	14	26,57	8,10	0,58	2,98	24,15
Total	257	Volume [µl]	90,43	min [ng]	1,72	
		min [ng/µl]	0,17	ng library	443,31	

Figure 16: qPCR quantification of the subpools and calculation of libraries. The figure shows the qPCR results of the subpools 1-13 that were included in library 1 for sequencing (A) and the equimolar pooling of library 1 and 2 (B and C). A: The efficiency of the standards was 92.35% with $R^2 = 0.991$. Cq values (y-axis) are plotted against the log(concentration in pM) of the standards seen in black color in the curve. The curve shows the detected fluorescence of the SD or subpools after each cycle with NCT in blue. B and C: At least two Cq values of subpool triplets were included in the calculation and the mean DNA concentration was calculated with the NebBiocalculator with adjusted fragment length of 470bp. 10µl of the lowest concentrated subpool 9 (library 1, B) and subpool 12.2 (library 2, C) were used as reference for equimolar pooling of all samples. The term “rep” refers to repeated subpooling due to insufficient amounts or quality of DNA within subpools.

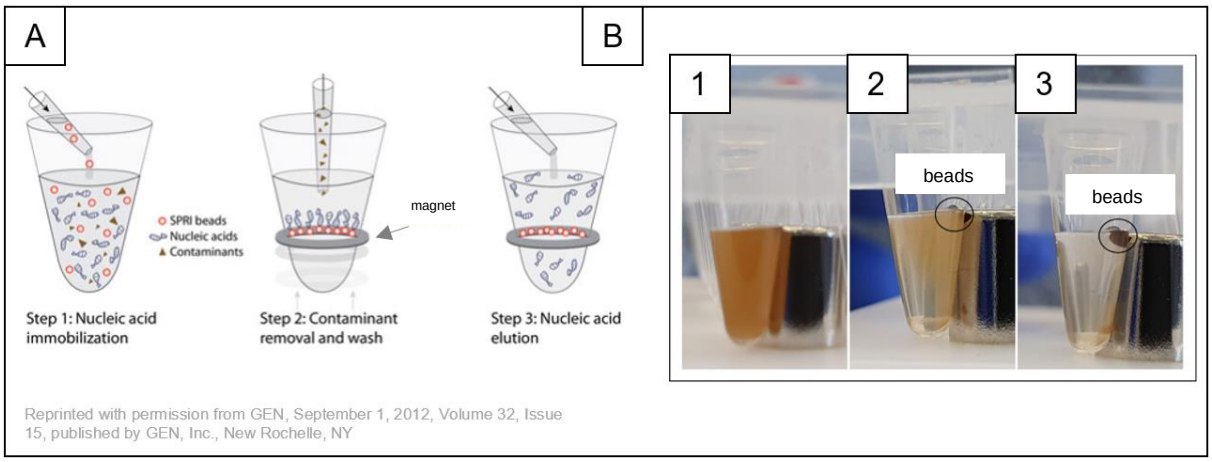


Figure 17: Library purification with magnetic beads. A: bead purification principle with step 1: binding of DNA on the solid magnetic beads and magnetic separation, step 2: ethanol washing and contaminant removal and step 3: elution of the DNA from the beads (reprinted with permission from GEN 2012, modified) B: The DNA-bead mix (1), clustering DNA-beads at the side of the reaction plate which are attracted by the magnetic stand (2) and clustered DNA-beads separated from the buffer and bound to the magnetic stand.

3.1.5.3 Quality control and quantification of the libraries

All libraries were quantified by qPCR and their average size and quality was measured with a bioanalyzer. The results are shown in figure 18. The bioanalyzer performs an automated gel electrophoresis of the sample library on a microfluidic chip within a fluorescently dyed gel. First, a DNA ladder is run and a SD curve of migration time against DNA size is plotted. Samples are then sized by normalization to a lower and upper marker fragment at 35 bp and 10380 bp and the SD curve based on their migration time. Using a highly sensitive bioanalyzer kit for double stranded DNA an average fragment size of 442 bp for library 1 and 463 bp for library 2 were calculated (figure 18A). Libraries were aliquoted right after AMPure bead purification in order to avoid repeated freeze and thaw cycles and directly measured in the bioanalyzer and qPCR. Libraries were run on the bioanalyzer undiluted and in 1:10 dilution according to the detection range. Next, the concentration of the library was determined by another qPCR run with correction by the average library size (figure 18B). Library 1 yielded a concentration of 86.7 nM which equals 24,91 ng/µl and library 2 yielded a concentration of 47,8 nM which equals 14.39 ng/µl. The qPCR run of library 1 had a

SD efficiency of 100.5 % at $R^2 = 0.999$ and the NCT was negative and started to appear only after cycle 36 (figure 18B, upper picture). Library 2 ran at a SD efficiency of 95.3 % at $R^2 = 1$ with NCT appearance after cycle 29 (figure 18B, lower picture).

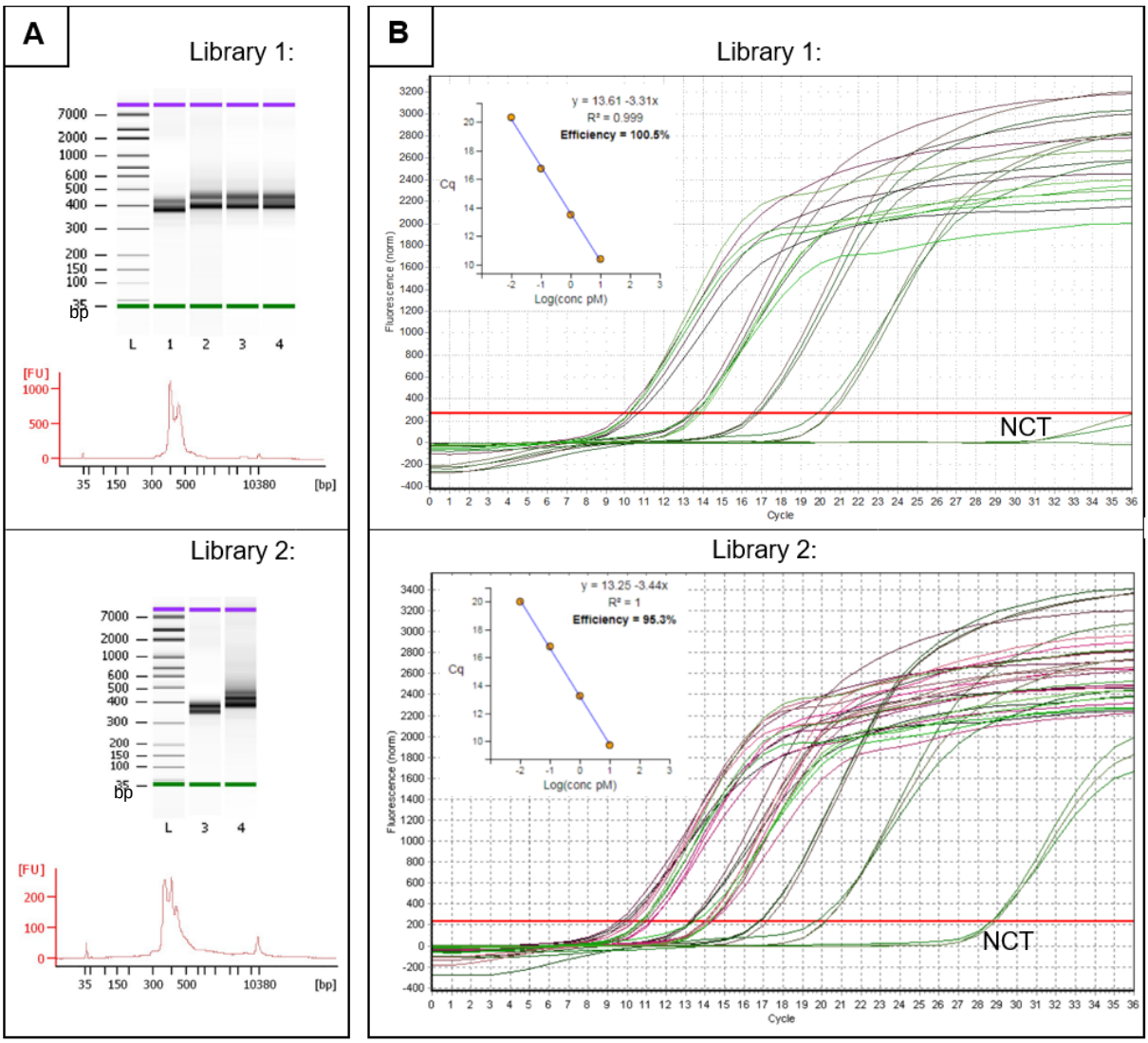


Figure 18: Bioanalyzer and qPCR results of the sequencing libraries 1 and 2. A: Gel electrophoresis showing the ladder fragments (L) and fungal ITS2 library 1 as 1: undiluted and 2-4: 1:10 dilution repetitions as well and library 2 as 3: undiluted and 4: 1:10 dilution. For both libraries the absorption spectra of a 1:10 dilution are shown as arbitrary fluorescence units (FU, y-axis) plotted against the bp length according to the marker. B: qPCR quantification of library 1 and library 2 in 1:10000 and 1:100000 dilutions with SDs in black and NCT in dark green. The SD efficiencies are shown in the upper left boxes as Cq values (y-axis) plotted against the Log(concentration [pM]). Library 1 has been run alone against the SD and presents in green lines. Library 2 (light green lanes) had been run together with a control library (pink lanes). Used marker was the high sensitivity DNA marker (35 and 10380 bp).

3.1.6 ITS2 region gene sequencing

In order to test the ITS2 sequencing, an Illumina MiSeq reagent kit v2 with 2 x 250 cycles was used and paired-end sequencing was performed on an Illumina MiSeq instrument. For that, library 2 was sequenced at a concentration of 17.5 pM together with 20 % PhiX control library as spike-in. The run yielded a cluster density on the flow cell chip of ~950 k/mm^3 and after a successful bioinformatic analysis for the identification of fungal taxa the library was run again on a MiSeq reagent kit v3 using paired-end 2 x 300 bp reads to cover the full fragment size of the amplicon product (see section 3.1.5.3). For the Illumina SBS technology workflow (see section 2.7.2.2 and figure 9) the library had to be diluted to 4nM. Table 4 shows the dilutions for the sequencing of library 1 and 2. Both libraries were sequenced at a concentration of 17.5 pM with 20% PhiX control library for 600 cycles.

Library 1 calculation:			**Library 2 calculation:**		
442	**bp**	average size (Bioanalyzer)	**463**	**bp**	average size (Bioanalyzer)
287300	daltons	650 daltons/bp	300950	daltons	650 daltons/bp
		10E6 daltons = 1			10E6 daltons = 1
0,2873	µg/pmol	µg/pmol	0,30095	µg/pmol	µg/pmol
287,3	ng/pmol		300,95	ng/pmol	
24,91	**ng/µl**	concentration (qPCR)	**14,39**	**ng/µl**	concentration (qPCR)
0,087	pmol/µl		0,048	pmol/µl	
0,087	nmol/ml		0,048	nmol/ml	
		true library			**true library**
86,70	**nmol/L**	**concentration**	**47,82**	**nmol/L**	**concentration**

Dilution of the library to 4nmol/L:		
	dilution	
21,68	factor	x2
0,92	µl template	1,85
19,08	µl water	38,15
20,00	µl total	40,00

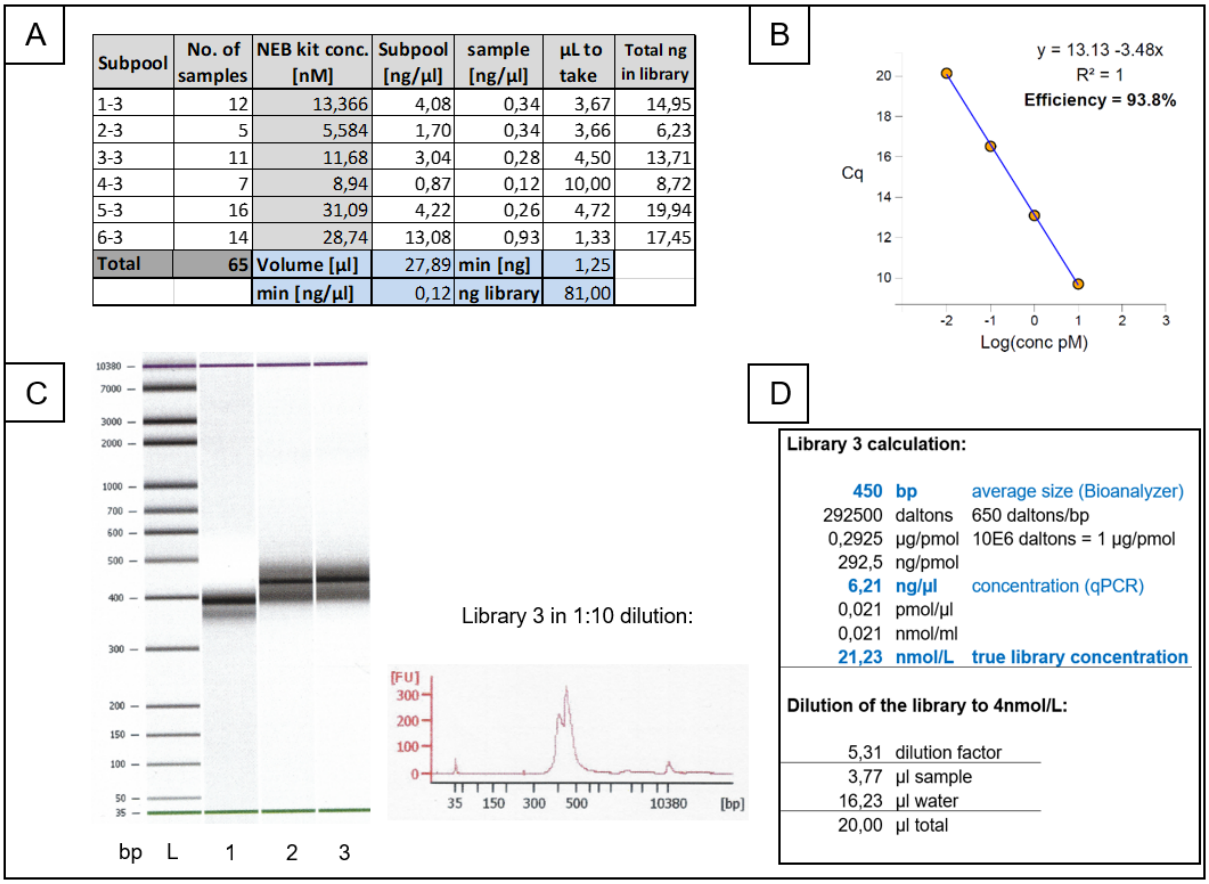
Dilution of the library to 4nmol/L:	
	dilution
11,95	factor
1,67	µl template
18,33	µl water
20,00	µl total

Table 4: Library 1 and 2 dilutions for sequencing. Libraries were diluted to a concentration of 4nM according to their measured concentration (qPCR) and their average fragment size (Bioanalyzer) in a total volume of 20µl or 40µl, respectively, to pipette a minimum amount of 1µl of the sample. A: Library 1 dilution, B: library 2 dilution.

Library 1 yielded a cluster density of ~867 K/mm^3 and library 2 yielded a cluster density of ~1000 K/mm^3. No over or under clustering happened and reads were distributed evenly among the samples supporting the chosen sample and PhiX library concentration. The overall yield were 8.41 GB for library 1 and 9.44 GB for library 2 with a total read count of ~20 million.

Illumina measures the accuracy of the correct incorporation of bases into the sequences by the Q_{30} score. Q scores are logarithmically related to the base calling error probabilities (Phred, P)[2] as in: $Q = -10 \log_{10} P$ and determine the probability of an incorrect base call. If a base is assigned a Q score of 30 this means that the probability of an incorrect base call is 1 in 1000 or 0.1 % error rate and respectively 99.9 % base call accuracy (Ewing et al. 1998; Ewing and Green 1998; Liao et al. 2017). The Q_{30} scores of both runs were sufficient (> 80 % of bases reached a Q_{30} score) and both runs passed the automated MiSeq platform quality check including the cluster passing filter (PF > 80 %).

After sequencing of both libraries, 65 samples with a read count lower than 1,0000 were combined in a third library (Library 3), after repetition of PCR and subpooling, and sequenced again under the above mentioned sequencing conditions. qPCR of library 3 yielded a SD efficiency of 93.8 % at $R^2 = 1$. An average library size of 450 bp was determined in the bioanalyzer and a concentration of 21.23 nM (equals 6.21 ng/µl) was measured in the qPCR (figure 19). Library 3 yielded a total of 10.01 GB of data with ~12 million reads and passed the MiSeq run quality check.



Subpool	No. of samples	NEB kit conc. [nM]	Subpool [ng/µl]	sample [ng/µl]	µL to take	Total ng in library
1-3	12	13,366	4,08	0,34	3,67	14,95
2-3	5	5,584	1,70	0,34	3,66	6,23
3-3	11	11,68	3,04	0,28	4,50	13,71
4-3	7	8,94	0,87	0,12	10,00	8,72
5-3	16	31,09	4,22	0,26	4,72	19,94
6-3	14	28,74	13,08	0,93	1,33	17,45
Total	65	Volume [µl]	27,89	min [ng]	1,25	
		min [ng/µl]	0,12	ng library	81,00	

Figure 19: Library 3 preparation. A: Pooling of the library from repeated samples pooled in new subpools. B: qPCR standard efficiency C: Gel electrophoresis picture of the Bioanalyzer with ladder (L), undiluted library (1) and 1:10 diluted library (2 and 3). FU are plotted against the fragment size in bp on the right. D: Calculation of library dilution for sequencing.

3.2 Impact of diet and genes on the gut mycobiome

3.2.1 Fungal taxonomic abundance

With the statistical analysis of the sequencing data of the AIL mice the impact of diet as well as the host genetics on the gut fungal composition should be ascertained. The sequencing output of the fungal DNA sequences was generated as gzipped fastq datasets. For each sample ID (one mouse) forward reads and reverse reads were present. By assigning the reads using the RDP classifier with the UNITE database to different taxonomical ranks (phylum to genus) an overview on the taxonomic composition of the three dietary groups could be constructed (figure 20). The phyla

Ascomycota and Basidiomycota were most abundant in the gut of AIL mice with Ascomycota making up 97.6 ± 3.6 % of all taxa in the mice fed the calorie-reduced diet, 97.8 ± 2.6 % in mice fed the control diet and 91.6 ± 15.6 % in mice fed the western diet. Basidiomycota made up 2.3 ± 3.5 % of all taxa in calorie-restricted mice, 2.1 ± 2.6 % in control mice and 8.3 ± 15.6 % in western diet mice. At genus level, *Penicillium* was with 53.3 % the most abundant genus found in the gut of all AIL mice, besides *Aspergillus* with 8.4 %, unknown Ascomycota with 7.8 % and *Candida* with 7.7 %. No significant differences could be observed between the three dietary groups.

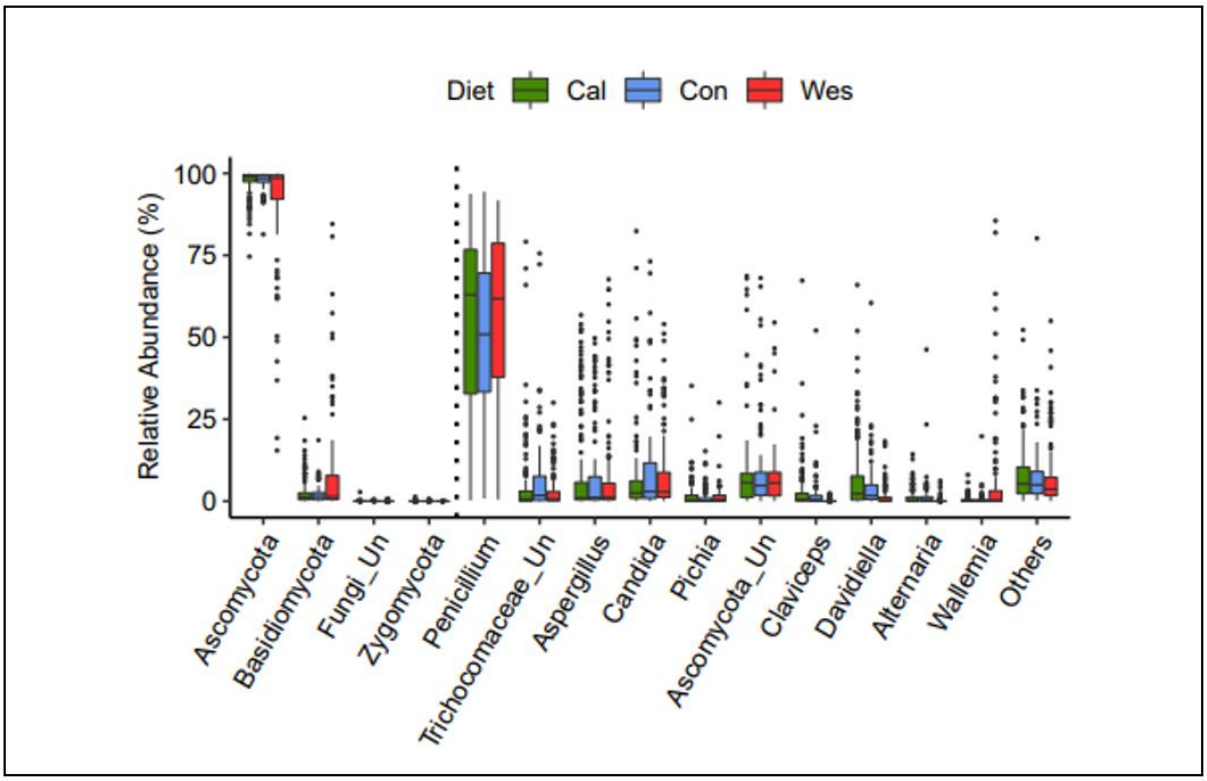


Figure 20: Taxonomic abundance of fungi in the three dietary groups. Relative abundance of fungal phyla (first 4 columns) and genera are shown with standard deviation using LEfSe algorithm. Green blocks: calorie-restricted group (Cal), blue: control group (Con) and red: western diet group (Wes).

The LEfSe algorithm was further used to correlate the found genera to the three different dietary groups. The phylum Basidiomycota with the genera *Wallemia* was more abundant in Western diet mice than in the calorie-restricted and control mouse

groups whereas the genera *Claviceps* and *Davidiella* from the phylum Ascomycota were more abundant in the calorie-restricted mouse group than in the Western diet group (figure 20).

3.2.2 Fungal diversities and indicator species

To further examine the gut fungal diversity and composition, sequences were clustered to species level OTUs at a similarity threshold of 97 % using the PIPITS pipeline. Samples were rarefied and normalized to 5,000 sequences per sample and alpha diversity and beta diversity in correlation to the three dietary groups were calculated in R. No significant difference in fungal alpha diversity (species richness and diversity) was found for the three dietary groups (figure 21A). However, significant differences (calculated with constrained analysis of principle coordinates (capscale)) were observed for the beta diversity (species composition and dissimilarity between groups). For simplification groups are named “Cal” for the calorie-restricted mice, “Con” for control mice and “Wes” for western diet mice from now on. The P-values calculated with the Kruskal-Wallis test for the beta diversity between two dietary groups ($p < 0.05$ counted as significant) were as follows: Cal vs. Con = 0.012, Con vs. Wes < 0.01 and Cal vs. Wes < 0.01 (figure 21 B).

More than 3,000 OTUs were found which were used to identify indicator species for the three dietary groups. Hit species with ≥ 99 % sequence redundancy in OTUs are shown in figure 21C. For Wes, OTUs assigned to *Wallemia sebi*, *Penicillium decumbens*, *Aspergillus rubrum*, *Fusarium culmorum*, *Kluyveromyces marxianus*, *Phanerochaete chryososporium*, *Lignincola laevis* and *Nannizzia gypsea* were identified as indicator species while *phoma herbarum*, *Aspergillus nidulans*, unclassified fungi species and *Neoascochyta paspali* were identified as indicator species in Cal and *Wallemia mellicola*, *Cryptococcus* sp., *Didymella glomerata*, *Fusarium CHlamydosporum*, *Alternaria metachromatica* and *Coniochaeta* sp. as indicator species in Con (figure 21C). Indicator species were dominantly from the phylum Ascomycota. Additionally, a cladogram from the LEfSe analysis was prepared including the most differentially abundant taxa found for Cal and Wes (figure 21D). Here, family members of the Basidiomycota with unidentified Helotiales, Mycenaceae and Wallemiaceae were more abundant in Wes. Unknown members of the Ascomycota as well as Nectriaceae were more abundant in Cal.

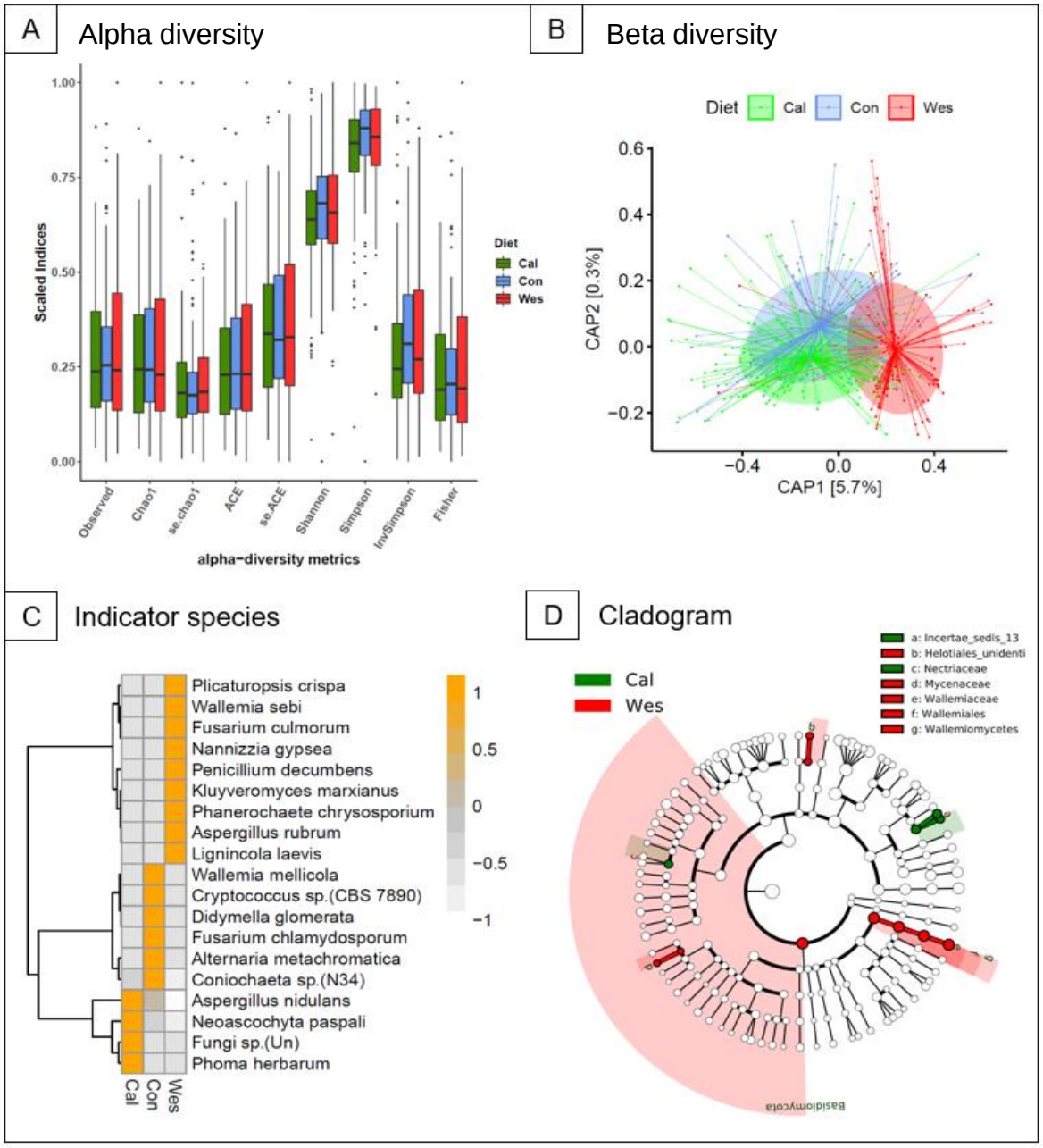


Figure 21: Diversity and indicator species analysis of the dietary groups. 477 samples were included in the analysis. Colors indicate the different dietary groups with green = Cal, green = Con and red = Wes. A: Alpha diversity of Cal, Con and Wes are shown with different metrics approaches and standard deviation. B: Beta diversity of the three dietary groups is shown in a capscale plot of the BrayCurtis distance. A and B: each dot represents one animal. C: Heatmap depicting the fungal indicator species for each diet. The dendrogram (hierarchical clustering) at the left side highlights the clustering among the indicator species. Species names are displayed on the right and dietary groups on the bottom. Color within each cell of the denotes mean scaled (1 to -1) counts for every species within the three dietary groups. D: LEfSe cladogram of Cal and Wes. The root denotes the fungal domain. Sizes of each node correspond to the relative abundance of the taxon. Upper half shows Ascomycota, lower half Basidiomycota. Highlighted Red and green parts describe the Western and calorie-reduced diet.

Because different generations among the mice (generation 18-20 were included) were found to be confounded with diet, generation was considered as a cofactor while accessing the statistical differences in alpha and beta diversity. Analysis was therefore performed with generation as condition. Also, in both LEfSe and indicator species analysis, all the taxonomical ranks were not considered significant for the different dietary groups if they were also significant for different generations.

3.2.3 QTL analysis

To further determine the relationship of host genetics with the mycobiota, residuals of taxonomical abundances were derived and used as traits with generation considered as fixed effect and cage as random effect. Traits were then investigated in three models to find additive QTL (further named “Add”), displaying the associations of the mycobiota with the host genetics only, QTL displaying host genetics interacting with diet (further named “Intdiet”) and QTL displaying host genetics interacting with sex (further named “Intsex”). All models considered diet and sex as additive covariate. To determine relatedness among the individual mice kinship was used. QTL were mapped using the different described models from phylum to species level. Table 5 shows a summary of the mapped QTL including LOD-score, SNP, used model and candidate genes. The full QTL table is attached in the appendix (see section 7.2, table 6).

A total of 52 QTL (genome-wide p-value < 0.05) for 43 taxonomic lineages could be mapped from the sequencing data of the AIL mouse cohort. Of these, 28 QTL were mapped for the Add model, 16 QTL for Intdiet and 6 QTL for Intsex (see table 5). The highest PVs in fungal lineages that were associated with host genetics were explained by cage with a mean percentage of 26 %, while host genetics explained on average 9.06 % of the PV in AIL mice. Diet, as environmental factor, explained 1.2 % of the PV and intrinsic factors such as generation and sex explained 4.9 % and 0.06 %, respectively (table 6).

3.2.3.1 QTL derived from the additive model

The following QTL were mapped with the Add model. On the order level two QTL for Russulales, a member of the Basidiomycota, could be mapped on the same intergenic region on chromosome 9 for the Add (~ 97 Mb; LOD = 7.7) and also for the Intdiet model (93-94.5 Mb; LOD = 11.7). On class level a QTL for Pucciniomycetes could also

be mapped to chromosome 9 (LOD = 8.42) with *Ube2cbp* (analogue = *Ube3d*) as candidate gene. The order Hypocreales (Ascomycota) was mapped to chromosome 8 (LOD = 7.36) containing the gene *Ttc9*. On family level, one QTL was mapped on chromosome 1 (LOD = 7.01), with an uncertain family of Pleosporales, that belong to the Dothideomycetes (Ascomycota). This QTL contained candidate genes such as *Fasl* or *Dnm3os*. Also, two QTL for Pucciniaceae and its genus *Puccinia* (Basidiomycota) were mapped on chromosome X (LOD = 7.89) with the candidate gene *Nox1* and on chromosome 5 (LOD = 7.7), respectively, with *Dpp6* as candidate gene. An unknown genus (LOD = 7.1) and the species *Yamadazyma Mexicana* (OTU1041; LOD = 6.8) from the order Saccharomycetales (Ascomycota) were mapped to the same region of chromosome 11. Candidate genes for this QTL were for instance *Nos2*, *Trp53i13* or *Vtn*. For an unidentified *Cryptococcus* genus (Basidiomycota) one QTL could be mapped on chromosome X (LOD = 6.95) containing the genes *Gpr174*, *P2ry10* and *P2ry10b*. Another QTL was mapped for the genus *Candida* (Ascomycota) to chromosome 9 (LOD = 8.65).

Further, on species level, *Nakazawaea holstii* (OTU925) from the phylum Ascomycota was mapped on chromosome 14 (LOD = 7.86) with *Fgf14* as candidate gene. The same OTU925 was also mapped for another QTL on chromosome 10 (LOD = 6.52) containing the gene *Plxnc1*. As members of the Ascomycota *Claviceps* and its species *C. purpurea* (OTU1298) could be mapped to chromosome 15 (LOD = 7.5 and 8.16) as well as *Talaromyces rugulosus* (OTU1512) to chromosome 13 (LOD = 6.58) containing the candidate gene *Cdk20*. One QTL for *Hypophichia burtonii* (OTU3016, Ascomycota) overlapped with *Candida* on chromosome 9 (LOD = 6.56). Within the genus *Aspergillus* (Ascomycota) the species *A. domesticus* (OTU1693) was mapped on chromosome 9 (LOD = 8.52) with the candidate gene *FLI1* and *A. nidulans* (OTU259) with the candidate gene *Abca13* on chromosome 11 (LOD = 6.45). An additional OTU (OTU298) for *A. nidulans* was mapped on chromosome 19 for the Intdiet model (see section 3.2.3.2). Within the genus *Penicillium* (Ascomycota) QTL were found for *P. canescens* (OTU2110) on chromosome 4 (LOD = 6.75) and another for *P. decumbens* (OTU2060 and OTU2044) on chromosome 2 (LOD = 8.45 and LOD = 6.36) including the candidate gene *mapkap1*. Furthermore, two QTL for unknown *Penicillium* species (OTU1832 and OTU1878) could be mapped on chromosome 2 (LOD = 7.63) and on chromosome 18 (LOD = 6.48).

3.2.3.2 QTL derived from the Intdiet model

The following QTL were mapped with the Intdiet model. Two QTL for the phylum Basidiomycota could be mapped to chromosome 11 (LOD = 10.35) and to chromosome 7 (LOD = 9.07) with identification of candidate genes such as *Tmc8* (see table 5). On the class level unknown Zygomycota (LOD = 11.13) were mapped for a QTL on chromosome 7. Polyporales (Basidiomycota) was mapped on chromosome 6 (LOD = 10.37) containing the candidate gene *Cracr2*. On the family level an unidentified family and genus from the order Hypocreales (Ascomycota) was mapped on chromosome 13 (LOD = 9.97). This QTL contained the *Adamts16* as candidate gene. Further, the genus *Geosmithia* from the phylum Ascomycota could be mapped for a QTL on chromosome X (LOD = 10.76) containing candidate genes such as *Magea6*.

Also, *Malassezia restricta* (OTU29) from the phylum Basidiomycota was mapped on chromosome 8 (LOD = 8.77) for a QTL containing for example the candidate gene *Ank1*. Within the genus *Penicillium* QTL were found for *P. citreonigrum* (OTU2352) on chromosome 1 including candidate genes such as *Kiss1* and *Atp2b4*. *P. spathulatum* (OTU2243 and OTU2213) was mapped for a QTL on chromosome 18 and for the genus *Aspergillus* the species *A. glabripes* (OTU941) and *A. nidulans* (OTU298) could be mapped on chromosome X (LOD = 9.77) and 19 (LOD = 8.87).

3.2.3.3 QTL derived from the Intsex model

The following QTL were mapped with the Intsex model. QTL from uncertain Basidiomycota classes (LOD = 8.95) were mapped on chromosome 9 on class level (table 5). On the order level Corticales (Basidiomycota) was mapped for a QTL on chromosome 18 (LOD = 9.16). Its family Corticiaceae and genus *Vuilleminia* were also mapped to chromosome 18 for all the three models with interweaving loci. The genus *Rhodotorula* from the phylum Basidiomycota was mapped on chromosome 11 (LOD = 10.61) for a QTL. Another genus level QTL for *Sporendocladia* (Ascomycota) was mapped to an intergenic region on chromosome 8 (LOD = 10.02).

RDP ID (UNITE)	Taxonomic Name	Taxonomic Rank	Chromosome	SNP	LOD	Model	Candidate Genes
2	Basidiomycota	phylum	7	UNC12630138	10,35	IntDiet	Gm23292
2	Basidiomycota	phylum	11	UNC20492040	9,07	IntDiet	Tmc8,6030468B19Rik, Gm20708,Tha1,Dnah17,Usp36
1616	Zygomycota;Incertae_sedis_10	class	7	UNC13835983	11,19	IntDiet	Plpp4
248	Basidiomycota;Incertae_sedis_4	class	9	UNC16770385	8,95	IntSex	Ttk,Bckdhb
2453	Pucciniomycetes	class	9	JAX00173799	8,42	Add	Ube2cbp
112	Polyporales	order	6	UNC12042546	10,37	IntDiet	Akap3,Rad51ap1,Cracr2a
244	Hypocreales	order	8	UNC15057374	7,36	Add	Ttc29
40	Russulales	order	9	JAX00174115	7,66	Add	Near to CTCF binding site
40	Russulales	order	9	UNC16882054	11,7	IntDiet	Gm24200
2313	Corticiales	order	18	UNC28780254	9,16	IntSex	Kctd1,Aqp4,Chst9
121	Cantharellales	order	18	JAX00081898	9,39	IntDiet	Myo7b
2454	Pucciniales	order	X	UNC31371673	7,57	Add	Gprasp1,Tceal5,Kir3dl2, Kir3dl1,H2bfm,Tmsb15l, Tmsb15b2,Tmsb15b1, Slc25a53,Esx1
1822	Pleosporales;Incertae_sedis_13;Incertae_sedis_13	family	1	UNC2069242	6,58	Add	Fasl, Suco, Pigc, Dnm3, Dnm3os
2156	Hypocreales_unidentified	family	13	UNC22815472	9,97	IntDiet	Ice1,Adamts16
2314	Corticiaceae	family	18	UNC28770047	9,39	Add	Psma8
2314	Corticiaceae	family	18	UNC28777523	13,46	IntDiet	Psma8
2314	Corticiaceae	family	18	UNC28780254	13,36	IntSex	Psma8
2455	Pucciniaceae	family	X	UNC31334791	7,89	Add	Nox1
3029	Puccinia	genus	5	UNC8846261	7,7	Add	Dpp6
13230	Sporendocladia	genus	8	UNC15386783	10,02	IntSex	Open chromatin and CTCF binding site
1752	Candida	genus	9	UNC16896353	8,65	Add	Nearest gene Gm5369
2636	Rhodotorula	genus	11	UNC19796517	10,61	IntSex	Hs3st3a1
1587	Saccharomycetales_unidentified_1	genus	11	JAX00315981	7,1	Add	Nsrp1,Efcab5,Trp53i13,Nufip2, Nek8,Sdf2,Pigs,Unc119,Vtn, Nos2
2157	Hypocreales_unidentified_1	genus	13	UNC22815472	8,86	IntDiet	Gm36607
9476	Nakazawaea	genus	14	UNC24942977	8,65	Add	Fgf14
2763	Claviceps	genus	15	JAX00409307	7,59	Add	Gm36480
5603	Vuilleminia	genus	18	UNC28770047	8,76	Add	Psma8
5603	Vuilleminia	genus	18	UNC28777036	9,63	IntDiet	Psma8
5603	Vuilleminia	genus	18	UNC28780254	12,38	IntSex	Kctd1,Aqp4,Chst9
2771	Cryptococcus_1	genus	X	UNC31185725	6,95	Add	Gm732
3029	Puccinia	genus	X	UNC31334791	7,45	Add	Nox1
1758	Geosmithia	genus	X	UNC31499614	10,76	IntDiet	Cypt3,Samt4,Magea6
OTU ID	**Nearest Species by BLAST**	**Taxonomic Rank**	**Chromosome**	**SNP**	**LOD**	**Model**	**Candidate Genes**
OTU2352	Penicillium citreonigrum	Species	1	CEAJAX00009745	11,7	IntDiet	Kiss1,Snrpe,Zc3h11a,Atp2b4
OTU2060	Penicillium decumbens	Species	2	UNC2857265	8,45	Add	Mapkap1
OTU1832	Penicillium sp.	Species	2	UNC2961404	7,63	Add	Arhgap15
OTU2044	Penicillium decumbens	Species	2	UNC2819709	6,36	Add	Nup188,Miga2,Dolpp1,Ptpa,Gm 14487,Tor1b
OTU2110	Penicillium canescens	Species	4	UNC8028591	6,75	Add	Trabd2b,Foxd2
OTU29	Malassezia restricta	Species	8	UNC080619407	8,77	IntDiet	Plat,Kat6a,Ank1
OTU3106	Hyphopichia burtonii	Species	9	UNC16899401	6,56	Add	Gm9621,Gm47165,Gm5369
OTU1693	Aspergillus domesticus	Species	9	UNC16111846	8,52	Add	Fli1
OTU925	Nakazawaea holstii	Species	10	UNC101431286	6,52	Add	Plxnc1,Gm24186
OTU259	Aspergillus nidulans	Species	11	UNC19053972	6,45	Add	Abca13
OTU1041	Yamadazyma mexicana	Species	11	JAX00029390	6,98	Add	Nsrp1,Efcab5,Trp53i13, Nufip2,Nek8,Sdf2, 2610507B11Rik,Pigs,Unc119, Vtn,Nos2,Crlf3,Atad5, Rnf135
OTU1512	Talaromyces rugulosus	Species	13	backupUNC130306089	6,58	Add	Habp4,Cdk20
OTU925	Nakazawaea holstii	Species	14	UNC24942560	7,86	Add	Fgf14
OTU1298	Claviceps purpurea	Species	15	JAX00409307	8,16	Add	Gm36480
OTU2243	Penicillium spathulatum	Species	18	UNC29116030	9	IntDiet	Nearest gene AC154172.2
OTU2213	Penicillium spathulatum	Species	18	UNC29082687	9,14	IntDiet	Nr3c1
OTU1878	Penicillium sp.	Species	18	JAX00450954	6,48	Add	Zfp438
OTU298	Aspergillus nidulans	Species	19	UNC30601828	8,87	IntDiet	Fam45a
OTU941	Aspergillus glabripes	Species	X	UNC200105170	9,77	IntDiet	Gm24706

Table 5: Summarized QTL table with candidate genes. QTL for fungal taxonomy (phylum to genus) classified with RPD were mapped on mouse chromosomes using different models (Add=host genetics-mycobiota interaction; Intdiet=host genetics-diet interaction; Intsex=host genetics-sex interaction) and gene candidates are shown. QTL are mapped for species level OTUs identified with BLAST.

3.2.3.4 Correlations between fungi and bacteria

For this part an additionally performed QTL analysis of the bacterial microbiome of the gut (cecum content) of the same AIL mice used in this work was taken for comparison with the found fungal QTL. The data derived from the bacterial 16S rRNA gene region (v1/v2) sequencing in the course of the doctoral thesis of Foteini Beltsiou. Figure 22 shows the QTL found with the three different models (Add, Intdiet and Intsex) that were also used to map active (sequences derived from bacterial 16s rRNA) and standing (sequences derived from bacterial DNA) bacterial microbiota.

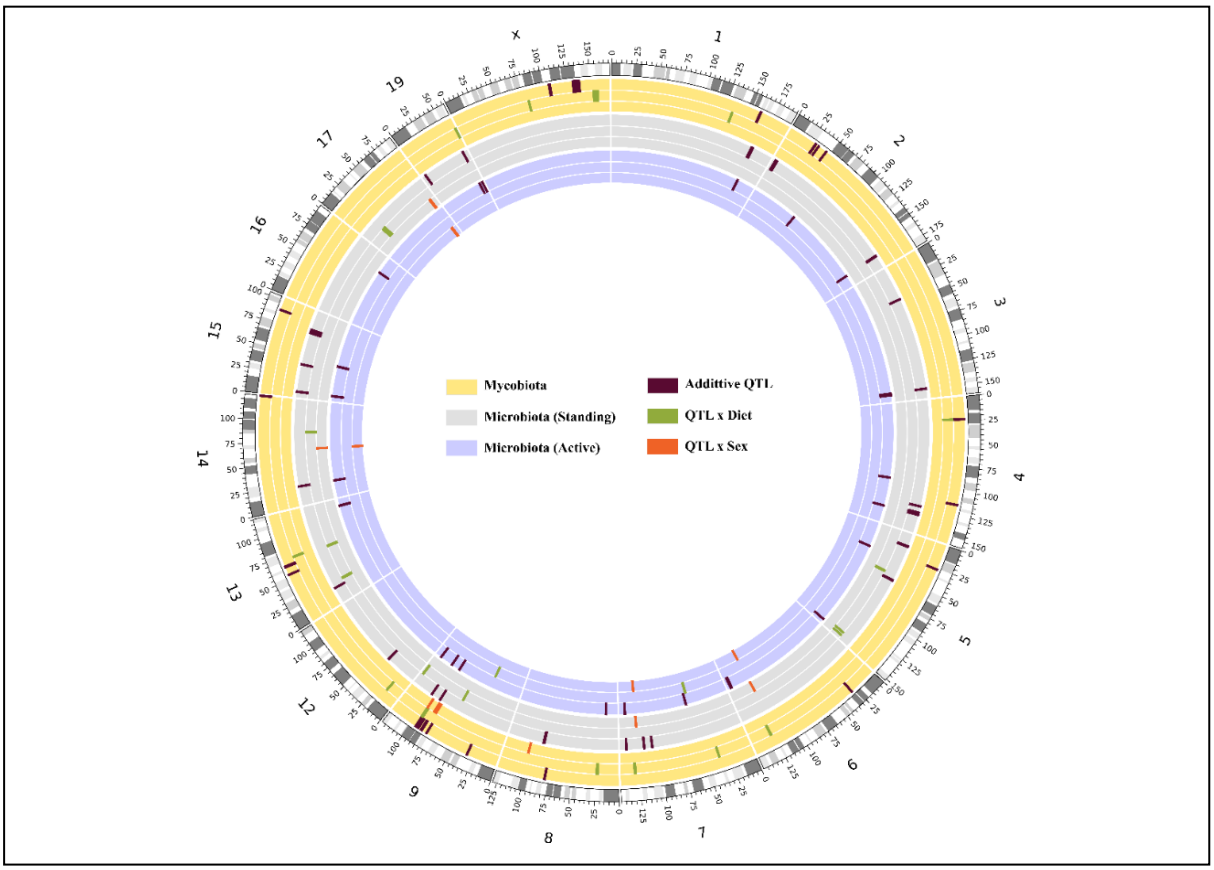


Figure 22: Circos plot of QTL in the AIL population associated with bacterial and fungal traits. The Circos plot shows QTL (αgw < 0.05) in AIL mice associated with bacteria (standing and active community) and fungi as traits. The outer most circle in the circular plot describes chromosomes with cytogenic bands in the C57BL/6J mouse genome (reference mouse assembly mm10). Every circle within each chromosome is color coded for fungi (yellow), bacterial standing (grey) and bacterial active (purple) communities describing the three models i.e. additive, interaction with diet- and sex from outwards to inwards. The QTL, marked as rectangles, with brown color represent the additive model, while green and red represent diet- and sex-interacting QTL, respectively.

Here, a total of 45 QTL (Add = 30, IntDiet = 10, IntSex = 5) for the standing bacterial communities and 38 QTL (Add = 31, IntDiet = 2, IntSex = 5) for the active bacterial communities could be mapped. The closest interaction between QTL was seen with the Add model in both fungi and bacteria with chromosome 9 and 13 showing QTL within a 50 cM range (see figure 22). On chromosomes 1 and 2, 4 to 9 and 12 to 15 as well as on chromosomes 19 and X fungal as well as bacterial QTL were found. No overlap between the fungal and bacterial QTL was found in this study.

4 Discussion

4.1 NGS protocol establishment

Traditional identification of fungi with culture-based methods is time-consuming and might not reflect the total fungal composition within a sample as to some fungi cannot be cultured or are very hard to grow under laboratory conditions. PCR and other culture-independent methods for fungal identification have greatly expanded, and so did the NGS (Nilsson et al. 2019). This study, including a large-sized experimental mouse cohort (see section 2.2), was designed for HT NGS. Successful bacterial 16s rRNA region gene sequencing from the same mouse samples should be expanded to fungal ITS sequencing. But no fungal DNA could be amplified in PCR from the DNA isolated with the Qiagen DNEasy Allprep (RNA and DNA) isolation kit (Qiagen, Venlo, NE) which was used for bacterial DNA and RNA extraction before the start of this work. Therefore, the need of a protocol for fungal DNA isolation and subsequent NGS was given. In order to be able to amplify ITS2 as target gene region, the extraction of fungal genomic DNA was essential.

During the establishment of the protocol, several steps needed special attention. First, the proper lysis of the sample material had priority. Due to the presence of the fungal cell wall, the bead - beating method together with strong homogenization was chosen for this protocol. The fungal cell wall has been found to be difficult to lyze because of its layered and firm structure (see section 1.1 and figure 1) and because of the fact that most of the fungi have an inner microcrystalline sleeve of chitin or concentrated chitin disks (Kumar and Mugunthan 2018). To break down resistant cell walls a variety of methods has been used so far including the use of liquid nitrogen rods, dry ice, magnetic beads, different mechanical devices or even microwave treatment (Lee et al. 1988; Griffin et al. 2002; Faggi et al. 2005; Zhang et al. 2010; Tendulkar et al. 2003). Off all, the use of thermolysis and lysis enzymes like Proteinase K became very popular in fungal molecular identification. It catalyzes the lysis of the fungal cell through β-1,3-glucanase and alkaline protease activity (Griffiths et al. 2006) and was therefore added as incubation step at 50 °C and 800 rpm for a minimum of 2 hours for the cell lysis prior to homogenization (see section 3.1.1 and figure 11).

Together with the enzymatic lysis, bead - beating is widely used in research for especially tough sample materials and microbes. Studies have already compared cell disruption methods for protein and DNA isolation from microorganisms and fungi and found that bead – beating is very efficient in fungal DNA extraction (Klimek-Ochab et al. 2011; Miller et al. 1999; Redanz et al. 2015; Aamir 2015). The Qiagen PowerLyzer Powersoil kit was chosen due to its ability to lyse tough microbes through the small diameter of beads (0.1 mm) and its PCR inhibitor removal technology. It has been demonstrated how important the selection of the bead size is for DNA extraction from microbes, both bacteria and fungi (Yang et al. 2020). At first, DNA isolates yielded a concentration below the determined threshold of 30 ng/µl. In order to overcome this problem, homogenization was changed from a 6,000 rpm (3 x 1 min) homogenization in a SpeedMill Plus (analytik jena, Jena (D)) to a 3 times 15 sec vacuum homogenization at 6,000 rpm in a Precellys 24 homogenizer, which was more efficient showing a higher DNA yield after isolation and also saved time during the HT isolation procedures (Dubacq 2016).

For the PCR the Phusion polymerase was chosen (see section 3.1.2) due to its high proof-reading capacity and stability as well as its low error rates. The enzyme was reviewed and tested intensively and was found to reduce nucleotide mismatches during reaction which are more likely the longer the sequence is. It yields a two to six fold higher efficiency and lower error rate, respectively, than other enzymes in the same category (Dolgova and Stukolova 2017; Li et al. 2006; McInerney et al. 2014). The manufacturer provides a fidelity calculator online which calculates the possible error rate (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/pcr-fidelity-calculator.html). For the usage in this work an estimated percentage of 0.693 (meaning the number of DNA molecules with one mismatch in 100) was calculated with an average fragment size of 450 bp and 35 PCR cycles. Also, purification of the used customized primers by a high performance liquid chromatography (HPLC) was shown to noticeably reduce mismatches (Andrus and Kuimelis 2001). Primers were therefore ordered accordingly. Additionally, barcoding within library construction was shown to suppress error in NGS while allowing detection of variant alleles that have an allele frequency below 0.1 % (Filges et al. 2019). The addition of these unique molecular identifiers can simplify the

identification of the majority of errors that occur during sequencing because of mistakes in library preparation or sequencer read errors by tracking the sequencing reads back to single DNA templates and further alignment of the reads with the barcode. True variants can therefore be identified separately from any type of introduced error (Brandariz-Fontes et al. 2015; Kinde et al. 2011).

During imaging of the gel electrophoresis misshaped DNA bands (from PCR products) were seen which made a correct quantification by volume and intensity impossible (figure 12A). This might have been due to an inequivalent salt concentration in the gel running buffer, the PCR Phusion 5xHF reaction buffer and the gel stain solution itself. PCR products might have run faster at the edges of the wells than at the centrum which could have resulted in the deformation of the gel bands. Straight bands were needed especially also for the extraction of the subpools from the gel (see section 3.1.4 and figure 14). Only appearing gel bands at around 450 bp were extracted because it was assumed that the construct of the ITS2 region gene together with the primer constructs (see section 1.5.3 and figure 6) would size in this range. In general, both the fungal ITS2 and ITS1 region products are described to range between 400 and 900 bp in size (Brasileiro et al. 2004). Due to difficulties in precisely cutting the gel under UV-light with a scalpel, gel extraction tips were used. This also saved time and lowered the exposure of the DNA fragments to the harmful UV-light. Nevertheless, small amounts of DNA may have remained in the gel (figure 14B). But the overall yield of subpool DNA was always sufficient for the pooling of the library (figure 16).

In order to successfully sequence a DNA library on a NGS platform such as Illumina MiSeq, the DNA library first has to be quantified. If the number of molecules within the library is underestimated by the quantification, sequencing may fail because the library concentration probably exceeds the capacity of the sequencing flow cell which results in a reduced yield of data due to cluster overlap. And if the concentration of the library is overestimated, one might also not use the full sequencing capacity and thereby the cost reduction of NGS might not be at its maximum. For this, not only the accurate quantification of the sequencing library, but also correct quantification of the subpools prior to library pooling has to be done equimolarly (Hussing et al. 2018). Studies have compared library quantification methods like light or UV-light spectrophotometry (for example NanoDrop or Qubit) and SYBR Green and TaqMan (fluorescent dyes) based qPCR assays. The qPCR assays gave more accurate predictions of DNA

concentrations (Pop et al. 2014; Dang et al. 2016). One big disadvantage of UV-spectrophotometers like the Qubit is that they do not only detect DNA, but also UV-molecules like RNA, proteins and phenols that absorb UV-light and thereby show a lower sensibility than qPCR assays for the detection of small DNA amounts (Nielsen et al. 2008). In this work, the DNA libraries were first quantified using a Qubit device but sequencing after failed due to a lack of cluster generation (data not shown). After having quantified the same library with qPCR and appropriate library dilution (figures 18 and 19; table 4), the sample could be sequenced without problems and also clustered nicely on the flow cell (see section 3.1.6). However, there are other qPCR preparation kits than the NEBNext library quantification kit, which may yield a higher coverage of for example regions with low content of guanosine and cytosine with also a higher rate of identified SNPs (Rhodes et al. 2014).

But the DNA can not only be quantified with the above mentioned methods, but also capillary electrophoresis on a chip can be done which is used by the Agilent Bioanalyzer (White et al. 2009; Panaro et al. 2000). The Bioanalyzer was chosen for determination of the average fragment size of the library and for quality approval prior to sequencing. Pop et al. (2014) evaluated the qPCR and the Agilent Bioanalyzer principles for quality control of NGS libraries and pointed out that the Bioanalyzer is the choice for quantification of high-quality libraries. However, as to the Bioanalyzer may underestimate the concentration of the DNA, this method was combined with the qPCR to obtain representative concentrations and sizes of the sequencing libraries. By SPRI purification of the library using the AmPure XP kit (see section 3.1.5.2 and figure 17) a high purity of the sample could be achieved which could be seen in the bioanalyzer later on and which led to a correct sizing of the libraries (figures 18 and 19). There are other quantification alternatives such as for instance the Nextera protocols directly available from the Illumina company that uses enzymatic cleavage (Adey et al. 2010). But the use of enzymatic fragmentation of for example genomic DNA was shown to introduce uneven sequence coverage in NGS (Marine et al. 2011).

Illumina MiSeq was chosen as sequencing platform for the DNA libraries obtained from the ribosomal ITS2 region gene derived DNA. Currently, the Illumina SBS technology is the dominant sequencing technology (Quail et al. 2009) and a MiSeq sequencer was available within the working area which additionally favored the use of this system. The SBS method is explained in more detail in section 2.7.2.2 and figure 9. Due to the

length of the ITS2 amplicon constructs of approximately 450 bp, sequencing was performed with paired-end 2 x 300 cycle chemistry (see section 3.1.6) which produces 300 bp of forward and 300 bp of reverse reads. The drawback is that a decrease in Q_{30} score within the reverse read could be seen in all three sequencing runs (data not shown) which is a given circumstance when sequencing 600 cycles or reads longer than 500 bp, respectively. Studies that analyzed the characteristics of errors occurring with Illumina sequencing showed that sequencing errors are systematic and the read quality is lower towards the end (Dohm et al. 2008). Some paired-end sequencing data also showed the presence of a read subpopulations within the reverse read with lower average qualities well below Q_{30}. Illumina currently does not provide explanations for this phenomenon but researchers suggest that the distributions of the fragment length within the library have a major impact on the quality drop of the reverse read. They could correlate fractions of low quality reverse reads to the fraction of long reads above 500 nucleotides (Tan et al. 2019). Still, Illumina was chosen as sequencing platform in this work due to the comparably high overall Q_{30} score that describes an average per base error rate of 1 in 1000 (Manley et al. 2016) and due to the compatibility with effective quantification methods (for example the NEBNext kit).

4.2 Diet influences gut fungal composition

Taxonomic composition within the human gut has been shown to be dominated by fungi from the phylum Ascomycota and Basidiomycota (Nash et al. 2017; Li et al. 2018; Hallen-Adams and Suhr 2017; Hibbett et al. 2007; Mar Rodríguez et al. 2015). The same observations were made in this work with Ascomycota composing more than 90 % of the found taxa in the gut of AIL mice (see figure 20). The genera *Penicillium, Aspergillus,* unknown Ascomycota and *Candida* were most abundant which also has been shown before for the human gut (Nash et al. 2017).

Although the intestines of human and mouse share physiological and anatomical similarities, for instance, in both a minimum of 14 fungal genera were found (Nash et al. 2017; Scupham et al. 2006), there are also dominant differences. It is thought that these differences evolved because of the diverging diets and feeding habits as well as difference in body size (Nguyen et al. 2015b). In mice, the cecum is larger than in

humans, relative to their total GT size, and ferments plant materials while in humans the function is not clear (Treuting and Dintzis 2012). Additionally, the majority of fungi found in the murine gut seems to be indigenous to the intestine because standard mouse food does not include many of the commonly found fungi (Scupham et al. 2006). Translating into the nutritional habits of humans one can be sure that a majority of gut fungi derive from food intake since it has been shown that the human gut mycobiome is relatively unstable and changes quickly upon changes of diet (Hallen-Adams and Suhr 2017; Nash et al. 2017). Also, many fungi become transient colonizers in the gut upon food digestion (Forbes et al. 2018) but do not persist over long periods of time (Hoffmann et al. 2013). A general discrepancy between study results is seen when using different sample types for fungal nucleic acid isolation and NGS. This might be due to the colonization of the GT either within the mucosa or within luminal or fecal contents where different overall microbial compositions could be observed (Savage 1977; Scanlan and Marchesi 2008). So one has to keep in mind that studies on the murine gut mycobiome may not fully represent that of humans, especially because many fungi and also bacteria derive from food intake and pass through the GT without colonization (Sam et al. 2017). Nevertheless, cecum content was chosen in this study to examine the mouse gut mycobiome because it was thought to well represent resident fungi within the mouse GT and lysis of the sample material was more efficient than for example with cecum tissue pieces.

That said, one of the major factors influencing the mycobiome in the gut is diet. For example could *Candida* already be positively correlated to the amount of dietary carbohydrates in the gut and also negatively associated with fatty acids (Li et al. 2018). In this work the phylum Basidiomycota with the genera *Wallemia* and *Mycena* were more abundant in Wes mice compared to the Con and Cal groups (figure 20) and the phylum Ascomycota with the genera *Phoma* and *Ascochyta* were more abundant in Cal and Con mice compared to Wes mice (figure 20). Western diet is associated with a high amount of fat, carbohydrates and cholesterol and was found to be one of the major causes for obesity in mouse studies, especially due to the high amount of fat (Turnbaugh et al. 2008; Cani et al. 2008; Heisel et al. 2017). The analysis of this work showed the effects of Western diet compared to a calorie-reduced diet on the gut mycobiome as to Wes mice were having a higher abundance of Basidiomycota (around 8 % of all taxa) than Cal mice (around 2 % of all taxa, figure 20.) which is in

line with studies that report Basidiomycota as emerging important pathogens causing a variety of clinical diseases and also invasive fungal infections (Singh et al. 2013). This might also be due to the increasing amount of risk factors like immunosuppressive drugs, modern invasive surgery, extreme weather and climate changes, the expansion of international travel as well as the tremendous usage of antifungals in agriculture (Brandt and Park 2013; Chowdhary et al. 2013). Some of these fungi might have been neglected as disease causing agents so far due to a lack of phenotypes when growing in the laboratory, where they don't show dikaryons or fruiting structures and hence, often cannot be correctly taxonomically identified (Sigler et al. 1995). Therefore, the identification through ITS sequencing displays a more precise method to investigate taxonomic abundances in for example clinical samples.

In this study no significant difference in fungal alpha diversity was found for the three dietary groups (figure 21A) but for the beta diversity (figure 21B). An increased fungal alpha diversity was found in diseased patients with inflamed mucosal tissues in IBD or CD compared to controls (Sokol et al. 2017; Ott et al. 2008). Fungal diversities in samples from diseased patients generally show a higher fungal load and less OTUs (Liguori et al. 2016) which could be partially confirmed in AIL mice (figure 21A) where Wes can be looked at as representative diet leading to obesity and thereby displaying disease. Alpha diversity for the Wes group was higher than in the Cal and Con groups but no significance was reached. Beta diversity analysis using PCoA (figure 21B) showed a significant difference between Wes and Cal-Con mice. Both the Cal and Con group clustered farther away from the nearly isolated Wes group indicating that mice fed the Wes show a lower beta diversity as in a lower dissimilarity between the fungal taxa. A lower beta diversity was also observed in obese patients compared to non-obese individuals (Mar Rodríguez et al. 2015).

OTU abundance analysis identified amongst others *Wallemia sebi*, *Penicillium decumbens*, *Aspergillus rubrum*, *Kluyveromyces marxianus* and *Nannizzia gypsea* as indicator species for Wes mice (figure 21C). *Wallemia sebi* which is now known as *W. mellicola* (Leong et al. 2015) represents together with the whole *Wallemia* spp. taxa the most xerophilic, osmophilic and halophilic described microorganisms (Zalar et al. 2005; Zajc and Gunde-Cimerman 2018). These physiological attitudes are normally rare within the Basidiomycota, thus, so far, *Wallemia* have been underestimated as health risk to humans. But the production of a variety of mycotoxins which are

increasingly produced at high salt concentrations (Jančič et al. 2016) has the potential to induce allergic reactions as well as infections of for instance the lung in humans (Guarro et al. 2008). An interesting study on allergic airways disease induction via the intratracheal house dust mite antigen showed how mice that had GT expansion of *W. mellicola* suffered from enhanced airway infiltrations with eosinophiles and other symptomatic pulmonary immune responses, while the fungus was not detected in the lung itself but in the gut (Skalski et al. 2018). The family Wallemiaceae was also identified as more abundant in Wes mice together with other members of the Basidiomycota like the Mycenacea, or unidentified Helotiales from the phylum Ascomycota, while unknown members of the Ascomycota as well as Nectriaceae were more abundant in Cal mice (figure 21D). While Mycena and Helotiales are mostly found in plants and in environmental samples (Thoen et al. 2020; Chew et al. 2015; Walker et al. 2011), their presence in the gut of Cal mice could be explained by the intake and digestion of fibres from the mouse chow together with fungi obtained during breeding. These fungi have not been associated with human diseases so far.

Penicillium and *Aspergillus* on the other hand belong to the most abundant fungal genera within the Ascomycota and the gut (Visagie et al. 2014) but they are also food-born organisms. Both are members of the Trichocomaceae family (Houbraken and Samson 2011) which also includes the genus *Talaromyces* (formerly *Penicillium*) that includes species with pathogenic behaviour leading to fatal mycoses in mostly HIV-infected patients (Chitasombat and Supparatpinyo 2013). *P. decumbens* and *A. rubrum* were found as indicator species for Wes in this analysis. While not much information is present concerning the virulence of *A. rubrum* (sometimes also referred to as *P. rubrum*), *P.decumbens* was already linked to a case of systemic infection in a patients with Acquired Immunodeficiency Syndrome (AIDS) or post-surgical inflammation (Alvarez 1990; Lyratzopoulos et al. 2002). Other *Aspergillus* species cause together with fungi from the genera *Candida, Cryptococcus* and *Trichophyton* acute and chronic infections in humans worldwide (Boral et al. 2018).

Two other Wes indicator species, *Kluyveromyces marxianus* and *Nannizzia gypsea* (priorly known as *Microsporum gypseum;* (Hoog et al. 2017)) are from the phylum Ascomycota. *K. marxianus* serves as model organism for enzyme production in industrial biotechnology (Fonseca et al. 2008; Lane and Morrissey 2010), while the soil-derived *N*. *gypsea* can be the rare cause of fungal infections of preferably

keratinous structures (Weitzman and Summerbell 1995) and other dermatomycoses in humans belonging to the tinea diseases or very rarely as onychomycosis (Dolenc-Voljč and Gasparič 2017; Romano et al. 2009).

In contrast, mice under caloric restriction had a higher abundance of *phoma herbarum*, *Aspergillus nidulans* and *Neoascochyta paspali* than in the Wes mice which were also assigned as indicator species (figure 21C). *Phoma* spp. are potentially pathogenic to humans but occurrence is rare and exclusively presented in immunocompromised people ranging from cutaneous infections to invasive diseases via for example consumption of contaminated foods (Young et al. 1973; Bennett et al. 2018; Paterson and Lima 2017; Oliveira et al. 2017). *A. nidulans* on the other hand, a potentially multidrug resistant fungus (Pereira et al. 1998), is used as model organism for gene regulation and cell development research in filamentous fungi (Pontecorvo et al. 1953). But together with *A. fumigatus*, *A. nidulans* has been characterized as common cause of chronic granulomatous disease (CGD) (Holland 2010). General abundance of the genus *Aspergillus* has been reported in obesity which is even increasing under anti-fungal therapy against for example *Candida* (Mar Rodríguez et al. 2015).

4.3 The influence of host genetics on the gut mycobiome

The majority of QTL were mapped using the Add model, which considered only interactions with fungal taxa and host genetics (table 5), while the lowest number of QTL were found with the Intsex model, where only interactions between sex and host genetics were considered. The Intdiet model mapped more than double the number of QTL compared to the Intsex model. These observations show that there is a connection of gut fungal taxa and host genes as well as an influence of diet. The association of fungal taxa with host genes could further be supported by the analysis of contribution to PV in QTL associated fungal lineages. Host genetics explained an average of 9.06 % of this variation and diet explained 1.2 %. But the highest PV was explained by cage (26 %). This finding is in line with previous microbial studies which observed high impact of the cage effect on gut microbiota (Srinivas et al. 2013; Franklin and Ericsson 2017; McCafferty et al. 2013). Additionally, also handling through different personnel as well as the location of the cages within the animal facility could

have an impact. QTL mapped with the Add model were mostly linked to fungi from the phylum Ascomycota but the number of identified candidate gene associated with these taxa was equally distributed among the two phyla (table 5). In this part of the work, the focus now lies on identified candidate genes and their role in human diseases as well as their association to the mapped fungal taxa. For comparison, please see table 5.

Tmc8 was identified through a QTL on chromosome 9 interacting with the order Russulales (Basidiomycota) as well as with diet. This gene has already been associated with respiratory diseases with emerging pathogenic Basidiomycota in a GWAS study (Chowdhary et al. 2014a). *Tmc8* (ENSEMBL: ENSG00000167895) encodes for the transmembrane channel like protein 8 and is expressed in various tissues as well as in different immune cells (Keresztes et al. 2003; Horton and Stokes 2014; Su et al. 2004). The gene mutations EVER1 and EVER2 have been reported in the rare autosomal recessive disease Epidermodysplasia verruciformis (EV) (Ramoz et al. 2002), in the promotion of human papillomavirus (HPV) gene expression (Lazarczyk et al. 2009) as well as in the induction of tumor necrosis factor (TNF) - α (Gaud et al. 2013). Another candidate gene found on chromosome 9 associated with the class Pucciniomycetes is a gene from the E3 ubiquitin ligase family which was shown to play an important role in fungal meningitis (Liu and Xue 2014), *Ube2d* (ENSEMBL: ENSG00000118420).

As candidate gene found on chromosome 8 for a QTL of the order Hypocreales the gene *Ttc9* encoding the tetratricopeptide repeat domain 9 can be named. It has been reported to be hormonally regulated in breast cancer cells where it also shows overexpression in the tissue (Cao et al. 2008). The upregulation of mRNA production was shown to be coupled with the growth inhibition through progesterone and also estrogen (Cao et al. 2006). Further, small RNAs from Hypocreales were observed to be abundant in human plasma while the *Ttc9* has been associated with plasma Aβ1-40 levels in GWA studies (Beatty et al. 2014; Chouraki et al. 2014).

Two important candidate genes were found amongst others when mapping QTL for uncertain families of the Pleosporales from the phylum Ascomycota to chromosome 1: *Fasl* (ENSEMBL: ENSG00000117560) and *Dnm3os* (ENSEMBL: ENSG00000230630), of which *Fasl* has been attributed a role in host defense against lethal fungal infections with *Candida albicans* and infections with

members of the Pleosporales (Chowdhary et al. 2014b; Rogge et al. 2015; Netea et al. 1999). *Fasl* encodes for the Fas ligand protein whose primary function as a receptor is the binding of the Fas antigen in order to deliver intracellular signals which then lead to apoptosis, but it has also been linked to tumor progression (Schneider et al. 1997; Lee and Ferguson 2003; Smith et al. 1994). *Dnm3os* encodes for the Dnm3 opposite strand/antisense RNA in humans and was shown to have critical effects on downstream signalling of the TGF – β in for instance the progression of lung fibrosis (Savary et al. 2019) but also different types of cancer (Zhang et al. 2019; Mitra et al. 2017).

On chromosome X and chromosome 5 two interesting candidate genes could be identified named *Nox1* and *Dpp6*. *Nox1* (ENSEMBL: ENSG00000007952) codes for the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a protein which is involved in DNA damage by the production of reactive oxygen species (ROS) (Babior 2004). But besides antimicrobial functions in host defense NADPH oxidase also modulates inflammation (Segal et al. 2012). NADPH oxidase is further reported in inherited CGD, during which recurrent severe fungal infections with for instance *Candida, Aspergillus* or *Cryptococcus* can lead to inflammation and CD-like IBD (Segal et al. 2012; Hogan and Wheeler 2014). Upon ROS production these fungi respond with their own production of ROS that together with the host-derived ROS trigger oxidative stress by simultaneously enhancing the fungal filamentous growth (Hogan and Wheeler 2014). *Dpp6* (ENSEMBL: ENSG00000130226) encodes the dipeptidyl peptidase like 6 transmembrane protein expressed in the brain (Sun et al. 2011) and was reported to be involved in a variety of neurodegenerative diseases like autism, progressive forms of multiple sclerosis or Alzheimer's disease (Prontera et al. 2014; Brambilla et al. 2012; Cacace et al. 2019).

Furthermore, genes identified through an Add QTL for Saccharomycetales on chromosome 11 were for instance *Nos2* and *Vtn.* For the nitric oxide synthase 2 encoding *Nos2* gene (ENSEMBL: ENSG00000007171) locus an increased expression has been reported in IBD patients (Dhillon et al. 2014). Normally, the produced nitric oxide (NO) acts as a messenger molecule for example for the host defense against invading pathogens such as fungi. But several fungi like *Cryptococcus* spp. or *Blastomyces dermatitidis* developed the ability to resist nitric oxide killing by the host (Hung et al. 2007; Rocco et al. 2011). Also, *Candida albicans* was shown to suppress

NO production through the secretion of specific mediators (Collette et al. 2014). Vitronectin is a glycoprotein that is present in the extracellular matrix and in the blood and it is encoded by the gene *vtn* (ENSEMBL: ENSG00000109072) (Schvartz et al. 1999). Through its characteristics to bind molecules like glycosaminoglycans or collagen, it interacts with β – glucans of the fungal cell wall of for example *P. carinii* or *C. albicans* which in turn stimulates macrophage release of TNF - α (Schvartz et al. 1999; Olson et al. 1996; Limper and Standing 1994).

On species level, *Nakazawaea holstii* from the phylum Ascomycota was mapped on chromosome 14 with *Fgf14* as candidate gene. *Fgf14* (ENSEMBL: ENSG00000102466) is a gene that encodes for the fibroblast growth factor 14. Mutations in *Fgf14* have been shown to be involved in ataxia which is a clinical syndrome associated with CNS histoplasmosis, a fungal infection of the CNS (Wang et al. 2002; van Swieten et al. 2003). A loss of function, on the other hand, was found to be associated with complex brain disorders like schizophrenia (Alshammari et al. 2016). *N. holstii* is described as a probiotic yeast which can survive gastric and pancreatic juices and is for instance found in olive oil (Zullo and Ciafardini 2019; Romo-Sánchez et al. 2010). *N. holstii* was also mapped for another QTL on chromosome 10 with the gene *Plxnc1* (ENSEMBL: ENSG00000136040) which codes for the transmembrane receptor Plexin C1. Plexin C1 can promote acute inflammation and is for example critical in acute lung injury, for which fungal infections are important risk factors (Granja et al. 2014; Kojicic et al. 2012; König et al. 2014). An association between this fungus and these two candidate genes therefore could be explained by the positive functions of the genes as well as the fungus.

Another QTL was mapped to chromosomes 15 and 13 which revealed the candidate gene *Cdk20* (ENSEMBL: ENSG00000156345). This gene encodes for the cyclin dependent kinase 20 (CDK20) which is involved in cell growth and which is also found in fungal species for example the budding yeast *S. cerevisiae* (Malumbres 2014; Malumbres et al. 2009). CDK20 has also been shown to promote radiochemoresistance in lung cancer cells (Wang et al. 2017).

A member of the genus *Aspergillus* was mapped on chromosome 9 with the candidate gene *FLI1* (ENSEMBL: ENSG00000151702). Invasive aspergillosis caused by *A. fumigatus* has gained tremendous importance due to the previously mentioned

increase in immunocompromised patients as well as the modern immunosuppressive therapies (Cohen et al. 1993). *A. fumigatus* causes fatal invasive lung infections with very low survival rates (Denning 1998; Bodey and Vartivarian 1989; Fukuda et al. 2003). *FLI1* (ENSEMBL: ENSG00000151702) encodes for the Friend Leukemia Integration transcription factor 1 (FLI1) which is involved in erythroleukemia induction through strains of the Friend virus (FRIEND 1957; Ben-David et al. 1990). FLI1 belongs to the E26 transformation-specific transcription factor family and regulates for example type 1 collagen gene expression in fibroblasts and it has been reported in autoimmunity and fibrosis (Hsu et al. 2004; Asano et al. 2010; Takahashi et al. 2017).

With *Abca13* (ENSEMBL: ENSG00000179869) on chromosome 11, a gene encoding for the ATP binding cassette subfamily A member 13 transmembrane protein (ABCA13), was identified (Prades et al. 2002). Defects in this gene were related by GWAS and other studies to several kind of diseases including autism, schizophrenia or ovarian carcinoma (Ueda 2011; Nakato et al. 2020; Iritani et al. 2018; Nymoen et al. 2015; Qian et al. 2020). *A. nidulans* who was associated with the chromosomal locus of *Abca13* was additionally mapped to chromosome 19 for an Intdiet model suggesting the interaction of diet with the mycobiome and further with host genetics.

Within the genus *Penicillium* several QTL were found for *P. canescens* with identification of the candidate gene *mapkap1* (ENSEMBL: ENSG00000119487). This gene encodes for the mitogen-activated protein kinases (MAPK) associated protein 1 and has been reported as predisposing gene for familial mixed mood disorder, cancer, obesity and diabetes as well as inflammatory diseases (Yang et al. 2019; Lawrence et al. 2008). In general, MAPKs are thought to play a role in fungal pathogenesis (Xu 2000).

For QTL mapped with the Intdiet model which only considered the interaction between diet and host genes several candidate genes could be identified. The QTL for the order Hypocreales from the phylum Ascomycota was mapped on chromosome 13 and contained the *Adamts16* gene (ENSEMBL: ENSG00000145536). This gene encodes for the ADAM Metallopeptidase with thrombospondin type 1 motif 16 protein (ADAMTS16). Members of the ADAMTS were reported to have anti-cancer or, in contrast, pro-tumorigenic characteristics (Mead and Apte 2018). A decreased expression of *Adamts16* has been reported in insulin-treated chondrosarcoma cells,

in the promotion of cardiac fibrosis and hypertrophy and in hypertension, and GWAS studies associated other ADAMTS loci further with complex traits such as urgency urinary incontinence in women or schizophrenia (Cakmak et al. 2015; Yao et al. 2020; Dubail and Apte 2015; Gopalakrishnan et al. 2012; Joe et al. 2009; Richter et al. 2015; McGrath et al. 2013).

The genus *Geosmithia* from the phylum Ascomycota could be mapped for an Intdiet QTL on chromosome X containing the candidate gene *Magea6*. *Geosmithia* causes invasive mycosis in patients with CGD due to a deficient ROS production which is a result of a mutated NADPH complex and has been described as an emerging pathogen for invasive mycosis in humans (Ravin et al. 2011). *Magea6* (ENSEMBL: ENSG00000197172) encodes for the MAGE family member A6 protein (MAGEA6) which has been shown to induce tumor regression in cancer patients that were immunized with MAGEA antigens (Thurner et al. 1999; Marchand et al. 1995). Also in mice MAGE genes have been identified where they are also expressed in tumor cells (Plaen et al. 1999).

Ank1 (ENSEMBL: ENSG00000029534) was identified on chromosome 8 with a QTL for *Malassezia restricta* (Basidiomycota) which has been reported in skin diseases and for example CD (Findley et al. 2013; Lam et al. 2019).(Lam et al. 2019) But *Malassezia* is also associated with a vegetarian diet (David et al. 2014). The protein Ankyrin 1, encoded by *Ank1*, plays a major role in cell motility, proliferation and activation and mediates the attachment of membrane proteins to the cytoskeleton (Smith and Penzes 2018). SNPs in *Ank1* have been associated with Late-Onset Alzheimer's Disease in a Chinese population (Chi et al. 2016).

Other genes that were identified with the Intdiet model were *Kiss1* (ENSEMBL: ENSG00000170498) and *Atp2b4* (ENSEMBL: ENSG00000058668) for different *Penicillium* and *Aspergillus* spp. The *Kiss1* gene encodes for the metastasis suppressor 1 protein or also named kisspeptin1 (Gottsch et al. 2009). The kisspeptins are amongst others expressed in the liver, the pancreas or the hypothalamus and act as tumor suppressors (Kotani et al. 2001; Lee et al. 1999; West et al. 1998). Gene expression as well as mutations of *kiss1* have been linked to puberty disorders and prostate cancer (Silveira et al. 2010; Wang et al. 2012). *Atp2b4* (also referred to as PMCA2) encodes for the ATPase plasma membrane Ca^{2+} transporting 4 protein (Brini

and Carafoli 2011). It was described in being associated in colorectal cancer, hearing loss and familial spastic paraplegia, lastly with more than 50 identified disease loci, in humans and as null mutations they are lethal to murine embryos (Geyik et al. 2014; Tempel and Shilling 2007; Blackstone 2012).

The family Corticiaceae and further genus *Vuilleminia* from the phylum Basidiomycota were mapped to chromosome 18 for all the three models (Add, Intdiet and Intsex). It could be shown that the abundance of Corticiaceae is inversely correlated with insulin levels in gut samples of obese people (Mar Rodríguez et al. 2015). As candidate gene *Kctd1* (ENSEMBL: ENSG00000134504) can be mentioned here. It encodes for the potassium channel tetramerization domain containing 1 protein (KCTD1) which has been reported in inhibition of adipogenesis (Pirone et al. 2019).

All of the described genes were associated with fungal taxa. Some of these associations have been reported before, while others give new hints on the interactions of host genes with mycobiota in the gut. But what could clearly be seen is that nearly all of the candidate genes bear the potential for further studies to investigate their roles in shaping the gut mycobial composition in more detail. Also, fungal-bacterial interactions within the total microbial gut community may have influenced fungal composition. It has been shown that bacteria and fungi can form consortia in which they mutually benefit (Tarkka et al. 2009; Kobayashi and Crouch 2009). Especially during infections these interactions can play a role, when fungi like for example *Candida* are accompanied by bacteria (Hermann et al. 1999; Peleg et al. 2010). Within this study, comparing QTL for fungal and bacterial traits in AIL mice, no overlap between fungal and bacterial QTL was found. This suggests a distinct role of the host genetics in the regulation of the two kingdoms in AIL mice. Interestingly, some of the fungal QTL overlapped with previously reported murine gut microbiota QTL. For example, QTL for *Penicillium* or different Basidiomycota overlapped with previously published QTL for species of the bacterial class Clostridia such as *Oscillospira* or *Coprococcus* (Bubier et al. 2020; Schlamp et al. 2019). It has been shown that *Penicillium* seem to negatively correlate with *Oscillospira* in Clostridoides infections (Stewart et al. 2019) and that a decreased abundance of *Coprococcus* has been observed in IBD patients together with an increased abundance of Basdiomycota (Sokol et al. 2017).

5 Conclusion and outlook

When compared to the bacterial microbiome, the human gut mycobiome shows a lower overall diversity while yeasts like Saccharomyces, Candida or Malassezia are the predominant colonizers. Many species of the Basidiomycota are hard to identify using culture-based methods due to the lack of laboratory phenotypes as well as the fact that many do not grow in culture. Fungal ITS region sequencing on NGS platforms is a fast and specific method to capture most of the fungal diversity and taxa in gut samples. In this work, a NGS protocol for the fungal ITS2 gene region was established including the isolation of fungal DNA from murine cecum content samples of AIL mice as well as the preparation and sequencing of DNA amplicon libraries. The protocol is effective and fast and suitable for NGS application due to multiple quality control steps along the process. Sequencing quality could be improved to a higher Q score for example and optimization of cluster densities by adjusting sequencing reagents. The decrease in read quality of the reverse read in Illumina 600 cycle sequencing technology has still to be clarified and also appeared in this work. With this problem solved, future ITS2 sequencing runs might be even more efficient in distributing reads more evenly and aligning the barcodes more precisely to the reads and thereby capturing the full fungal diversity in the sample.

QTL analysis of the gut mycobiome of the AIL mice revealed associations with fungal taxa with host genetics as well as a significant influence of the environmental factor diet on the gut mycobial composition. The identification of SNPs by NGS studies with identification of candidate genes which interact with specific microbiota is important to understand complex diseases and their phenotypic variations that are shown to be driven by changes in the microbiome. The susceptibility of a host to develop a certain disease phenotype might be highly influenced by the composition of the gut mycobiome. What still remains unclear is the possible effect of the bacterial gut communities on the gut fungi and whether changes in the gut mycobiome happen due to the influence of environmental factors like diet or the host genetics alone or if an imbalanced bacterial microbiome might change the fungal mycobiome through interactions. This relationship could also be mutual and should be investigated further.

The statistical analysis of the gut mycobiome in AIL mice revealed several candidate genes which were associated with certain fungal taxa. Of these, *Nox1* displays an

interesting target for further studies due to its antimicrobial functions in host defense as well as its role in inherited CGD which can lead to CD-like IBD. Further SNP identification in this gene might reveal reasons for the susceptibility for recurring fungal infections. Another candidate gene is *vtn.* Through its interactions with components of the fungal cell wall functional studies on this gene might be beneficial for accessing the question whether dietary induced changes in the gut mycobiome can have a significant impact on gene expression. The model organism *C. albicans* could be used as to it was already shown to be interacting with expressed proteins of this gene. Next, it could be promising to take a closer look at the gene *Kctd1* which has been reported in the inhibition of adipogenesis. The associated fungal family found in this work has already been correlated with obesity and could display a basis for human studies, in which abundances of Corticiaceae could be monitored over time upon a dietary intervention. Additional murine knock-out studies on *Kctd1* might reveal genetic impact on gut mycobial compositions.

Taken together, research on genetic associations with fungal abundances in the gut continuously drives understanding of complex phenotypes. The identification of polymorphisms in immune function regulating genes can help to determine the susceptibility of certain genotypes of host populations to fungal infections. With fungal infections increasing through immunosuppression in the modern world, the need of the development of alternative treatment options other than antifungal drugs and corticosteroids is present and could be addressed through further genetic studies on genes identified in this work. What remained unclear were bacterial-fungal interactions. No overlaps between bacterial and fungal QTL on different chromosomes of the mouse genome were found in this work but fungal QTL might be linked to previously found bacterial QTL. Further investigations may include gnotobiotic mouse studies together with fecal transplantations to verify whether the observed changes in the gut mycobiome were caused by only diet or rather induced changes within the bacterial composition and subsequent effects on the fungal communities through interactions within the overall microbial community. Additionally, fungi produce a wide range of metabolites that have the potency to influence gut biosis and regulatory pathways within the immune system. Therefore, the characterization of metabolic parameters of these mice in correlation to fungal abundances in the gut would be another interesting future investigation.

6 Literature

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7 Appendix

7.1 Full protocol for NGS of fungal DNA from murine gut samples

DNA isolation

Material needed:

DNeasy PowerLyzer PowerSoil Kit (Qiagen, KatNo# 12855-100), Qiagen Proteinase K (Qiagen, Kat.No# 19133), heatblock at minimum 800RPM, Homogenizer

Before starting:

Clean all working surfaces, pipettes and racks with 70% EtOH and RNAse away.

- Add up to 0,25 g of gut sample (with a sterile tip) to a PowerBead Tube and add 750 µl of PowerBead solution to the tube.
- Add 60µl of Solution C1 to the tubes, invert several times, vortex briefly and incubate at RT for 10 min.
- Add 20 µl Proteinase K to each tube, invert and vortex for 5 sec.
- Incubate the tubes at 800 rpm and 50 °C for minimum 2 h.
- Put the tubes in a homogenizer at min. 6000 rpm for 3 x 15 sec (adjust time according to the power of the homogenizer).
- Centrifuge the bead tubes at 12.000 x g for 30 sec.
- Transfer 400 µl of supernatant to a clean 2 ml collection tube (provided). The supernatant may still contain some soil particles.
- Add 250 µl of Solution C2, vortex for 5 sec and incubate 5 min at 4 °C.
- Centrifuge the tubes for 1 min at 13.000 x g.
- Transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
- Add 200 µl of Solution C3, vortex briefly and incubate 5 min at 4 °C.
- Centrifuge 1 min at 13.000 x g.
- Transfer up to 700 µl of supernatant into a clean 2 ml tube.
- Add 1200 µl of Solution C4 and vortex for 5 sec.
- Load up to 700 µl of supernatant to a MB Spin Column and centrifuge at 13.000 x g for 1 min. Discard the flow-through and repeat to a total of three times per sample.
- Add 500 µl of Solution C5 and centrifuge for 30 sec at 14.000 x g, discard the tube with flow-through and place the filter in a new 2 ml collection tube (not provided) and repeat centrifugation step at 14.000 x g for 1 min to get rid of the C5 solution.
- Carefully place spin filter in a clean 2 ml tube while avoiding any splashing of the C5 solution onto the MB Spin Column.
- Add 100 µl of Solution C6 to the centre of the white filter membrane (alternatively DNA-free PCR grade water) and elute the DNA for 30 sec at 13.000 x g.
- Store the isolated DNA at -20 to -80 °C for further analysis.

ITS2 region PCR

Material needed:

ITS-region forward and reverse primers with indexes (barcodes), Phusion Hot Start II DNA High Fidelity Polymerase (Thermofisher Scientific, Kat.No# F530L), DNAse, RNAse-free PCR-Grade water, Thermocycler, UV-light cabinet

- Prepare the following Master Mix (MM, template is not included in the MM):

Reagent	**Volume**
5x HF Phusion Buffer	5µl
dNTPs (10mM)	0.5µl
Forward Primer number (2µM)	4µl
Phusion	0.25µl
Template DNA	2µl
H_2O	9.25ml
Total reaction volume	**25µl**

- Chose 1 forward primer for each run and combine with different reverse primers.
- Dilute the primers to a concentration of 2 µM.
- Keep the enzyme at -20 °C until usage.
- Pipette 19 µl of MM to all tubes under a UV-hood.
- Add 4 µl of reverse primers under a UV-hood.
- Remove the PCR plate or tubes from under the hood and pipette 2µl of DNA samples (minimum concentration of DNA should be 30 ng/µl).
- Run primer controls, negative isolation control and PCR control with each run.
- Set the thermocycler to the following program:

Step	**Degrees**	**Time**	
Initial denaturation	98°C	30 sec	
Denaturation	98°C	9 sec	x 35 cycles (~ 1.5 h)
Primer Annealing	50°C	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	10 min.	
Keeping cold	4°C	∞	

Gel electrophoresis of PCR products and quantification

Material needed:

Gel chamber, agarose powder (Biozym, Kat.No# 0000520836), 1X TAE buffer (freshly prepared), gel dye HD Green (https://www.intas.de/dna-rna-faerbereagenzien/255-

dna-dye-hdgreen-plus), DNA gel loading dye 6x (Thermofisher Scientific, Kat.No# R0611), 100bp DNA ladder (Thermofisher Scientific, Kat.No# SM0323), microwave, nitrile gloves, gel imager and imaging program

1.) Gel preparation:

- Prepare a 1.5 % agarose gel in 1X TAE buffer with 4 µl of HD Green dye in 100 ml of dissolved gel.
- Wear protection gloves and remove bubbles from the gel. Cover the gel while hardening.
- Avoid long loading times of the PCR products into the gel pockets.

2.) Preparation of PCR products:

- Mix 5.5 µl of each PCR product and 1 µl of 6x loading dye.
- Load 5µl of DNA ladder and 5µl of each PCR product mix into the gel.
- Run the gel at 120 V for 5 min followed by 60 min at 110 V.
- Image the gel under UV-light.
- Negative, primer and PCR controls should not show any bands.

3.) Quantification of DNA in the gel and preparation of subpools:

- Quantify the DNA fragments in the gel with a gel imaging software.
- Use the marker band as reference.
- The ng of DNA fragments can be calculated from correlation to the reference marker.
- Prepare subpools from PCR products with one forward primer on ice. Subpools have to be equimolar with 100 ng/µl concentration of each PCR product.
- Store the subpools at -20 °C.

Subpool and library preparation

Material needed:

Qiagen MinElute Gel Extraction kit (Qiagen, Kat.No# 28604), ultrapure agarose (Thermofisher Scientific, Kat.No# 16500100), Gel Extraction Tips (Biozym, Kat.No# 615935), UV-light plate, NEBNext Library quantification Kit for Illumina (New England Biolabs, Kat.No.# E7630S), clean absolute Ethanol, Agencourt AmPure XP kit (Beckham Coulter, Kat.No# A63880), Magnetic stand, Agilent High Sensitivity DNA kit (Agilent, Kat.No# 5067-4626), SYBR SAFE DNA gel stain (Thermofisher Scientific, Kat.No# S33102)

1.) Subpool extraction from the gel:

- Prepare a 1.5% ultrapure agarose gel with 10 µl of SYBR SAFE gel dye in 100 ml of dissolved gel and load the complete subpools into the gel.
- Run the subpools as described for the gels above.

- Cut only the bands at approx. 450bp under UV-light and collect in clean 1.5 ml tubes and proceed with the gel extraction according to the manufacturers protocol (Qiagen, 28604).
- Take 5 µl aliquots from each subpool for analysis and store subpools at -20 °C

2.) qPCR of subpools:

- scale down the protocol to 10µl per reaction volume to save reagents
- Prepare the reaction volumes according to the manufacturers protocol.
- Calculate subpool concentrations with the NEBiocalculator (https://nebiocalculator.neb.com/#!/qPCRlibQnt).
- Use the calculated concentrations for library pooling

3.) Library pooling and AmPure bead purification:

- Equimolarly mix the subpools into libraries (maximum sample amount = 300 ± 20 amplicons) according to the lowest measured subpool concentration.
- Before starting: Freshly prepare 75 % Ethanol and set aside the magentic beads at RT for about 30 min to prewarm.
- Clean up the library with the Agencourt AmPure XP kit on a magnetic stand following the manufacturers protocol.
- Take a 3 µl aliquot of the purified library before storing at -20 °C.

4.) Library characterization for sequencing:

- Run the NEBnext qPCR quantification with your library according to the manufacturers protocol.
- Run your library on the Bioanalyzer and calculate the average size of the library according to the manufacturers protocol.

Important: Vortex the DNA ladder and marker before loading onto the bioanalyzer chip.

Illumina MiSeq V3 – NGS

Material needed:

MiSeq Reagent Kit v3 600 cycles (Illumina, Kat.No# MS-102-3003), freshly prepared NaOH (0.2 N), Sequencer

1.) Cartridge, HT1 buffer preparation:

- Thaw the HT1 (Hybridization Buffer) at RT. When thawed, store at 2° to 8°C or on ice.

- Thaw the reagent cartridge in a water bath (1 – 1.5 h needed) or at RT (3 h needed) and gently mix thawed reagents. Do not allow the water to exceed the maximum water line.
- Before loading, gently tap the cartridge to dislodge water from the base and dry. Make sure that no water has splashed on the top of the reagent cartridge.

2.) Dilution of samples:

- Prepare a fresh dilution of NaOH: 80 µl laboratory-grade water + 20µl 1 N NaOH
- Denature the library:
  5 µl of 4 nM library + 5 µl of 0.2 N NaOH
- Vortex briefly and centrifuge at 280 x g for 1 min and incubate for 5 min at RT to denature the DNA into single strands.
- Then add the following volume of pre-chilled HT1 to the denatured library:
  10 µl denatured DNA + 990 µl pre-chilled HT1
- Dilute the library (now 20 pM) to a final sequencing concentration (final volume 600µl) of 17.5 pM:
  525.4 µl denatured (20 pM) library + 74.6 µl of pre-chilled HT1 buffer
- Store library on ice until loading.

- Dilute the PhiX control library to 4 nM:
  2 µl PhiX (10 nM) + 3 µl Tris-Cl buffer (10 nM, pH 8.5 with 0.1 % Tween 20)
- Denature PhiX control:
  5 µl PhiX control (4 nM) + 5 µl freshly prepared 0.2 N NaOH
- Vortex briefly and centrifuge at 280 x g for 1 min and incubate at RT for 5 min.
- Dilute the denatured PhiX control to 20 pM:
  10 µl denatured PhiX control (2 nM) + 990 µl pre-chilled HT1 buffer
- Dilute the denatured PhiX control to 17.5 pM (final volume 300 µl):
  262.7 µl denatured PhiX control (20 pM) + 37.4 µl HT1 buffer
- Invert several times to mix.

- Prepare 660µl of the final load mix (17.5 pM library with 20 % PhiX control):
  132 µl PhiX control (17.5 pM) + 528 µl library (17.5 pM)
- Store sample on ice until loading.

- Dilute the custom primers forward, reverse and index to 0.5 µM:
  3 µl primer (100 µl) + 597 µl HT1 buffer
- Store on ice until loading.

3.) Loading of the cartridge:

- Open well 17, 18, 19 and 20 with a tip.
- Transfer the volume of well 12 into a new tube.
- Combine 60µl read1 primer mix from position 12 and add 540 µl of diluted custom forward primer

- Load 600 µl of sample/primer as follows (avoid air bubbles):
  Position 17: Final load (denatured 17.5 pM library with 20 % denatured PhiX control)
  Position 18: Diluted custom forward primer containing 10 % read1 primer mix from position 12)
  Position 19: Diluted custom index primer
  Position 20: Diluted custom reverse primer

4.) Start run:

- Save the sample sheet on the desktop and press SEQUENCE
- Login to the Basespace sequencing hub
- Clean flow cell with ampure water from both sides to remove storage buffer and carefully remove all water. The flow cell should be completely clean and dry.
- Proceed with the orders displayed on the device.
- Start the run.
- The v3 chemistry run takes ~65h

5.) After the run:

- Remove the formamid solution from well 8 of the cartridge and discard as special waste.
- Start a postrun-wash.

7.2 Full QTL table

RDP ID (UNITE)	Taxonomic Name	Taxonomic Rank	Chromosome	SNP	LOD	START	END	%R² (LOD)	%R² (Sex)	%R² (Gen)	%R² (Diet)	%R² (Cage)	Model	Candidate Genes
2	Basidiomycota	phylum	7	UNC12630138	10,35	36217417	36377617	10,8	0,2	2	0,79	35,85	IntDiet	Gm23292
2	Basidiomycota	phylum	11	UNC20492040	9,07	117545132	118530429	9,53	0,2	2	0,79	35,85	IntDiet	Tmc8,6030468B19Rik, Gm20708,Tha1,Dnah17,Usp36
1616	Zygomycota;Incertae_sedis_10	class	7	UNC13835983	11,19	129034744	130149978	11,63	0,1	2,12	0,53	1,21	IntDiet	Plpp4
248	Basidiomycota;Incertae_sedis_4	class	9	UNC16770385	8,95	83855900	84473161	9,41	0,02	0,75	0,37	19,16	IntSex	Ttk,Bckdhb
2453	Pucciniomycetes	class	9	JAX00173799	8,42	86373943	86400299	8,88	<0.01	7,92	0,55	46,36	Add	Ube2cbp
112	Polyporales	order	6	UNC12042546	10,37	126848048	127850668	10,82	0,13	0,31	0,93	15,06	IntDiet	Akap3,Rad51ap1,Cracr2a
244	Hypocreales	order	8	UNC15057374	7,36	78235223	78903753	7,81	0,05	3,38	5,91	26,67	Add	Ttc29
40	Russulales	order	9	JAX00174115	7,66	90677892	90681049	8,11	0,08	4,08	0,48	9,95	Add	Near to CTCF binding site
40	Russulales	order	9	UNC16882054	11,7	93022854	94468212	12,12	0,08	4,08	0,48	9,95	IntDiet	Gm24200
2313	Corticiales	order	18	UNC28780254	9,16	14974151	15527411	9,62	<0.01	1,52	0,3	7,06	IntSex	Kctd1,Aqp4,Chst9
121	Cantharellales	order	18	JAX00081898	9,39	31199252	32034289	9,85	<0.01	0,75	0,23	5,22	IntDiet	Myo7b
2454	Pucciniales	order	X	UNC31371673	7,57	135196467	138968699	8,02	<0.01	6,26	0,45	55,22	Add	Gprasp1,Tceal5,Kir3dl2,Kir3dl1, H2bfm,Tmsb15l,Tmsb15b2, Tmsb15b1,Slc25a53,Esx1
1822	Pleosporales;Incertae_sedis 13;Incertae_sedis_13	family	1	UNC2069242	6,58	161321524	162280389	7,01	0,1	0,28	4,09	11,34	Add	Fasl, Suco, Pigc, Dnm3, Dnm3os
2156	Hypocreales_unidentified	family	13	UNC22815472	9,97	70348004	71076189	10,43	0,01	3,32	0,1	13,54	IntDiet	Ice1,Adamts16
2314	Corticiaceae	family	18	UNC28770047	9,39	14326515	15059635	9,85	0,07	0,94	0,45	5,92	Add	Psma8
2314	Corticiaceae	family	18	UNC28777523	13,46	14637698	15762396	13,81	0,07	0,94	0,45	5,92	IntDiet	Psma8
2314	Corticiaceae	family	18	UNC28780254	13,36	14729484	15865163	13,72	0,07	0,94	0,45	5,92	IntSex	Psma8
2455	Pucciniaceae	family	X	UNC31334791	7,89	132100158	134157164	8,35	<0.01	5,57	0,4	57,99	Add	Nox1
3029	Puccinia	genus	5	UNC8846261	7,7	26884871	27134429	8,15	<0.01	6,43	0,46	54,64	Add	Dpp6
13230	Sporendocladia	genus	8	UNC15386783	10,02	101783657	101909615	10,47	0,13	4,35	0,92	4,13	IntSex	Open chromatin and CTCF binding site
1752	Candida	genus	9	UNC16896353	8,65	93952827	94131153	9,11	0,03	6,27	1,08	20	Add	Nearest gene Gm5369
2636	Rhodotorula	genus	11	UNC19796517	10,61	64412443	64724846	11,06	<0.01	0,43	0,03	8,55	IntSex	Hs3st3a1
1587	Saccharomycetales_unidentified_1	genus	11	JAX00315981	7,1	76679070	79530680	7,54	0,22	9,6	0,18	31,48	Add	Nsrp1,Efcab5,Trp53i13,Nufip2, Nek8,Sdf2,2610507B11Rik, Pigs,Unc119,Vtn,Nos2
2157	Hypocreales_unidentified_1	genus	13	UNC22815472	8,86	70693059	70712481	9,32	<0.01	3,43	0,11	13,64	IntDiet	Gm36607
9476	Nakazawaea	genus	14	UNC24942977	8,65	124577548	124769406	9,11	0,01	11,19	1,12	21,13	Add	Fgf14
2763	Claviceps	genus	15	JAX00409307	7,59	90026716	90401640	8,04	0,03	2,9	7,29	38,82	Add	Gm36480
5603	Vuilleminia	genus	18	UNC28770047	8,76	13954968	14974151	9,22	0,13	1,23	0,06	4,33	Add	Psma8
5603	Vuilleminia	genus	18	UNC28777036	9,63	14637698	16134939	10,09	0,13	1,23	0,06	4,33	IntDiet	Psma8
5603	Vuilleminia	genus	18	UNC28780254	12,38	14938022	15724201	12,78	0,13	1,23	0,06	4,33	IntSex	Kctd1,Aqp4,Chst9
2771	Cryptococcus_1	genus	X	UNC31185725	6,95	106716393	108056584	7,39	<0.01	1,71	4,15	9,52	Add	Gm732
3029	Puccinia	genus	X	UNC31334791	7,45	132100158	134157164	7,9	<0.01	6,43	0,46	54,64	Add	Nox1
1758	Geosmithia	genus	X	UNC31499614	10,76	153533292	158259643	11,2	0,02	1,15	0,08	13,96	IntDiet	Cypt3,Samt4,Magea6
OTUID	**Nearest Species by BLAST**	**Taxonomic Rank**	**Chromosome**	**SNP**	**LOD**	**START**	**END**	**%R² (LOD)**	**%R² (Sex)**	**%R² (Gen)**	**%R² (Diet)**	**%R² (Cage)**	**Model**	**Candidate Genes**
OTU2352	Penicillium citreonigrum	Species	1	CEAJAX00009745	11,7	133222151	133859717	11,86	0,01	7,31	0,23	19,14	IntDiet	Kiss1,Snrpe,Zc3h11a,Atp2b4
OTU2060	Penicillium decumbens	Species	2	UNC2857265	8,45	34420744	34658977	8,71	0,01	0,56	0,49	5,25	Add	Mapkap1
OTU1832	Penicillium sp.	Species	2	UNC2961404	7,63	44226155	44322324	7,89	0,01	7,02	0,08	29,39	Add	Arhgap15
OTU2044	Penicillium decumbens	Species	2	UNC2819709	6,36	30326924	31196516	6,63	0,01	7,56	0,04	14,52	Add	Nup188,Miga2,Dolpp1,Ptpa, Gm14487,Cstad, 1700001O22Rik,Tor1b
OTU2110	Penicillium canescens	Species	4	UNC8028591	6,75	114570777	114926583	7,02	0,03	11,99	0,1	38,87	Add	Trabd2b,Foxd2
OTU29	Malassezia restricta	Species	8	UNC080619407	8,77	22671231	23905921	9,03	<0.01	1,28	0,33	16,16	IntDiet	Plat,Kat6a,Ank1
OTU3106	Hyphopichia burtonii	Species	9	UNC16899401	6,56	93099549	94273181	6,83	0,02	11,15	0,01	37,76	Add	Gm9621,Gm47165,Gm5369
OTU1693	Aspergillus domesticus	Species	9	UNC16111846	8,52	32457417	32463381	8,78	0,07	10,26	0,08	57,01	Add	Fli1
OTU925	Nakazawaea holstii	Species	10	UNC101431286	6,52	94794407	94943563	6,79	0,06	10,67	0,66	20,99	Add	Plxnc1,Gm24186
OTU259	Aspergillus nidulans	Species	11	UNC19053972	6,45	9173438	9716170	6,72	0,03	2,9	5,15	38,49	Add	Abca13
OTU1041	Yamadazyma mexicana	Species	11	JAX00029390	6,98	76679070	80617602	7,26	0,09	8,86	0,16	33,57	Add	Nsrp1,Efcab5,Trp53i13,Nufip2, Nek8,Sdf2,2610507B11Rik, Pigs,Unc119,Vtn,Nos2,Crlf3, Atad5,Rnf135
OTU1512	Talaromyces rugulosus	Species	13	backupUNC13030 6089	6,58	63210381	64505358	6,85	0,08	6,17	0,04	35,26	Add	Habp4,Cdk20
OTU925	Nakazawaea holstii	Species	14	UNC24942560	7,86	124577548	124769406	8,12	0,06	10,67	0,66	20,99	Add	Fgf14
OTU1298	Claviceps purpurea	Species	15	JAX00409307	8,16	89997323	90401640	8,43	0,02	1,95	7,59	44,36	Add	Gm36480
OTU2243	Penicillium spathulatum	Species	18	UNC29116030	9	41708067	41733945	9,25	<0.01	12,97	0,54	29,59	IntDiet	Nearest gene AC154172.2
OTU2213	Penicillium spathulatum	Species	18	UNC29082687	9,14	39047223	39715173	9,38	<0.01	13,05	0,44	31,97	IntDiet	Nr3c1
OTU1878	Penicillium sp.	Species	18	JAX00450954	6,48	5264642	5526503	6,75	0,12	1,97	0,26	14,3	Add	Zfp438
OTU298	Aspergillus nidulans	Species	19	UNC30601828	8,87	60383903	61103683	9,13	0,04	3,97	3,84	34,48	IntDiet	Fam45a
OTU941	Aspergillus glabripes	Species	X	UNC200105170	9,77	82219367	82464595	10	0,04	3,97	0,01	86,09	IntDiet	Gm24706

Table 6: Full QTL table with candidate genes. The table shows all mapped QTL using the three different models Add (host genetics-mycobiome interactions only), Intdiet (host genetics-diet interactions only) and Intsex (host genetics-sex interactions only) with taxonomical assignment of fungi from phylum to species level on chromosomal loci. Percentages of explanations for phenotypic variation are given as R^2 LOD, Sex , Genetics (Gen), Diet and Cage.

8 Curricuclum vitae

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Education

06/2021:	Dr. rer. nat. at University of Lübeck (D)
10/2018-06/2021:	PhD candidate of the RTG1743 at CAU Kiel (D)
01/2017-06/2021:	Doctorate candidate at LIED, University of Lübeck (D)
12/2016:	Master of Science Infection Biology at University of Lübeck (D)
09/2013-07/2014:	DAAD Exchange student at Zhejiang University Hangzhou (CHN) in Chinese
07/2013:	Bachelor of Science Biology at CAU Kiel (D)
06/2009:	High School degree at Leibniz-Gymnasium Bad Schwartau (D)
1996-2000:	Primary school Sereetz (D)

Languages German (native)

English (fluent written and spoken, Cambridge CAE certificate)

French (fluent written and spoken, DELPH A2 certificate)

Chinese (HSK Level 4, written and spoken)

Portuguese (written and spoken, A2 Level)

Latin (Big Latinum)

Work and internships

From 07/2021:	Consultant at Ecker + Ecker GmbH, Hamburg (D)
01/2017-06/2021:	Scientific assistant at LIED, UKSH Lübeck (D)
01-02/2020:	Guest scientist at Fiocruz Institute in Curitiba (BRA)
10/2015-03/2016:	Intern at EUROIMMUN Medizinische Labordiagnostika AG (D)
2014 until now:	Teacher (Englisch, German, Biology), Nachhilfecoach Lübeck (D)
2012-2013:	Student scientific assistant at Botanical Garden at CAU Kiel (D)
2009-2011:	Journalist/author at the advertisement agency EWA, Bad Schwartau (D)
2009-2011:	Salesperson for Karls Erdbeerhof, Sereetz/Niendorf/Kiel (D)
04-05/2010:	Intern at J.H. von Thünen-Institute in Trenthorst (D)
01-03/2010:	Intern dramaturgy and Public Relations at Theater Lübeck, Lübeck (D)
10-12/2009:	Intern at Lübecker Nachrichten, Lübeck (D)
07/2009:	Intern communication design at the advertisement agency EWA, Bad Schwartau (D)

Skills	MS Office, GraphPad Prism, Photoshop/Gimp
	R, pearl, python, Ruby (all: basic statistical programming)
Hobbies	Music (classical piano education), travelling and languages, yoga and acrobatics, arts and movies

Declaration

I hereby confirm that this thesis is my own work and was completed without any unauthorized assistance. I have not used any sources apart from those indicated in the list of references.

Ich versichere an Eides statt, die vorliegende Arbeit selbstständig und nur unter Benutzung der angegebenen Hilfsmittel angefertigt zu haben.

Lübeck, 05.07.2021 _____

Anna Lara Ernst