Kasper Schei

Early Gut Fungal and Bacterial Microbiota in Growth and Allergy-Related Diseases

Doctoral thesis

NTNU
Norwegian University of
Science and Technology

Kasper Schei

Early Gut Fungal and Bacterial Microbiota in Growth and Allergy-Related Diseases

Thesis for the Degree of Philosophiae Doctor

Trondheim, June 2021

Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Clinical and Molecular Medicine

NTNU
Norwegian University of
Science and Technology

TIDLEG SOPP- OG BAKTERIEMIKROBIOTA I VEKST OG ALLERGIRELATERTE SJUKDOMMAR

BARNETARMFLORA I VEKST- OG ALLERGIUTVIKLING

Det er ikkje tvil om at tarmflora er i vinden for tida. Endeleg har forskarar kunna djupdukke ned i mikrobemiljøet i tarmen, for no har ein fått mye betre metodar å gjere det på. Tarmfloraen er *det gløymte organet* fordi ein har visst om det lenge, men først dei siste åra har forstått at desse 1-2 kiloa av tarmbakteriar, tarmsoppar og andre tarmmikrobar faktisk påverkar oss.

Til no har ein sett mest på korleis bakteriar i tarmen påverkar oss, men hos dei aller minste barna er dette ikkje fullt utforska. Om tarmsoppen, som berre utgjer ein brøkdel av tarmmikrobane, veit ein enda mindre, og knapt noko hos barn. Denne avhandlinga omfattar tre studiar kor den første har skildra barnesoppfloraen hos barn frå 10 dagar til 2 år og mødrene deira. I studie to og tre har ein sett på om det er samanheng mellom sopp- og bakterieflora hos desse barna, med høvesvis vekstutvikling fram til 9 år og astma- og allergiutvikling fram til 6 år. Vi har analysert resultat frå 298 (studie 1) og 278 barn (studie 2 og 3) og deira mødrer frå ProPACT-studien som vart gjennomført i Trondheim på starten av 2000-talet. Der målte vi mengda sopp- og bakterie-DNA, og vi prøvde å finne ut kva for sopp- og bakteriearter det DNA-et kom frå ved å sekvensere det.

I første studie såg vi at 88 % av mødrer og 56-76 % av barna hadde påviseleg sopp i avføringsprøvene sine. Litt usikkerheit rundt utvinninga av sopp-DNA-et gjer at det kan vere meir. Dessutan hadde barn høgare sjanse for å ha påviseleg tarmsopp om mor hadde det. Den vanlegaste tarmsoppen hos spedbarna var *Debaryomyces hansenii*, ei mjølkegjær som òg finst i Jarlsberg. Hos dei større barna var brødgjæra vanlegast (*Saccharomyces cerevisiae*). Vi klarte berre å finne soppartar hos 4 % av barna, som gjer at desse resultata er usikre.

I andre og tredje studie fann vi at toåringane med mye tarmsopp vart høgare mellom 2-9 år, og desse hadde òg høgare sjanse til å utvikle astma og høysnue fram til 6 år. Dessutan fann vi at meir sopp og bakteriar i tarmen hos eittåringar var tynnare første leveåret. Vi har med dette funne ein samanheng, men det tyder ikkje direkte at ein blir høgare og allergisk av å ete ein godt gjæra bolledeig. Ein har likevel føreslått at visse korte feittsyrer og hormon han henge i hop med vekst- og astmautviklinga.

Dette er heilt nye eksperimentelle funn som ein treng å stadfeste i nye studiar for å kunne stole fullt på dei. Likevel peiker dei på at tarmfloraen, og særleg tarmsoppen, kan ha innverknad på den utviklande kroppen til barna som veks opp blant oss.

Den ovannemnde avhandlinga er funnen verdig for offentleg forsvar for graden philosophiae doctor (ph.d.) i medisin.

Disputasen finn stad digitalt 11. juni 2021.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

LIST OF PUBLICATIONS

The thesis is based on the following three scientific papers:

Paper I
K. Schei, E. Avershina, T. Øien, K. Rudi, T. Follestad, S. Salamati and R. A. Ødegård.
'Early gut mycobiota and mother-offspring transfer.' *Microbiome* 5(1): 107. (2017)

Paper II
K. Schei, M. R. Simpson, E. Avershina, K. Rudi, T. Øien, P. B. Juliusson, D. Underhill, S. Salamati and R. A. Ødegård.
'Early Gut Fungal and Bacterial Microbiota and Childhood Growth*.' Frontiers in Pediatrics* 8: 572538 (2020).

Paper III
K. Schei, M. R. Simpson, T. Øien, S. Salamati, K. Rudi, R. A. Ødegård.
'Allergy-related diseases and early gut fungal and bacterial microbiota in children'.
Manuscript submitted.

ABBREVIATIONS

ANCOM – Analysis of Composition in Microbiomes
AR – Allergic rhinoconjunctivitis
BMI – Body mass index
CFU – Colony-forming units
CI – Confidence interval
CT – Cycle threshold
DNA – Deoxyribonucleic acid
FABP2 – Fatty acid-binding protein 2
FMT – Faecal microbiota transplant
HMP – Human Microbiome Project
IBD – Inflammatory bowel disease
IgA – Immunoglobulin A
IGF – Insulin-like growth factor
IOTF – International Obesity Task Force
ITS – Internal Transcribed Spacer
LBP – Lipopolysaccharide-binding protein
LEfSe – Linear Discriminant Analysis Effect size
LGG – *Lactobacillus rhamnosus* GG
LPS - Lipopolysaccharide
MetaHIT – Metagenomics in the Human Intestinal Tract
miRNA – micro-RNA
NGS – Next-generation sequencing
OR – Odds ratio
OTU – Operational taxonomic unit
OWOB – Overweight and obesity
PACT – Prevention of Allergy among Children in Trondheim
PCoA – Principal Coordinates Analysis
PCR – Polymerase Chain Reaction
PPR – Pattern-recognition receptors
ProPACT – Probiotics in PACT
QIIME – Quantitative Insights into Microbial Ecology
qPCR – Quantitative PCR
RDP – Ribosomal Database Project
RNA – Ribonucleic acid
rRNA – Ribosomal RNA
SCFA – Short-chain fatty acids
SD – Standard deviation
SDS – Standard deviation score
sp., spp. – species (singular and plural)
T_H – T helper-cell
THF – Targeted Host-Associated Database
UNITE – User-friendly Nordic ITS Ectomycorrhiza Database
WHO – World Health Organisation

1 INTRODUCTION

The microbes in the gastrointestinal tract, the gut microbiota, are sometimes called *the forgotten organ*. This is because we have known its presence for a long time, but its effect on the human body has not been known until recently. Although not formed by our own DNA, this microbial community live with us in an inherent manner. With novel technologies developed the latest years, researchers could suddenly explore the composition and effects of the microbiota. The bacterial part of the microbiota has been most extensively explored as these, by far, are the most abundant species in the gut. The fungal microbiota has also lately gained scientific interest, but has remained less explored, especially in children.

Childhood growth and development of allergy are important aspects of paediatrics. Children's height and weight are closely monitored by the child itself, parents, and society to ensure normal development and to detect diseases that impair growth. Furthermore, a global surge of childhood overweight and allergy development is seen the last decades. There is now great interest if some of these childhood pandemics could be associated with the fungal or bacterial gut microbiota. If so, potential therapeutic agents could be developed to lower the prevalence of these diseases.

This thesis has explored the development of the gut fungal and bacterial microbiota in children and its associations with longitudinal growth, overweight and development of allergy-related diseases.

2 BACKGROUND

GUT MICROBIOTA

The human *gut microbiota*, colloquially referred to as the *gut (micro)flora,* encompasses the living microorganisms that co-exist within the human gastrointestinal tract. These microorganisms include bacteria, archaea, protists, fungi, and viruses, and these organisms thrive well within the gut since the gastrointestinal tract provides them nutrition and a protective environment.

Over the last two decades, the gut microbiota has received increasing attention. Recent calculations estimate the gut microbiota to some 40 trillion cells, which outnumbers the number of human cells by approximately 1.3, [1] substantially lower than previously believed. [2] The vast majority of these cells are bacterial (99 % of the gut microbiome is bacterial, [2]) and the adult gut microbiota is composed of approximately 160 different bacterial species per individual with large variations between individuals. [2, 3] Astonishingly, this microbial gene pool (called the *gut microbiome*, related to the last syllable of 'genome') provides a gene count multitude 150-fold larger than the human genome. [2] These properties make the gut microbiota a bank of metabolic pathways that aid in the metabolism of non-degradable substances.

The gut microbiota has probably co-existed with *Homo sapiens* since the human emergence. Man and microbes live in a cunning symbiosis in the gut; the microbes are pathogens in the proximal gastrointestinal tract, and yet they live in mutualism in the distal gut. [4] Since many gut microbes are opportunistic pathogens, the human body depends on an active immune system and shrewd barriers so that the microbes only get their share of the food and do not invade the host. In return, the microbiota cleaves indigestible fibres to energy, produces vitamins, aids in the immunological defence maturation, and finally protects the host against pathogenic microbes. Hence, the description *commensal* (i.e., microbes benefit, whereas the host is unaffected) might be unsuitable, at least for some species, and the term *mutualism* (i.e., both species benefit) could seem more appropriate. [4] However, the price to pay is that the microbes may turn pathogenic when the host immune control weakens. Abnormal gut microbiota compositions among fungi and bacteria have been observed in people with diseases, and this state of abnormal gut microbiota composition is termed *dysbiosis*. [5]

In 2001, the Human Genome Project sequenced the human genome for the first time. [6] As an extension of this project, the Human Microbiome Project (HMP) was founded in 2007, [7] with the aim to sequence the human microbiome and discover whether there is a core microbiome in all humans. Following HMP, in 2008, the EU sister project *Metagenomics in the Human Intestinal Tract* (MetaHIT) was started with the objective to find associations between the human microbial community structure and human health and disease. [2] As the sequencing technology has improved and has become less costly over the last decades, these and other studies have revealed some puzzle pieces as to how the gut microbiota interacts with the human body. Still, there are many unanswered questions, and microbiota variations between individuals and geography complicate the creation of a comprehensive and uniformly processed database that can represent the human gut microbiome. [8]

MICROBIOME METHODS

The microbial life was first revealed through the lens of Leeuwenhoek's microscope, illustrating the need for technology to study fungi and bacteria. The traditional method to study these microbes has been with so-called *culture-dependent methods*. Isolation of the microbe in a growth medium was needed before further investigations with microscopy, biochemical testing, and growth media selectivity to determine the taxonomy. [9, 10]

In the last two decades, *culture-independent methods* have emerged and revolutionised the field of microbiota research. Culture-independent techniques are primarily based on microbe identification by recognising the specific microbe's DNA sequence and thereby omitting the culturing bias. New DNA sequencing methods, called next-generation sequencing (NGS) or high-throughput sequencing, have further developed conventional Sanger sequencing into a faster and cheaper form of DNA sequencing, like Illumina, Roche pyrosequencing and Ion Torrent technologies. RNA products from bacteria itself can also be sequenced and show the presence of living microbes in the samples; however, it requires meticulous sample preparations and more challenging analyses.

In principle, we divide microbiome sequencing into *metabarcoding* and *metagenomic sequencing* (also called shotgun sequencing). [10] In metabarcoding, a specific DNA segment (a 'barcode') is selected by choosing the appropriate primers for the actual study. In microbiome studies, the ribosomal RNA gene segments that code for the ribosomal subunits are used. These are relatively preserved within the biological kingdom and yet variable enough within hypervariable regions to work as barcodes for each species. Since bacteria are prokaryotes, their DNA codes for a bacteria-only 16S subunit of the ribosome (Figure 1). Eukaryotes like fungi have an 18S ribosomal subunit instead. The hypervariable V3-V4 region of the 16S RNA gene is widely used in bacterial sequencing, by which eukaryotic DNA is also
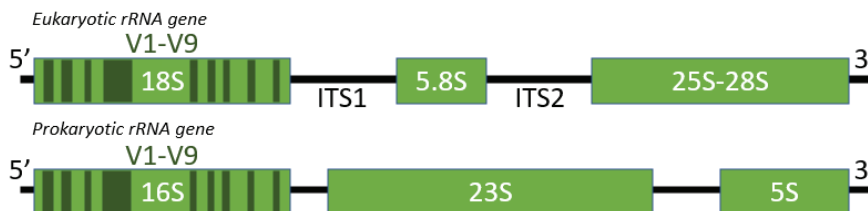
Figure 1. Illustation of the ribosomal DNA used as identity markers in metabarcoding. The green boxes show the DNA coding for the subunits in the ribosomes, and these are different in eukaryotes and prokaryotes. V1-V9 are the hypervariable regions of the 16S/18S ribosome DNA sequence. The Internal transcribed spacers (ITS) are intercalated between the 18S and 5.8 S ribosomal subunits and the 5.8S and 25S-28S.

excluded. In fungal sequencing, however, there has been no agreement on which primer pairs are preferred, so sequencing of the 18S gene and the spacer regions between the subunit genes, called internal transcribed spacer (ITS) 1 and 2, has been widely used. [10, 11] By sequencing a smaller part of the DNA as is done in metabarcoding sequencing, DNA from more microbes could be sequenced and identified quickly and less costly than in metagenomic sequencing.

In metagenomic sequencing, there is no selection of DNA segments to be sequenced. Here, all sample DNA is cut into small pieces and sequenced. Thereby all sorts of DNA in a faecal sample are sequenced, including DNA from human host cells, bacteria, archaea, fungi, and plants. The DNA quantity to sequence becomes much larger, and the identification of rare species, like fungi, would require a very deep sequencing that is not readily available. Therefore, metagenomic sequencing is not widely used in gut mycobiota research yet. [10, 11] However, it could solve several of the challenges in mycobiota research.

The bioinformatic process comprises the conversion from short sequencing reads to *operational taxonomic units* (OTUs) that are unique for each microbial strain. The OTUs are then matched up with already known sequences in taxonomic databases; this process is called *annotation*. The bacterial databases are now thoroughly processed, whereas the fungal databases still contain several misclassifications. [10, 12]

FUNGAL MICROBIOTA IS CALLED MYCOBIOTA

A fungus (Latin and plural *fungi)* is a collective term for all yeast and mould organisms. Since the 1960s, biologic taxonomy has defined fungi as a distinct biologic kingdom with several characteristics that separate fungi from other kingdoms like bacteria, protists, archaea, plants, and animals. *Taxonomy* is the study of species classification, and a *taxon* (plural *taxa*) is a classification entity like a species or a genus.

Fungi are evolutionarily related to plants and animals as they all are developed from bacteria, and thus they share specific characteristics. Like plants, animals and protists, fungi are *eukaryotic* organisms (Greek *eu* – 'well, good', *karyon* – 'kernel, core') because the fungal cell holds a nucleus, in which the majority of the cell's genome is. The fungal genome contains mainly intranuclear DNA, arranged in haploid chromosomes of various numbers (3-40). In contrast, bacteria lack a cell nucleus and are defined as *prokaryotes* (Greek *pro-* – 'before', *karyon* – 'kernel, core'). Fungi contain cellular organelles like the energy-producing mitochondria; the ribosomes, endoplasmic reticulum, and Golgi apparatus essential for protein synthesis; and lysosomes necessary in cellular degradation. Like animal cells, fungi cannot convert energy from light in photosynthesis and are therefore dependent on absorbing external energy. However, the fungal cells have a rigid cell wall like plant cells, made by polysaccharides like *chitin* or *chitosan* and cross-linked with *glucans*.

Fungi are divided into *yeasts* and *moulds* based on their microscopic appearance. Yeasts are unicellular organisms that grow by splitting the cell in half (called nuclear fission) or by extruding a part of the cell membrane into a new cell (called budding). This growth is seen in Brewer's yeast, *Saccharomyces cerevisiae*. Some yeasts may even form longer chains of yeast cells called pseudohyphae (seen in Candida spp.). Moulds, on the other hand, grow in string-like structures called hyphae. These hyphae form a macroscopic root structure called mycelium, and this kind of growth is seen in *Aspergillus* spp. Some of the most pathogenic fungi (genera *Histoplasma*, *Blastomyces*, *Paracoccidioides* and *Coccidioides*) may even take the shape of both yeasts and moulds and are therefore called *dimorphic fungi*. Furthermore, both yeast and moulds can reproduce asexually where new fungal spores are made my mitosis or through sexual division where two cells undergo meiosis in spore formation.

Unknown fungi continue to be discovered at a high rate, and calculations show that only about 5 % of all fungi seem to have been found, implying a total number of 2-4 million fungal species out there. [13] Like bacteria, fungi are ubiquitous in our environment, being

present in the soil, organic materials, and the air. They have been important throughout human history; their abilities were discovered early and exploited in human food culture like bread, alcoholic beverage, ripened cheese, and other fermented products [14]. The discovery of penicillin, an antibacterial product of the air-borne mould *Penicillium*, was a medical turning point [15]. Also, the discovery of fungi as potential human pathogens revolutionised clinical medicine.

Gut mycobiota

Being ubiquitous in our environment, fungi do readily get in contact with the human body surface. Because the skin and mucosa are faced continuously with fungi, the epithelial surfaces (skin, gastrointestinal tract, and urogenital tract) have developed their distinct mycobiota. Similarly, bacterial microbiotas have established at different human epithelial surfaces. [9] Yet, the implications of mycobiota colonisation have not been fully elucidated.

The fungi that populate the gastrointestinal tract are called the *gut mycobiota* (Greek *mykes* – 'fungus'; *biota* – 'life'). The gut mycobiota has recently been recognised as a substantial part of the gut microbiota and can be important for human health. [5] The gut mycobiota was discovered over a century ago, [16] but has remained largely unexplored until culture-independent methods were developed. Using a culture-independent method, called shotgun sequencing, of the adult gut microbiome, one has shown that only 0.1 % of the microbial DNA is fungal. [9] Although this percentage is debated and might be underestimated, gut fungi are part of the gut's *rare biosphere* (microbial species of low quantity in an ecosystem). [5, 9] Nevertheless, being eukaryotes, fungal cells are about 5 µm in diameter, whereas bacteria reach about 0.5-1.0 µm across (although there is significant variation in size). Assuming the volume of a sphere ($\frac{4}{3}\pi r^3$), the fungal cell are at least 100 times larger than a bacterial cell and could comprise approximately 10-15 % of the gut microbiota volume. A larger cell could produce more substances and affect its environment more than a small cell. Furthermore, as eukaryotes, fungi have a larger repertoire of biochemical pathways than bacteria. Therefore, the mycobiota influence on the host and remaining gut microbiota appears more significant than the actual number of fungal cells implies.

The gut mycobiota consists of a species selection from approximately 100-200 different fungal genera [17, 18]. Two extensive mycobiome studies with healthy American adults have investigated the normal human gut mycobiota with culture-independent methods. In the HMP cohort of 307 adults, the most prevalent gut fungi were *Saccharomyces cerevisiae,*

Malassezia restricta and *Candida albicans,* and in total 177 genera were detected (ITS2 as primer target). [19] In another study of 96 sequenced samples, the genera *Saccharomyces*, *Candida* and *Cladosporium* were the most present, with a total of 63 genera (ITS1 as primer target). [20] Only 29 fungal genera were present in both studies, indicating that several detected genera might not be actual colonisers of the gut but mere transient microbes from food or the environment. [14] Studies using culture-dependent methods have proved the presence of living microbiota in faecal samples, although with a surprisingly poor concordance of detected species between culture-dependent and culture-independent techniques. [21, 22]

The human gut mycobiota offers several physiological abilities to the human body by facilitating nutrient extraction and digestion through enzyme and vitamin production. [23, 24] The gut mycobiota is also essential as a form of antigen exposures to train the immune system and its responses. By activating the fungus-specific pathogen-recognition receptors (PRRs) and adjacent mechanisms, defences against harmful pathogens and tolerance towards helpful commensals are formed. [5, 25-27]

FUNGAL AND BACTERIAL GUT MICROBIOTA IN CHILDREN

Microbiota diversity

The *alpha diversity* (α diversity) describes how many taxa that prevail in each sample. The bacterial alpha diversity within each child's gut increases as the offspring ages. [28, 29] It is often calculated upon the species *richness* (number of species in a limited area) and the relative distributions of the present species within the same ecosystem. [30] The alpha diversity is commonly expressed in Shannon-index (H') or Simpson's reciprocal index.

The *beta diversity* (β diversity) expresses how diverse two samples appear in terms of number and abundances of OTUs. [30] The offspring's taxa distributions become more like each other as the offspring grow older. [28, 29] This means that the beta diversity decreases by age and is expressed with the Bray-Curtis dissimilarity index. Together, these diversity terms show that bacterial communities become more complex yet more alike as the offspring age.

In contrast to bacteria, fungal diversity has been poorly investigated until recently. The fungal alpha diversity is smaller than for bacteria as there are fewer gut fungal species, [31] and an American study showed that fungal alpha diversity (Shannon index) actually decreased from 1-11 months. [32] There are yet no description of the development of fungal abundance from birth to two years of age.

Foetal microbiota

The womb might not be as sterile as previously imagined. Bacteria have been detected in the placental tissue, umbilical cord blood, amnionic fluid and meconium. [33-35] Placental microbiota and amniotic fluid seem to contain mostly Proteobacteria (*Escherichia coli*) and Actinobacteria (*Propionibacterium acnes*), [33, 35] and interestingly, the placental microbiome is most similar to the oral microbiome of the same pregnant women. [33] This indicates microbial transmission from the mouth to the placenta. The umbilical cord and meconium microbiota show a different microbial profile with more Firmicutes (*Enterococcus*, *Staphylococcus* and *Streptococcus*), but also *P. acnes.* However, the amniotic fluid and the meconium share more than half of the same microbial species. [35] Summarised, the foetus is possibly colonised already before birth and may be equipped with a microbial dowry provided by its mother, although of relatively low counts. Alternatively, these findings could merely represent contamination as the samples were collected. As to the presence of other microbial kingdoms, including fungi, this has not been investigated yet.

Maturation of the infant microbiota

After birth, the new microbiota establishes and forms a new ecosystem within the infant's gut. The establishment is stepwise, and the steps are affected by mode of delivery, breastfeeding or formula-feeding, the introduction of table food, weaning and diet. [3, 28, 36] The initial microbiota of vaginally delivered neonates is characterised by *Enterococcus*, *Escherichia/Shigella*, *Streptococcus* and *Rothia*. [28, 35], illustrating an oxygen-rich environment as several of these are facultative anaerobic bacteria. *Bifidobacterium* and *Bacteroides* also establish (albeit at lower abundances) in the neonate gut, [28] two bacterial genera that will increase in abundance later in life. Concerning fungi, a Puerto Rican neonatal study (0-30 days of age, ITS2-sequenced) showed that the most prevalent neonatal gut fungi were *C. albicans, C. parapsilosis, C. orthopsilosis, C. tropicalis, S. cerevisiae* and *Cryptococcus pseudolongus*. [37] Culturing in neonates has shown a *Candida* spp. prevalence at 23 %, and it more than doubles to 50 % within four months. [12] Even in premature infants, Saccharomycetales seemed to be the most abundant fungi. [38, 39] *Candida albicans* and *Malassezia* spp. are shown to be partly transferred vertically from mothers to their offspring, [12, 40, 41] supporting the theory that fungi colonise the neonatal gut through the birth canal. Interestingly, highly abundant gut fungi, like *Malassezia*, *Candida* and *Saccharomyces*, were found alive in most breastmilk samples, implying a reasonable path of gut fungi colonisation from the mother to the offspring [42]. This illustrates that fungi and bacteria are present in neonates, yet the significance of this is not established.

Immediately after birth, infants are breastfed (or get formula). This relatively homogenous substrate forms a new environment, to which the gut microbes must adapt. Facultative anaerobic microbes pave the way for anaerobic bacteria by depleting the intraluminal oxygen, and by four months of age, infants host bacteria that are well-adapted to breast milk. [28] Four-month-old infants tend to host greater abundances of Actinobacteria (especially *Bifidobacterium*), but also *Bacteroides*, *Enterococcus* and *Streptococcus*. [28, 29] The breast-milk contain antimicrobial molecules like IgA and miRNA, [43] non-digestible oligosaccharides that promote the growth of bacteria like *Bifidobacterium*, which also are found to grow in breast-milk and functions as a living gut inoculum. [44] In a study with 18 healthy Italian children at 0-2 years of age, faecal culturing showed fungal presence in 89 % and a higher fungal richness than in adults. The most prevalent genera were *Penicillium*, *Aspergillus* and *Candida*. Most of these isolates could stand physiological conditions like body temperatures, bile acids, acidic environment and stress testing with hydrogen peroxide,

indicating that they are true commensals. [45] An American study showed that Malasseziales were more abundant in the first three months of life, followed by a gradual succession of Saccharomycetales. [32]

As children are weaned and consume an adult-like diet, microbiota changes occur. [28, 36, 46, 47] Firmicutes and Bacteroidetes, like *Clostridia* and *Bacteroides*, respectively, become more prominent, [28] together with bacteria that produce short-chain fatty acids (SCFAs) acetate, propionate and butyrate. [28, 48] The gut epithelium can then absorb these compounds as energy and signalling substances. [49] During the chaotic 2-5 first years of life, the gut microbiota finally stabilises to a consistent distribution that may last to adult age. [3]

Adult-like or *mature microbiota* usually encompasses approximately 160 distinct species with higher proportions of Bacteroidetes and Firmicutes, and lower proportions of Actinobacteria, Proteobacteria and Verrucobacteria. [28, 46, 47, 50, 51] In adulthood, the microbial species distributions show a significant inter-individual variation. [50, 51] There have been attempts to find core microbiota patterns similar between individuals, and by ordination, similar microbiotas have been clustered together in *enterotypes*. [51] The presence of enterotypes has been debatable and suggested renamed to *biomarkers*. [52]

Summarised, the bacterial microbiota succession in infancy is well described, whereas the establishment of the mycobiota needs to be further explored. Also, the health consequence of the early microbiota needs to be established.

DETERMINANTS OF THE EARLY GUT MICROBIOTA

Two of the most studied modifiers of the early microbiota are *mode of delivery* and *diet*. Other modifying factors include genetics, gestational age, antibiotics, and health status. [3]

Mode of delivery. Vaginally delivered children are exposed to the mother's vaginal and gut microbiota in the birth process, in contrast to children delivered by Caesarean sections. This is visible in the neonatal gut microbiota composition pattern. Neonates delivered by Caesarean sections are enriched with *Enterobacter*, *Haemophilus*, *Staphylococcus*, *Streptococcus* and *Veillonella* genera, [28] implying a more aerobic environment like the skin or mouth microbiome. This contrasts with those delivered vaginally, who harbour more *Bacteroides*, *Bifidobacterium*, *Parabacteroides* and *Escherichia/Shigella*, [28] species readily found in the anaerobic adult gut microbiota. There can still be demonstrated differences by 12 months, [3, 28] particularly the *Bacteroides* genus show a delay in colonisation in the Caesarean section group, indicating that at least some bacteria are primarily transferred through birth. [28, 47, 53]

Breast milk or formula. By four months, exclusively breastfed offspring harbour taxa like *Lactobacillus* and *Bifidobacterium*, [28] bacteria that are specialised in utilising milk as an energy source. A more adult-like microbiota is shown in formula-fed offspring, including *Clostridium*, *Akkermansia muciniphila* and *Desulfovibrio*. [28, 46] At 36 months, however, no difference is found. [46] Human breast milk contains several antimicrobial peptides. One of these is lactoferrin, an iron-binding glycoprotein that inhibits the growth of several bacteria and fungi, partly by the protein itself and proteolytic cleavage of this protein to other antimicrobial compounds. [54, 55] Possibly, these peptides could impact the growth of microbes in the gut during breastfeeding.

Antibiotics. Not surprisingly, antibiotic administration affects microbiota composition by eliminating larger parts of the bacterial community. The effect is especially strong in early life when the community is still unstable. [56] Overgrowth of *Clostridium difficile* is an established side-effect of broad-spectrum antibiotics, but a loss of essential bacteria in early life, like *Bifidobacterium*, is also seen. [56] Furthermore, a transient faecal overgrowth of fungi is seen after the use of broad-spectrum antibiotics. [57, 58] Several authors even suggest that the association between antibiotic administration and obesity and allergy-related diseases could go through gut dysbiosis due to antibiotics. [56]

GUT MICROBIOTA IN HEALTH AND DISEASE

Efforts have been put into investigating if the microbiota has a role in human diseases. From previous research, restoring a disrupted microbiota with faecal microbiota transplantation (FMT) has been successful for recurrent *Clostridium difficile* infections. [59] In inflammatory bowel diseases (IBD), a lower microbial diversity is shown, together with reduced proportions of Firmicutes (Roseburia, Faecalibacterium) and increased Proteobacteria, and currently undergoing studies with FMT against IBD could indicate promising results. [59, 60]

Mycobiota has been associated with physiological and pathophysiological processes. For some humans, fungi can have an unfavourable influence, and the term *fungal dysbiosis* describes a state of unbalanced mycobiota related to disease. [61] This phenomenon is most extensively studied in immunocompromised patients who regularly contract opportunistic commensal fungal infections, [5] and in patients with diseases like asthma, obesity and IBD. [60, 62-66]. Lately, fungal dysbiosis was also seen in children who later developed type-1 diabetes mellitus. [67] These findings imply that gut mycobiota could be aetiologically important in human diseases. In the following section, the association between gut microbiota and human longitudinal growth and development of overweight and allergy-related diseases will be elaborated.

CHILDHOOD GROWTH AND EARLY GUT MICROBIOTA

Physical growth is a hallmark of childhood, and monitoring children's growth regularly is an essential clinical parameter to find chronic diseases in childhood. [68] Linear and healthy childhood growth velocity and weight increase are important markers of a child's development and depend on the genetic potential, healthy nutrition, functioning endocrine systems, and disease absence. [69, 70]

Childhood height development has been extensively studied and divided into four different growth phases, with specific hormonal growth regulators in each phase. [69, 70] The first phase, *foetal growth*, describes rapid intrauterine growth and is regulated by insulin-like growth factor I and II (IGF-1 and IGF-II) and insulin itself. From birth, *infancy growth* is mainly programmed from the intrauterine environment and somewhat due to growth hormone (GH) and thyroid hormones. Subsequently during the first year of life, *childhood growth* takes over with a steady, annual growth declining from 25 cm in the first year of life to 4-6 cm towards puberty. This growth phase is mostly dependent on GH, IGF-1, and thyroid hormones. Infancy growth and childhood growth have a fascinating capability of *catch-up* or *catch-down growth* so that a newborn small for gestational age can adjust to its genetic potential within this period. Lastly, *pubertal growth* describes the final growth spurt, which is mainly regulated by sex hormones. [69]

Faltering growth may indicate ill-health including chronic disease or severe undernutrition in childhood. Consequently, repeated childhood measurements plotted on percentile charts are implemented at children's health centres worldwide to identify children who grow too fast or too slowly. [71] Height and *body mass index* (BMI) deviations can be described as the deviation from a reference population by standard deviation scores (SDS, also called z-scores) by which the deviation is adjusted for age and sex. [72]

Longitudinal growth

Recently, a link between childhood growth and early gut microbiota has been suggested, either through increased energy extraction or other metabolic interactions with the host. [73, 74]

In the livestock industry, low-dose antibiotic administration has been widely used as growth promotors. [75] Due to increasing antibiotic resistance, the European Union banned using growth-promoting antibiotics in animal production in 1999. Consequently, the use of yeast cells and cell wall products of *Saccharomyces cerevisiae* emerged as an important growth promotor. [76] Active dried yeasts (especially *S. cerevisiae*) as feed additive increased feed

intake and the average daily weight gain in especially young ruminants and showed similar growth-promoting abilities as low-dose antibiotic supplementation. [76] These abilities seem particularly important in the weaning process, allegedly protecting gut microbiota from pathogenic species. Furthermore, in mice, SCFAs, prominent bacterial metabolites, are shown to increase the serum level of IGF-1, an important growth hormone. [77] Animal studies could therefore indicate an association between gut fungal and bacterial microbiota, higher SCFAs and longitudinal growth.

In children, gut microbiota dysbiosis is seen in environmental enteropathy and kwashiorkor, two paediatric malnourishment conditions that lead to stunted height growth. [73, 74] Here, malnutrition and stunting (impaired growth) in Bangladeshi children at 6-20 months of age have been associated with immature gut microbiota. [78] Accordingly, in healthy pre-school children, height velocity was associated with certain Firmicutes spp. at three months and higher gut bacterial diversity at 0–3 years. [74, 78-80] While early human antibiotic use may predispose to later childhood obesity, [81] its possible effect on height velocity is less elucidated. A *Helicobacter pylori* eradication study with one-week administering broad-spectrum antibiotics in 6-10-year-old children showed increased height SDS by 20 % in the intervention group, compared to the control group within one year, even when *H. pylori* was not eradicated. [82] The same antibiotics-height association was observed in a large Finnish infancy cohort. [81] In two randomised-controlled trials in which preterm neonates (28-32 weeks and 30-37 weeks of gestational age, respectively) were supplemented with a probiotic *S. cerevisiae* strain (*S. boulardii*), probiotic groups experienced greater weight gain than the control group (length was not measured in one study and increased non-significantly in the other). [83, 84] Whether these growth-promoting associations are mediated by fungal growth, SCFA or other aspects of the microbiota and its metabolites remain unexplored.

Weight development

Childhood weight is also supposed to increase linearly as the child ages, and missing weight increase or rapid increase warrants investigation. Since weight is highly dependent on height, BMI describes the relative body size as weight per square meter according to the formula $BMI = \frac{weight\ (kg)}{\left(height\ (m)\right)^2}$. In adults, BMI 18.5 to 25 kg/m^2 represents normal weight, 25 to >30 kg/m^2 is overweight, and 30 kg/m^2 or more is obesity. These BMI cut-offs cannot be applied in children because the normal BMI ranges decrease from one year to school-age before a gradual increase up to adulthood. BMI cut-offs corrected for age and sex that represent the

growth trajectory leading to BMI 25 and 30 kg/m^2 at 18 years of age have been developed, so-called *iso-BMI categories*. [85] Yet a continuous variable for BMI deviation is often sought in research questions, and *BMI standard deviation score* (BMI-SDS) is widely used. The score describes how far the BMI deviates from the age and sex-specific mean BMI, and recently a Norwegian childhood growth reference was made. [72, 86]

Accelerated longitudinal growth in the first four years of life has been associated with increased body size at nine years. The same connections are seen between higher birth weight and rapid weight gain in infancy. [87] Paediatric (as well as adult) obesity is a global epidemic, and childhood overweight and obesity (OWOB) have steadily been rising over the last 30 years, threatening the health of our young successors [88]. In 2019, 38 million 0-4-year-old children had OWOB and 340 million children from 5-19 years had OWOB in 2016; several countries are approximating or even exceeding 30 % OWOB. [89] Although the previously soaring figures now suggest stabilisation in at least the Western part of the world, the stabilising levels of childhood OWOB are far too high and need to be lowered [88-90]

Association with gut microbiota and OWOB. Studies on germ-free mice have established a link between gut microbiota and the development of obesity. In 2006, by colonising germ-free mice's gut (raised under sterile conditions) with faeces from obese or lean mice donors, those receiving obese-type faecal transplant developed increased body fat compared to those receiving lean-type faecal transplants. [91] Faecal transplant studies from human twins (discordant for obesity) into germ-free mice have shown similar results. [92] This implies a causal link to the gut microbiota in obesity development. The pathophysiological hypotheses are several. Microbiota can increase the energy extraction from undigested fibres and affect molecular cascades that result in increased lipid storage, reduced lipid metabolism and appetite hormone stimulation. It may also induce a systemic low-grade inflammation called *metabolic endotoxaemia* that promotes fat accumulation and insulin insensitivity. [93]

Great efforts have been put into investigating the bacterial diversity and taxonomy in humans with weight gain, and human studies have reported both supporting and conflicting results. [94] Metabolic diseases like obesity and diabetes mellitus type 2 have been associated with aberrations in the gut microbiota. In adult humans (as well as in mice), reduced bacterial diversity, a relative absence of the bacterial phylum *Bacteroidetes,* and a greater abundance of *Firmicutes* seemed to correlate with obesity, although the results vary. [92, 95-97] In a meta-analysis, however, the bacterial gut microbiota of adults with obesity had lower alpha

diversity, evenness, and richness. Still, they did not find evidence for taxonomic patterns associated with obesity. [94]

Although not consistently associated with adult gut microbiota taxonomy, childhood OWOB could be linked to early gut microbiota. Fewer studies have been conducted in children, but some early gut microbiota patterns have been associated with childhood obesity. At three months of age, the relative abundances of Firmicutes and Lachnospiraceae were positively with early overweight and obesity, whereas *Bifidobacterium* spp. showed a negative association. The microbiota's rapid maturation by six months of age renders a higher risk of obesity at 18 months. [98] At two years of age, the families Ruminococcaceae and Lachnospiraceae (all adult-type bacteria) have been positively associated with later obesity, whereas *Faecalibacterium prausnitzii* has been correlated to leanness. [79, 99-101]

Concerning gut mycobiota, obesity and metabolic disorders have been associated with the increased presence and abundance of Saccharomycetes, Dipodascaceae and Tremellomycetes. [62]. Both obesity and metabolic syndrome are pro-inflammatory states, and Tremellomycetes are associated with higher inflammation levels. Accordingly, a lower abundance of the ascomycotic Eurotiomycetes and notably less of the zygomycotic *Mucor* spp. might protect against an unhealthy metabolic profile. [62, 63]

The gut microbiota may therefore be associated with longitudinal growth and development of overweight, although no distinct compositional patterns are found. In particular, the relation to the mycobiota ought to be investigated.

ALLERGY-RELATED DISEASES AND GUT MICROBIOTA

Allergy-related diseases comprise conditions like asthma, allergic rhinoconjunctivitis (AR) and eczema. Like obesity, these conditions are also characterised by chronic low-grade inflammation. They are aetiologically linked to allergy development, where the human immune system develops a state of hypersensitivity against supposed harmless substances. The allergy-related diseases usually emerge in a specific order, known as *the atopic march*. Atopic children may typically develop eczema and food allergy during the first years of life, whereas asthma and AR can often emerge later in life, before or during school-age. [102]

Over the last decades, the prevalence of allergy-related diseases has increased, rendering these conditions the most prevalent chronic diseases in childhood. [103, 104] In 6-7-year-old children, the global prevalence of asthma, AR and eczema was 11.7 %, 8.5 % and 7.9 %, respectively, and the increase has been particularly steep in developing countries. [104] Although genetic disposition is important, environmental factors are suspected to explain the global surge in allergy-related diseases since the rise has happened over only a few decades. Changes in the gut microbiota may be one of these. [105]

Several risk factors for allergy-related diseases are also associated with the gut microbiota, including mode of delivery, formula-feeding, antibiotics, urban living, less animal contact and a Westernised diet with high fat and high carbohydrate intake. [106-108] These risk factors could also reduce or alter the gut microbial composition and thereby change the immunologic exposure. [108]

Reduced gut bacterial alpha diversity and richness at one month of age have been associated with eczema in the first year of life and later asthma. [102, 109, 110] Furthermore, some bacterial taxa associated with breast-feeding, like *Bifidobacterium* and *Lactobacillus*, are underrepresented in children by 1-3 months with later development of asthma or wheeze; [32, 111] however, *Bifidobacterium* has also been seen overrepresented in allergic children at 8 years of age. [112] Furthermore, lower abundance of *Prevotella* and *Coprococcus* (potential propionate- and butyrate-producers, respectively) is seen from infancy to school age in allergic children. [112, 113] This is in line with evidence that increased levels of the immunoregulatory SCFAs butyrate and propionate in early life could protect against development of allergy-related diseases. [114] Therefore, a timely succession from milk-adapted bacteria to adult-associated bacteria seems to be present in those without allergy-related diseases.

The knowledge on mycobiota and development of allergy-related diseases are scarce, however. Murine studies have hypothesised a link between gut fungi and asthma, as gavage with specific fungi into the murine gut deteriorated allergic airway disease. [115] In a small Ecuadorian human study, increased fungal abundance at three months was associated with atopic disease at 4-5 years. [111] In neonates, an increase of *Candida* was seen in those with increased asthma risk at four years. [32]

Fungi have a large repertoire of metabolite production; in fact, thousands of metabolites have been isolated. Many of these fungal metabolites affect humans, some of the most well-known being penicillin, cyclosporin A and statins. [116] Fungi can also produce metabolites that affect the human immune system. *Candida* and *Cryptococcus* (and probably others) have been shown to produce prostaglandins and leukotrienes, possibly to improve the fungal environment. These could inhibit a T_H1 immune response and promote a T_H2 response, although not proven in humans. [13, 117-120] If these substances also are produced by the gut mycobiota, they could potentially affect allergy development. There could therefore be an association between the early mycobiota and allergy development, but more research is needed.

GUT PERMEABILITY AND INFLAMMATION

The gut mucosa needs to be impermeable for microbes to keep all opportunistic pathogens away, and at the same time be permeable to absorb all available nutrients. Tight junctions bind the mucosal cells together, and much of the nutrient absorption goes through active and passive cellular channels. Since the gut mucosa is the place of contact between the gut microbiota and the host, the gut mucosa immune system must be optimally adapted to tolerate the commensal microbes in the lumen whilst simultaneously eliminate pathogenic and invasive microbes.

A permeable gut mucosa, colloquially known as a *leaky gut*, signifies that more microbial products cross the epithelial lining and produce a low-grade inflammatory response. This has been observed in both asthma and overweight development, yet a causal association has not been proven. [121-124] However, obesity confers a chronic low-grade inflammation, which is closely related to *metabolic endotoxaemia*. [123] This inflammation is an immune response towards high levels of lipopolysaccharide (LPS, or endotoxin), [125] a part of the cell membrane of Gram-negative bacteria. If LPS or other bacterial products cross the gut barrier and enter

the circulation, such an inflammation could be formed and might contribute to obesity development.

Two of the biomarkers for gut permeability are *lipopolysaccharide-binding protein* (LBP) and *fatty acid-binding protein 2* (FABP2, also called intestinal-type FABP (I-FABP)). LBP is part of the innate immune response that opsonises LPS when LPS crosses the gut barrier. Thereby, LBP serves as a marker of metabolic endotoxaemia. [126] FABP2 is expressed in the gut cells from the duodenum to the caecum and is believed to contribute in long-chain fatty acid metabolism or transport. Being an enterocyte constituent, it serves a marker of gut permeability at enterocyte turnover or damage. [127] Higher circulating levels of both markers are observed in neonatal necrotising enterocolitis, [128] but it remains to be investigated whether markers of permeable gut could be connected to allergy development and microbiota.

3 AIMS

This thesis aimed to study the early human gut mycobiota and the bacterial microbiota and investigate their associations with longitudinal growth and development of overweight and allergy-related diseases, two highly prevalent paediatric conditions associated with low-grade inflammation. More specifically, the aims of each study were:

- To investigate the development of the early gut fungal and bacterial microbiota from ten days and up to two years of age and to evaluate the transfer of mycobiota from mother to offspring (Paper I)
- To study associations between early gut fungal and bacterial microbiota from ten days to two years in a prospective cohort, and
    - childhood height-SDS and BMI-SDS in a longitudinal cohort of healthy children up to the age of nine years (Paper II)
    - development of allergy-related diseases (Paper III),
    - explore correlations between allergy-related diseases and biomarkers of increased gut permeability (Paper III).

4 MATERIALS AND METHODS

THE STUDY POPULATION

The Prevention of Allergy among Children in Trondheim (PACT) study is a large prospective birth cohort of unselected healthy mothers and their offspring who were followed to identify important aspects in the development of allergy-related diseases. Nested within the PACT study, a selection of the participants joined the ProPACT study, a randomised, double-blinded, placebo-controlled trial, in which one-half of the participants were randomised to drink probiotics, and the other half to consume placebo probiotics. [129, 130] In this study, 415 pregnant women who attended the regular Norwegian Antenatal Care Programme at their general practitioner or mid-wife from September 2003 to September 2005, were asked to complete questionnaires on their health and risk factors and collect faecal samples from themselves and their offspring. The study also included a clinical follow-up at 2 and 6 years. Because of only minor short-term differences in the children's gut microbiota, the two arms of the ProPACT randomised-controlled trial were merged (more under 'Merging the two arms') and the participants were followed like a prospective cohort study.

In Paper I, we selected 298 mother-offspring pairs with at least one pair of faecal samples from mothers and offspring who participated in the ProPACT study (Table 1A). In Paper II and III, we selected the 278 offspring that attended the clinical evaluation at two years of age, together with their parents (Table 1B).

Table 1A. Participant characteristics in Paper I (N = 298)

Maternal age at delivery (years (SD))	29.6 (+/- 3.9)
Caesarean sections	12.8 %
Probiotics users	49.4 %
Antibiotic therapy during pregnancy	7.2 %
Male offspring	46.4 %
Gestational age (weeks (SD))	40.4 (+/- 1.5)
Birth weight (kg (SD))	3.6 (+/- 0.4)
Birth length (cm (SD))	50.7 (+/-3.3)
Breast-fed at three months	97.1 %
Formula-fed at three months	6.5 %

Proportion of children receiving antibiotic treatment within	
- 6 weeks:	6.3 %
- 1 year:	17.2 %
- 2 years:	44.4 %

Table 1B. Participant characteristics in Paper II and III (N = 278)

Participant characteristics		n[†]
Girls, n (%)	149 (53.6)	278
Caesarean section, n (%)	35 (12.8)	273
Birth weight, g, mean (SD)	3633 (486)	278
Birth length, cm, mean (SD)	50.5 (1.9)	242
Gestational age at birth, weeks, mean (SD)	40.3 (1.6)	274
Months of breastfeeding, months, mean (SD)	11.0 (4.6)	260
Antibiotics administration, n (%)		
- within 6 weeks	6 (2.5)	241
- within 1 year	35 (13.6)	258
- within 2 years	166 (41.7)	278
Glucocorticoid inhalations before 2 years, n (%)	4 (1.4)	278
Eczema within 6 years, n (%)	53 (24.7)	215
Allergic rhinoconjunctivitis within 6 years, n (%)	22 (10.6)	207
Asthma within 6 years, n (%)	10 (4.0)	221

[†] Number of observations included in the analysis. SD – standard deviation.

The parents completed questionnaires concerning the child's home environment at 36 weeks of gestation, after 6 weeks, 1 and 2 years after birth, and questionnaires on child health at 1, 2 and 6 years. These included mode of delivery, length of breastfeeding, antibiotics administration to mothers and offspring and gestational age. Although we do not have details on the antibiotic administration in the offspring before 10 days of life, no offspring delivered by Caesarean section received antibiotics during labour, but one developed septicaemia afterwards and was treated accordingly. Also, a clinical evaluation of allergy-related diseases was performed by a paediatrician at 2 years of age and by research assistants at 6 years of age.

The probiotic milk administration was double-blinded and randomly provided to one-half of the pregnant population from 36 weeks of gestation until 3 months after birth. The probiotic milk contained 5×10^{10} colony-forming units (CFUs) of *Lactobacillus rhamnosus* GG (LGG), 5×10^{10} CFUs of *Bifidobacterium animalis* subsp. *lactis* Bb-12, and 5×10^{9} CFUs of *Lactobacillus acidophilus* La-5 per day. The remaining half received placebos in the form of heat-treated fermented skimmed milk with no probiotic bacteria.

Merging the two arms

Data from the probiotic and placebo arm of the ProPACT trial were included in the current study. Previous analyses of the ProPACT study have shown a 49 % reduction of cumulative incidence of eczema within 2 years of age, but not for asthma or AR. [129] Previous analysis of the infant stool samples has indicated that only LGG appears to be transferred from mother to child. Although we observed greater abundance and presence of LGG at 10 days and 3 months in infant stool samples from the probiotics group, the relative abundance of LGG was still low and did not persist to 1 and 2 years of age. [130] Apart from that, there was no observed difference in the bacterial microbiota composition nor the bacterial alpha diversity between the intervention and control groups. Since the stool samples' overall microbial compositions were unaffected by the maternal supplementation, we pooled the probiotic and placebo arms of the ProPACT trial.

Faecal samples

A total of 1516 faecal samples from mothers and offspring were selected in Paper I (Table 2), and 1015 samples from offspring were used in Paper II and III. Maternal samples were collected at 35-38 gestational weeks of pregnancy and 3 months postpartum. Offspring faecal samples were obtained at 10 days, 3 months, 1 year and 2 years, and they were sampled from the diapers. Faecal samples were collected from the diaper by parents, with an enclosed spoon as sampling equipment and stored in a tube with 10 mL Cary Blair transport medium (approximately 20 times dilution). Samples were immediately frozen to -18 °C at home and held in a frozen state before transport to the laboratory for storage at -80 °C before further analyses.

Anthropometrics

The information on height and weight in childhood were collected at routine visits in child health centres and school health centres as a part of the Norwegian Child Growth Monitoring Programme.

Height was measured supine when less than 2 years of age and standing thereafter with a stadiometer. Weight was measured with a digital weight, according to Norwegian guidelines for child health centres. We collected height, weight, and BMI measurements, which were converted to SDS (z-scores) based on a sizeable Norwegian child population reference. [72] The SDS were adjusted for age and sex, so the expected value for a given age at a given sex was 0. Accordingly, values within 2 and -2 SDS correspond to 95 % of the normal population. We identified those measurements where height-SDS and weight-SDS were ≤ -3 and >3, and those where height decreased in two consecutive measurements. These measurements were evaluated against the growth curve, and 82 data points were excluded when it was obvious that the identified measurements were wrongly recorded. Furthermore, we modelled all growth curves for inspection to secure good-quality data.

Allergy-related diseases

Current eczema (within the last year) at 2 or 6 years was defined according to The U.K. Working Party's diagnostic criteria for atopic eczema. [131, 132] Ever eczema at 2 or 6 years was defined using the questionnaire responses if the parents reported that the child had 'ever had eczema' and 'ever had a recurring itchy rash during 6 months'. Similarly, asthma and AR were defined using the questionnaire if the parents reported that the child was 'ever diagnosed with asthma by a physician' for asthma and 'ever had hay-fever, allergic rhinitis or allergic conjunctivitis' for AR.

Ethical considerations

The parents signed an informed consent at inclusion and were once more informed when the anthropometry data were drawn, with the ability to withdraw, which two participants did. The study protocol was approved by the Regional Ethical Committee of Central Norway (2014/1796). Trial registration at Clinicaltrials.gov NCT00159523, registered 08.09.2005.

METHODS

Table 2B. DNA quantification and ITS1 rRNA gene region sequencing of faecal samples (study I)

	Pregnant	Postpartum	10 days	3 months	1 year	2 years	Total
All faecal samples (count)	248	253	274	246	247	248	1516
Detected fungal ITS1	221 (89 %)	220 (87 %)	153 (54 %)	148 (60 %)	163 (66 %)	189 (76 %)	1094 (72 %)
Sequenced samples	47 (19 %)	27 (11 %)	28 (10 %)	4 (2 %)	7 (3 %)	12 (5 %)	125 (8 %)
Passed rarefaction and taxonomic classification	28 (11 %)	25 (10 %)	15 (6 %)	4 (2 %)	7 (3 %)	11 (4 %)	90 (6 %)

Table 2B. rRNA gene quantification and 16S/ITS1 rRNA gene region sequencing of faecal samples (Paper II and III)

	10 days	3 months	1 year	2 year	Total
All faecal samples (count)	274	246	247	248	1015
Detected bacterial DNA (16S rRNA gene region)	266 (97 %)	243 (99 %)	247 (100 %)	243 (98 %)	999 (98 %)
Sequenced 16S rRNA V3-V4 gene region amplicons (after rarefaction) [a]	178 (65 %)	193 (78 %)	216 (87 %)	170 (69 %)	757 (75 %)
Detected fungal DNA (ITS rRNA gene region)	153 (54 %)	148 (60 %)	163 (66 %)	189 (76 %)	653 (64 %)
Sequenced ITS gene region amplicons (after rarefaction)[a]	15 (6 %)	4 (2 %)	7 (3 %)	11 (4 %)	37 (4 %)

[a] Samples were sequenced if the qPCR cycle threshold was < 35 cycles to provide reliable results in the sequencing procedure. Few samples were excluded due to rarefaction.

DNA extraction and quantification

The faecal samples were homogenised before DNA was extracted using a bacterial DNA extraction protocol that involved mechanical and chemical cell lysis, [47] as no fungal protocols for faecal DNA extraction were validated. The stool samples were homogenised by bead-beating with acid-washed glass beads (Sigma). We isolated the DNA with an LGC mag nucleic extraction maxi kit (LGC Genomics, Middlesex, UK) together with a KingFisher FLEX magnetic particle processor (ThermoScientific, Waltham, MA) according to the manufacturer's recommendations, including a negative control as contamination control.

We used different primers for bacteria and fungi to quantify the bacterial and fungal abundances with quantitative PCR (qPCR). The primers targeted against bacteria (V3-V4 part of the 16S ribosomal RNA gene region) [133] were PRK341F (CCTACGGGRBGCASCAG) and PRK806R (GGACTACYVGGGTATCTAAT). We used LightCycler qPCR (Roche)

with thermocycles comprising 95 °C in 15 min, then (95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s) × 40. For each qPCR plate, we included positive and negative controls (*Escherichia coli* and sterile water, respectively).

The fungal internal transcribed spacer 1 (ITS1) amplicons, placed between the 18S and 5.8S rRNA gene regions, were constructed using the primer pairs ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC), according to Tang et al., [11] with a LightCycler qPCR. We used thermocycles of 95 °C in 15 min, then (95 °C in 30 s, 56 °C in 30 s, 72 °C for 45 s) × 50. Positive and negative controls (*S. cerevisiae* and sterile water, respectively) were included for each qPCR plate. The qPCR cycle threshold (CT) value cut-off for fungal detection was set to either within the value of the negative control or 45 cycles because our experience suggests that DNA quantification beyond 45 cycles may produce misleading results. The qPCR CT values were converted to fungal and bacterial DNA concentration using standard curves for CT values with measured DNA concentrations (described in the Statistics section). Ultimately, these DNA concentrations were used as markers for absolute abundance in these studies.

Sequencing of bacterial and fungal rRNA gene amplicons
We used the bacterial 16S rRNA gene region amplicons that were sequenced with Illumina MiSeq in three runs in a previous study by Avershina et al. [47] Next, we sequenced a selection of the fungal ITS1 rRNA gene region amplicons with Illumina MiSeq. Since many of the samples had low ITS DNA quantities, we chose to sequence the ITS1 rRNA gene region in only those with sufficiently high ITS DNA quantities. An upper cycle threshold (CT) cut-off value of 35 was applied to ensure good quality amplicons.

For the sequencing preparation, we measured the ITS DNA concentrations of the 125 selected ITS DNA samples from mothers and offspring (in addition to four positive and four negative controls) with FLx 800 cse (Cambrex), normalised with a Biomek 3000 (Beckman Coulter) and prepared for amplicon sequencing using Illumina MiSeq v3 600-cycle chemistry, according to the producer's instructions. Four positive and four negative controls were also included in the library. The library was quantified using a Droplet Digital PCR (ddPCR, BioRad) and then diluted to a concentration recommended for sequencing. We then performed gene paired-read sequencing of the ITS1 rRNA gene region on a MiSeq platform (Illumina).

The sequencing of the ITS1 rRNA gene region produced 3 722 830 quality-filtered reads (median 16 355, minimum 3, maximum 119 46). The children samples included 1 576 611 quality-filtered reads (median 16 367, minimum 3, maximum 119 463). From the bacterial sequencing of children, we obtained 21 018 190 quality-filtered reads (median 7 172, minimum 10, maximum 169 024).

We used the QIIME (Quantitative Insights into Microbial Ecology) pipeline for quality-filtering and diversity estimation, and the UPARSE algorithm was used for OTU clustering for both bacterial and fungal reads. [134] For fungal results, we applied a rarefaction cut-off of 6000 reads per sample to retain as many samples as possible whilst maintaining an even representation. For bacterial sequencing results, a rarefaction cut-off of 2000 reads was applied. [47] The final dataset comprised 100 fungal samples with more than 6000 sequences per sample and a total of 214 OTUs. Ten of the samples were later discarded due to incorrect inclusion criteria, resulting in the inclusion of 90 samples in the analysis (Table 2). Only 37 samples from offspring were sequenced for fungi since they contained lower abundances of ITS1, and only three of the participating children had repeated sequencing results, all at 10 days and 2 years. 1315 OTUs were obtained from 26 bacterial classes.

Annotation

Since there was no well-established quality annotation database designed for mycobiota taxonomy assignment at present, and because fungi are often subject to misclassification, we used a conservative concordance system for the taxonomic annotation. We compared the OTU sequences with four databases as follows: GenBank (NCBI, ≥ 97 % identity and E-value $< 10^{-50}$); the Warcup Fungal ITS and UNITE Fungal ITS (User-friendly Nordic ITS Ectomycorrhiza Database with a bootstrapping threshold of 80 %) through the Ribosomal Database Project (RDP) Classifier (https://rdp.cme.msu.edu/classifier/classifier.jsp); and the Targeted Host-Associated Fungi ITS Database (THF), [11] which was specially curated for the gut mycobiome. A concordance of at least three of these databases at the lowest taxonomic level or two databases together with a justifiable reason was determined as sufficient qualification for final assignment of each OTU. We followed the recent taxonomic reclassification for the fungi by manually curating the classification of OTU-representative sequences with Index Fungorum as the reference. [135] Bacteria were read-assigned with Greengenes v13.8. [47]

STATISTICS

Microbial abundances

The fungal ITS copy concentrations were right-skewed and therefore log-transformed to obtain a normal distribution.

For samples without quantifiable fungus levels (CT ≥ 45, 260 samples), the abundance per mL was set to half of the negative control for the purpose of analyses in Paper III, as the information of low fungal abundance in these samples was meaningful in the study. A further 102 fungal samples could not be analysed due to technical issues and were thus excluded.

Standard curve calculation

A standard curve was made to convert the qPCR CT values into bacterial 16S and fungal ITS copy concentrations in the faecal samples. Establishing the standard curve for bacterial and fungal concentrations was done separately. We created a positive control of known bacterial or fungal concentration that was integrated into the analyses. For bacteria, the positive control was purified DNA from a cultured *E. coli* strain, solved in nuclease-free water. For fungi, the positive control was made by carefully dissolving a commercially produced fresh baking yeast block consisting of a *S. cerevisiae* strain into nuclease-free water. Then, we extracted the DNA in the same manner as the DNA from the faecal samples. Next, we quantified the DNA concentration of the positive controls with Qubit (Thermo Fisher Scientific). Finally, we calculated the concentration of 16S or ITS copies in the positive controls based on its mass.

Then, we made three dilutions of the positive control, which we included in all qPCR plates. We quantified the CT values of these dilutions with the qPCR in the same way the faecal sample CT values were quantified, and after that we used the average of the CT values to make a linear standard curve. Since the positive controls were diluted by tenths, we used the logarithmic function of the ITS concentration to obtain a linear standard curve. The standard curves made it possible to convert CT values to 16S concentrations and ITS concentration.

Statistics of microbial compositions

The research on the gut microbiome has expanded quickly in recent years, with the development of statistical analysis for microbial data. [136] OTUs, which are compiled DNA reads from the bioinformatic process originating from a specific microbial strain, were analysed against parameters of interest. In Paper I, the diversities and OTU abundances between the groups were analysed with non-parametric Mann-Whitney *U* tests and Kruskal-

Wallis tests, as abundance distributions of the taxa were not normally distributed. By this, we disregarded the potential within-subject correlations; nevertheless, a non-parametric test for repeated measurements, i.e. Friedman's test was not applicable due to the low numbers of observations in some groups. We controlled the false discovery rate of the non-parametric analyses through the Benjamini-Hochberg procedure.

The newly developed *Analysis of Composition of Microbiome (*ANCOM) method is a more sophisticated statistical composition analysis that considers that the faecal sample only constitutes a small sample from the total gut microbiome. In simple terms, it uses the log-ratio of all taxa to provide a reference against the specifically analysed taxon and controls the false discovery rate to 0.05. Zero-prevalence cut-off was at 0.9, corrected for multiple comparisons by the Benjamini-Hochberg procedure and dichotomised into high and low SDS for height and weight at 0 SDS. This was applied in Paper II. Lately, the ANCOM analysis has also been extended to handle repeated samples as well. [136, 137]

In Paper III, the *Linear discriminant analysis effect size* (LEfSe) was used. This is specially made for microbiome analyses to determine which taxa will likely explain differences between groups using the relative abundance. It combines non-parametric tests (Kruskal-Wallis test and Mann-Whitney *U* test) with a linear discriminant analysis, [136, 138] and estimates the effect size of the taxa that statistically differ significantly. The threshold LDA score was 2.0, and a correction for multiple testing was included.

Ecological ordination methods separated the microbial taxa in a principal coordinates analysis (PCoA). This method tries to explain the multi-dimensional taxonomic variability of the samples into usually two dimensions. Samples of similar taxonomic composition will be put closer together than those of mostly different taxonomic compositions.

Mixed model analysis for microbial abundance
The fungal DNA data from the offspring samples were analysed using a *linear mixed model* for the fungal DNA concentration, and a *mixed logistic regression* for the presence of fungal DNA. A linear mixed model is a multi-level statistical model akin to linear regression. However, the model can handle non-independent data like repeated measurements using random effects, meaning that the model can be adjusted for random variables (like individuals having several consecutive measurements). Furthermore, a mixed model could also be adjusted by fixed effects, i.e. non-random parameters over which the group varies. Like a linear mixed model that needs continuous outcome values, a mixed logistic regression

is a multi-level model that handles dichotomised outcome values. The models included a random intercept for mother-offspring pair and age, maternal fungal DNA concentration/presence, mode of delivery, and maternal probiotic intervention were used as covariates. The effect of antibiotic use was studied in a separate model because we lacked information on the use of antibiotics within 10 days of life. The interaction terms were investigated and included in the final analyses if significant.

Multiple linear regression analyses were performed for independent data to test the associations between the fungal DNA abundance and clinical characteristics in Paper I.

Anthropometric analyses

Linear mixed models were used to evaluate the association between fungal and bacterial abundance and diversity, and height-SDS and BMI-SDS. Then, we tested microbial abundances and diversity against mode of delivery, breastfeeding, and duration of breastfeeding with linear mixed models. We tested the models for interaction between abundance or diversity, antibiotic administration, and age, which did not change estimates and were therefore not included in the final model. Furthermore, neither probiotic supplementation nor Caesarean section did show associations with height- or BMI-SDS and was not in the model. We divided the growth data into three age groups based on the Karlberg model (0-1 year, 1-2 years and 2-9 years), [70] as these growth periods have different growth characteristics.

Allergy-related diseases

The associations between fungi and bacteria abundances at all four time points were evaluated against the development of asthma, AR, and eczema within 6 years. We used logistic regressions since the outcome measures (allergy-related diseases) were dichotomised values (healthy or affected).

To explore the causal direction between fungal abundance and development of allergy-related disease, we developed a model to approach the associations between fungal abundance and allergy-related diseases. Because we had collected faecal samples from four different time points and information on during which period the allergy-related diseases were developed, we could estimate which of these came first. Although this analysis could not determine causality, it could indicate the direction of the association. This type of sub-group analysis required a large sample size. Therefore, we chose to use eczema development as the model for allergy-related diseases since this was the most prevalent condition in our study sample. In the first part of the analysis, we used logistic regressions to investigate whether those with higher bacterial or fungal abundances had higher risks of developing eczema by 2 years, if they had not developed eczema at the time the faecal sample was collected. This is illustrated in Figure 2 by grey arrows. For instance, is there a higher risk of developing eczema between 3 months and 2 years of age if there were higher fungal abundance levels at 3 months? A result in this part of the analysis would indicate that higher microbial abundance preceded eczema development. For the last part of the analysis, the outcome measure was development of eczema from 2 years and up to 6 years in those without eczema up to 2 years of age, as the last faecal sample was collected at 2 years of age.
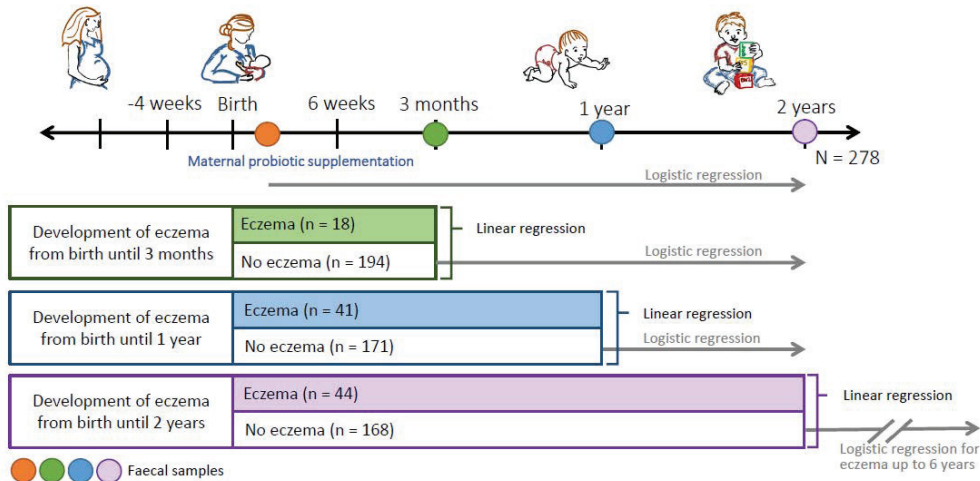
Figure 2. Causality analysis. Overview of how the causality analysis between fungal and bacterial abundances and eczema was performed. Linear regressions were used to estimate if eczema diagnosed at or prior to each timepoint predicted fungal or bacterial abundance at that timepoint. Logistic regressions were used to estimate if fungal or bacterial abundance at each timepoint predicted eczema up to 2 years (6 years for the lowest analysis) among those with no history of eczema by that timepoint. The figure is modified from Paper III, which is only submitted and not subject for copyright.

The opposite situation was considered in the second part of the analysis. Here, the change in microbial abundances was evaluated by linear regressions, based on whether the child had developed eczema by the time of the faecal sample collection. The coloured braces illustrate

this in Figure 2. For instance, are there higher fungal abundances in the faecal samples at 3 months of the children that already had developed eczema within 3 months, than those that had not?

Statistical programmes

The statistical analyses were conducted using MATLAB 2016a (The MathWorks, Inc.), STATA 14 and 16 (StataCorp) and SPSS Statistics version 23.0 (IBM). The diversity indices were computed using the Norwegian programme PAST. [139] The microbial calculations and heatmaps were conducted in R using PhyloSeq. [140] The OTU analyses in article II were performed in R, and in Paper III at the online Galaxy module. [138]

Significance level

The significance level was set to $\alpha = 0.05$. In the two first articles, the statistical significance was presented with 95 % CI and a p-value together with the effect estimate. However, a p-value is redundant in the presence of a 95 % CI. Due to the tendency to omit the p-value in scientific papers, the p-values were excluded in Paper III to emphasise the CI. The p-value only describes the probability that the test shows the observed result or more extreme, given that the null hypothesis (H_0) is true. In addition to this probability, a CI also illustrates the analysis's precision by a narrower range indicating a closer approximation to the effect estimate (if it does not involve 0/1, depending on the type of statistical test).

5 SYNOPSIS OF RESULTS

FUNGAL ABUNDANCE AND DIVERSITY IN OFFSPRING AND MOTHERS

In Paper I, we sought to explore the gut mycobiota of pregnant mothers and their offspring and look at the mother-offspring relation to mycobiota transfer.

In the subgroup of 298 participants with faecal samples in mothers and offspring, 88 % of mothers and 56-76 % of children had detectable gut fungi in their faecal samples (Figure 3, left). The fungal abundance was highest in samples of pregnant mothers, whereas there was a tendency to a higher fungal abundance at 10 days and 2 years of age, than at 3 months and 1 year of age, although this did not reach statistical significance (Figure 3, right).
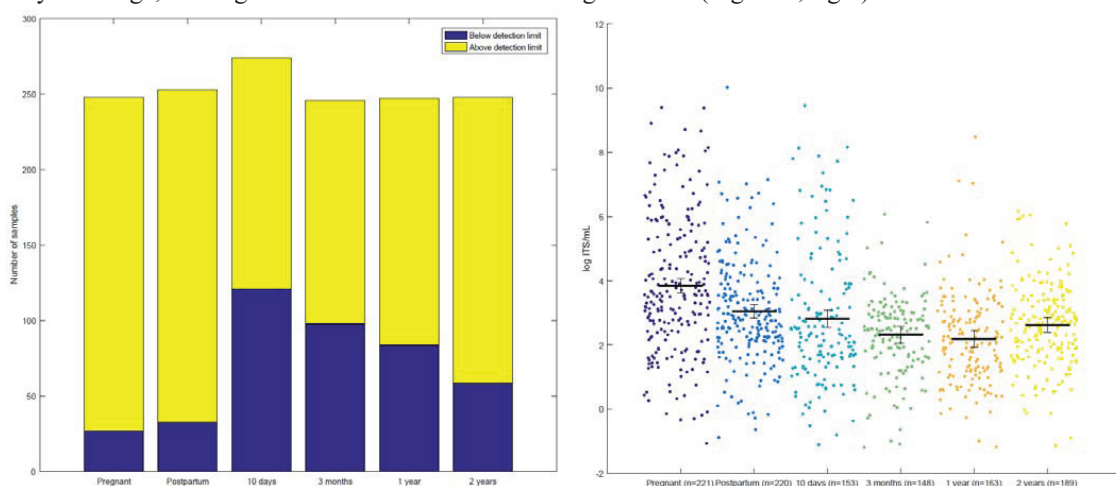

Figure 3. Detection of fungal ITS DNA (left). The counts of samples with detected and non-detected fungal ITS DNA for each age group. The detection limit was set to a higher fungal ITS concentration than the negative control or within a CT value of 45 cycles. **Fungal ITS DNA concentration in maternal and offspring faecal samples (right).** A scatter plot of the fungal ITS DNA concentrations (log ITS copies per mL, mean and 95 % CI). The concentration of the ITS copies quantifies the amounts of fungi in the samples. The figures are printed from Paper I, distributed under Creative Commons Licence.

We also showed that an offspring had increased odds (OR = 1.54, 95 % CI 1.01 to 2.34) of having detectable gut fungi if the mother also had detectable gut fungi. The association was strongest 10 days after birth. In the sequenced samples, the mycobiota alpha diversity was smallest at 10 days and increased with age. However, the beta diversity was at its highest in faecal samples 10 days after birth. At 10 days and 3 months after birth, *Debaryomyces hansenii* was the most abundant species, and *Saccharomyces cerevisiae* became the most abundant species after weaning. The compositional diagrams are shown in the appendix (Figures S1A-C).

EARLY GUT MICROBIOTA AND CHILDHOOD GROWTH

In Paper II, we studied the relationship between the gut fungal and bacterial microbiota from birth to 2 years of age, and height and BMI development from birth to 9 years of age.

In the subgroup of 278 children with faecal samples and clinical follow-up, we found that fungal abundance at 2 years of age was positively correlated with height-SDS between 2 and 9 years of age (β = 0.11 height-SDS; 95% CI 0.00 to 0.22) (Figure 4A). Fungal abundance did not, however, show an association with BMI-SDS in the same age group. In the first year of life, we observed a lower BMI-SDS in those children with higher fungal abundance in faecal samples collected at 1 year of life (β = -0.10 BMI-SDS, 95% CI -0.20 to 0.00) (Figure 4B). Similarly, higher bacterial abundance and bacterial diversity at 1 year of age were associated with lower BMI-SDS in the first year of life (β = -0.13 BMI-SDS, 95% CI -0.22 to -0.04; and β = -0.19 BMI-SDS, 95% CI -0.39 to -0.00, respectively). We found no relation to the use of antibiotics or probiotics.

Figure 4. Height-SDS and BMI-SDS and microbial abundance as predicted linear associations. A) Prediction of height-SDS at 2-9 years for fungal and bacterial abundance. B) Predictions of BMI-SDS at 0-1 year for fungal and bacterial abundances. The predictions are shown as lines and the coloured areas cover the 95% CI. The bacterial abundance prediction model for height-SDS remains statistically non-significant. The figures are printed from Paper II, distributed under Creative Commons Licence.

Compositional diagrams are shown in the appendix (Figures S2A-B). For microbial taxa ANCOM analyses, we found a negative association between the relative abundance of *Bifidobacterium longum* at 2 years of age and height-SDS from 2 to 9 years.
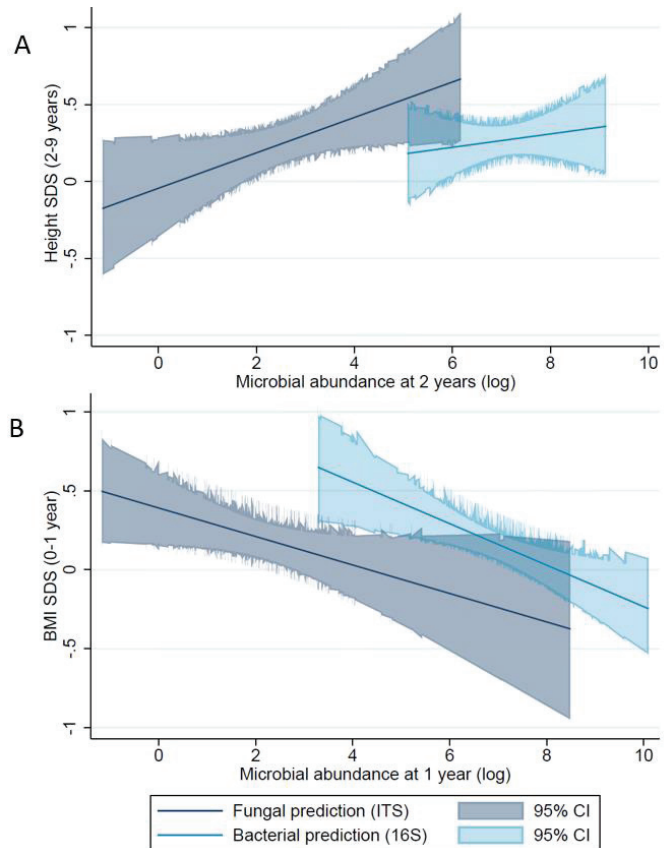
EARLY GUT MICROBIOTA AND DEVELOPMENT OF ALLERGY-RELATED DISEASES

In Paper III, we explored the associations between the gut fungal and bacterial microbiota from birth to 2 years of age and the development of allergy-related diseases from birth to 6 years of age.

In the subgroup of 278 children with faecal samples and clinical follow-up, we observed positive associations between higher fungal abundance at 2 years of age and the cumulative incidence of ever asthma (OR 1.70, 95 % CI 1.06 to 2.75) and AR (OR 1.41, 95 % CI 1.03 to 1.93) up to 6 years (Figure 5). A similar association between eczema up to 6 years of age and fungal abundance at 2 years was also found (OR 1.18, 95 % CI 0.99 to 1.40), but the estimate was imprecise due to a wide CI so that we cannot exclude that there is no association.
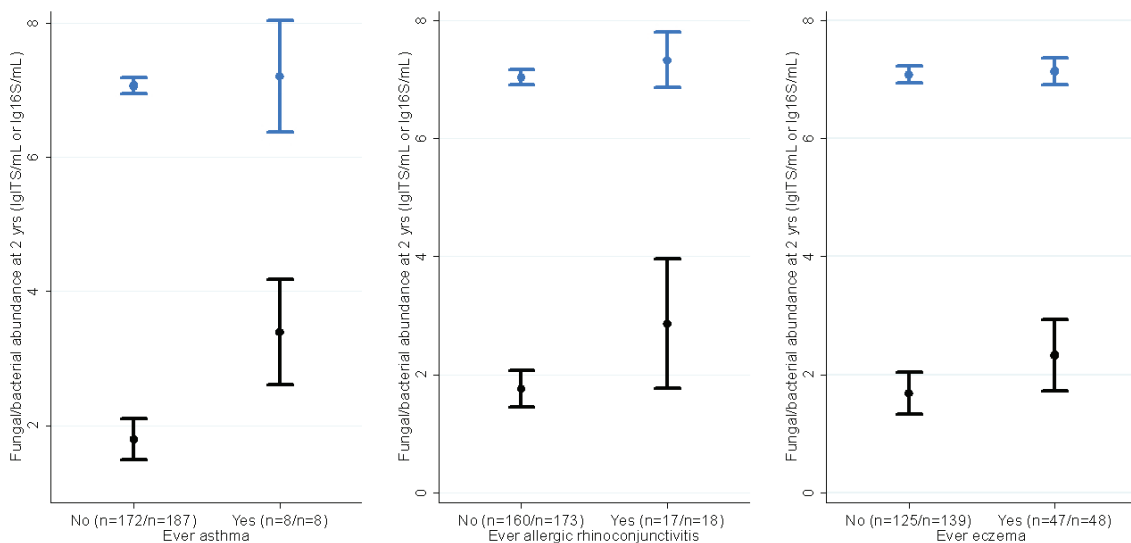
Figure 5. Fungal and bacterial abundance and allergy-related diseases. These figures depict the mean and 95 % CI of fungal (black) and bacterial (blue) abundance at 2 years of age for children with ever allergy-related disease at 0-6 years, supplementing the logistics regression analyses. The numbers within brackets represent the number of participants in each group for fungal and bacterial analysis, respectively. The figures are printed from Paper III, which is only submitted and is not subject for copyright.

When searching for the potential directions of causality between microbiota and eczema, we found that there was an association between fungal abundance at 2 years and the development of eczema between 2 to 6 years of age (OR 1.82, CI 0.97 to 3.44); however, the finding cannot be regarded as statistically significant. We did not find support for any other associations in the opposite direction of causality.

Principal coordinates analysis ordination plots for fungi and bacteria showed that faecal samples from 1-2-year-old children with current eczema were overrepresented in the region that represented the 2-year-old microbiota composition. (Figure 6AB). A taxonomic LEfSe analysis of the fungal and bacterial gut microbiota showed higher abundances of *Enterococcus* sp. at 2 years in children without eczema.

Evaluation of the gut permeability markers FABP2 and LBP against allergy-related diseases showed a lower risk of ever eczema with increased FABP2 at 1 year; however, no other associations were found with allergy-related diseases and FABP2 or LBP. There was no association between FABP2 and fungal abundance.
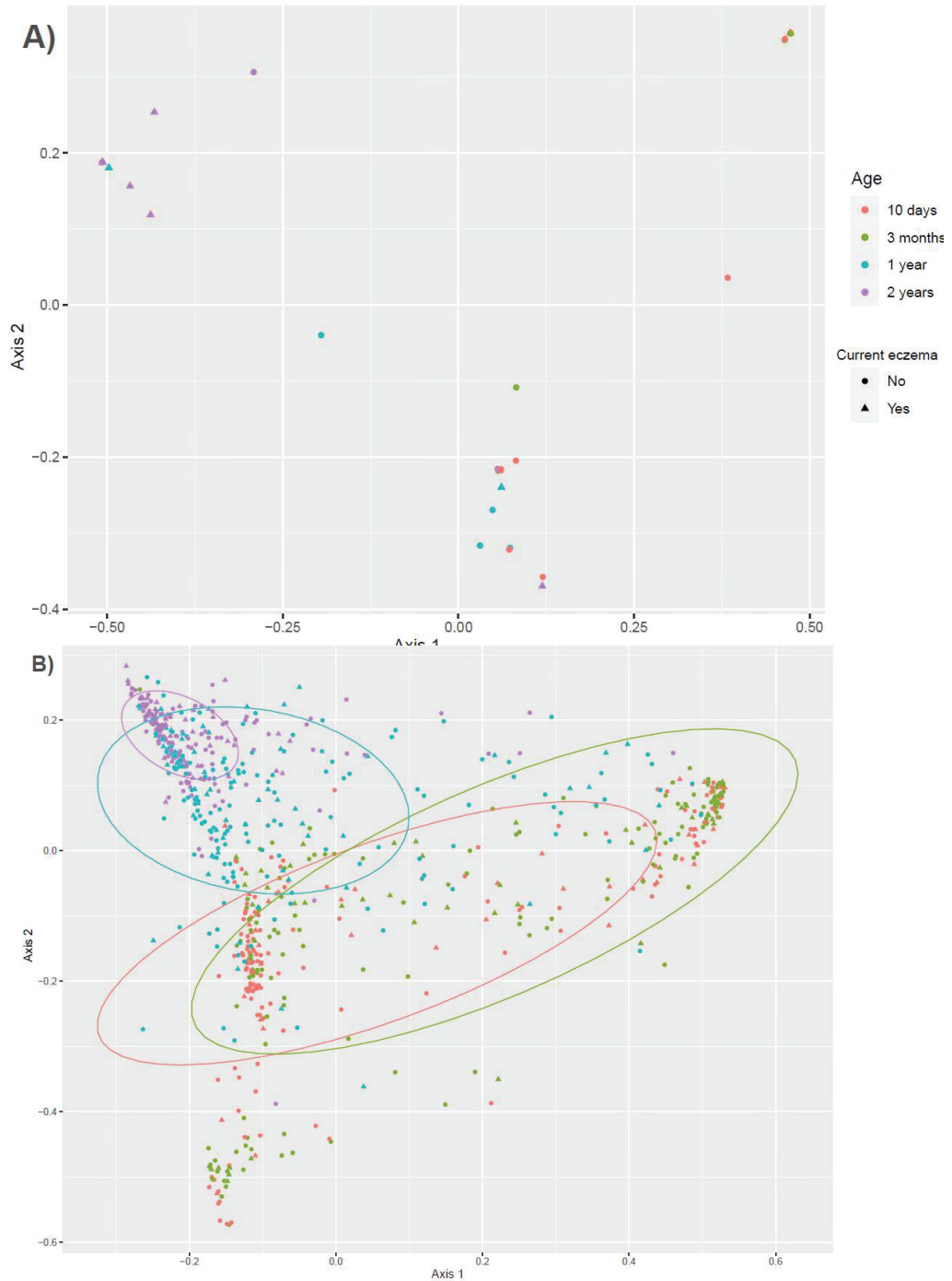
Figure 6. Principal Coordinates Analysis (PCoA) of fungi and bacteria. PCoA is a multivariate ordination method that depicts the all-over taxonomic variation between samples, in which two samples with similar taxa and abundances are put closer and dissimilar samples would be placed far apart. The 2-year samples cluster top-left for both fungi and bacteria, whereas neonatal samples are more wide-spread. Figure 6A describes fungal taxa distribution. Figure 6B describes bacterial taxa distribution. Ellipses contain 70 % of each age group. The figures are printed from Paper III, which is only submitted and is not subject for copyright.

6 DISCUSSION

DISCUSSION OF METHODS, STRENGTHS AND WEAKNESSES

Material and study design

The inclusion criteria to the ProPACT study were living in Trondheim (the third-largest city of Norway), filling in a questionnaire and willingness to consume probiotic milk once a day. The chance for selection bias is therefore limited; however, we observed that many of the participants were highly educated (78 % of mothers had higher education). This sampling bias could impair the external validity by underrepresenting the lower socioeconomic groups. Prevalence of childhood OWOB are higher in families with a low level of parental education, [141], as might be the case for asthma as well. [142] Apart from this skew, the newborn children were of normal weight and height, close to equal inclusion of boys and girls and only slightly less Caesarean sections than the general Norwegian newborns (13 % in our study vs. 14-15 % in the general population). [143] With the rather unselected sampling, a relatively high external validity was thereby attained in this cohort study. Repeated faecal sampling enabled us to investigate the variation between participants and follow everyone's development.

The study design gave us limited control of faecal sampling. Although the parents were well informed about collecting the samples and quickly freezing them to avoid contamination and improve preservation, we did not control the amount of faeces put into the sampling containers, so the sample dilution of the Cary-Blair medium could vary between the samples. This could represent a random sampling misclassification that weakens the result precision. Allergy outcomes were based on self-reporting questionnaires based on the ISAAC study, [104] but questionnaires are inherently prone to recall bias. For instance, might the parents with allergic children more clearly remember that their children received antibiotics as this could be their explanation for allergy development. Although we did not find an association between antibiotics and microbial characteristics, this could introduce a type II error in our study. Due to the nine years follow-up time, frozen DNA was stored for a long time. This could somewhat have impacted the quality of the samples, but no sample age difference between the groups. There was no freeze-thawing of DNA samples before the current study.

Methodological considerations

Extraction. The microbial DNA from the faecal samples were extracted with a lysis protocol optimised for bacteria due to the lack of well-established fungal DNA extraction protocols. The extraction method involved chemical and mechanical lysis. Still, because fungal cell

walls are inherently tougher than the bacterial cellular membrane, the risk was that some fungal DNA remained within intact fungal cells. The DNA extraction efficacy of fungal cells also appeared to be strain-specific, which could overestimate some species and underestimate others. [144] In a pilot study at the NMBU laboratory where the microbial experiments were carried out, we showed that a bacterial lysis protocol was inferior to a fungal lysis protocol for the amount of extracted fungal DNA (not yet published). A recent study showed that although the fungal DNA varied between extraction protocols and some variations in some bacterial species' relative abundance, the fungal and bacterial alpha diversity remained the same between extraction methods. [144] Moreover, a large HMP-based study showed that the fungal taxonomy and diversity stayed the same with bacterial or fungal extraction method, although not stating whether the DNA quantity differed. [19] Therefore, one could assume that a DNA extraction method optimised for fungi, like the non-commercial IHMS Protocol Q, (Fiedorova, Radvansky et al. 2019) would be preferrable for future microbiome studies involving bacterial and fungal DNA. In this way, we could potentially have obtained higher fungal quantities and would make us able to sequence a greater proportion of samples. This could increase the internal and external validity of the study and increase precision. So, even though we believe in the results, non-optimal extraction methods could reduce the reproducibility of this study. We have thus focused on a transparent visualisation of the data so that future research could build on our methods and results.

Quantification. We used the 16S rRNA gene section as a measure of bacterial abundance. This gene region is a universal region of all bacteria, and the quantity of this primer region would therefore quantify the total number of bacteria. Quantification of fungi remains more complicated. In contrast to bacteria containing less than seven of the 16S rRNA gene in their one chromosome, eukaryotes like fungal cells could have numerous copies because of tandem loops of the ribosomal RNA gene (*rrn*, the DNA coding for the ribosomes), with up to some thousand copies per cell. [11, 145] Furthermore, there could be larger variation in rRNA gene copy numbers within a species. [145] Caution should therefore be taken when using these parts for quantification. Fungal quantification of the *rrn* gene (including the ITS1 gene region) has been performed previously in bovine rumen studies. [146] A study by Taylor et al. presented that the quantification of fungal abundance by the ITS region strongly correlated with fungal abundance estimations. [147] Therefore, we concluded that the ITS1 section could work well as a fungal quantification marker in addition to the fungal barcode marker. Future studies should involve quantifying fungi and preferably consider the number of *rrn* copies for

each species, either by developing databases or through estimations by metagenomic sequencing. Also, in metabarcoding a standard sequencing target, like ITS1, ITS2 or another target, should be established.

Selection of samples to sequence. To ensure enough fungal DNA for the ITS1 sequencing, we set an ITS cut-off at 35 cycles. This strict ITS quantity cut-off was essential to only sequence samples with a reliable fungal DNA amount that did not represent sample contamination. Whilst fungal abundance was quantifiable for approximately two-thirds of the stool samples, this trade-off made us able to sequence only a minority of the samples for fungal DNA, due to low levels of faecal fungal DNA in the first two years of life. Thus, we had limited opportunity to investigate the role of the mycobiota composition on childhood growth and allergy-related disease. It cannot be excluded that the low-abundance samples had different mycobiota compositions. Nevertheless, in this relatively unselected study population of mothers and offspring, our selection could reflect the gut mycobiota of a healthy gut.

Sequencing and annotation. The mycobiome was characterised using metabarcoding sequencing (Illumina MiSeq) of the ITS1 gene region. Since some fungi are more cultivable than others, the relative distributions of fungi would be more representable by a culture-independent technique. Being uncertain if metagenomic sequencing could sequence deeply enough to describe the low-abundance mycobiota, [10, 11] metabarcoding was reckoned the most appropriate method. However, the interpretation must be cautious since the method only depends on finding intact DNA segments and not living microbes. Therefore, contaminants from food could easily be found in low abundances, like *Ustilago* spp. (obligate corn pathogens) or Agaricomycetes like the edible white button mushroom *Agaricus bisporus*. Moreover, the gut mycobiota is known to be more unstable than the bacterial gut microbiota, and so the transient (yet living species) species from food could also be easily interpreted as a part of the autochthonous mycobiota. However, repeated samples over time helped us see which species that remained in several samples. As for primer choice, the ITS1 region adjacent to the 18S rRNA gene is the most described in fungal databases and most diverse, meaning it would give the best annotation solution. [11] In the last years, there has been a tendency to use the ITS2 segment instead of ITS1. Use of ITS1 may estimate a larger fungal diversity whereas ITS2 tends to show more Ascomycota, [19, 32] which partly could explain differing taxonomic findings between studies with various methods. However, the ITS segments are not precise enough for good species classification, and a disparity in ITS length

(100-550 base pairs) between samples could also cause PCR bias towards shorter reads. [11, 148] Thus, a consensus on primer use needs to be established.

Taxonomic databases. Sequencing of rRNA gene fragments like ITS1 depends on good-quality taxonomic databases where ITS reads can be aligned against known fungal taxa to annotate the OTUs. Several such fungal databases are still under development (UNITE, RDP, GenBank and other specially curated databases) and there are still many errors in taxonomic names that could overestimate the diversity and decrease the precision of the sequencing. [146] For instance, many fungi have had two taxonomic names, one for its asexual form (anamorph) that looks different from its sexual form (teleomorph), yet their genetic structure is the same. With the advent of DNA-based identification methods, the idea of *One fungus–one name* became prevailing, and one has tried to use one taxonomic name per species. [148] Still, misclassifications do occur and are present in the databases. [148] It is estimated that as much as 20 % of all fungal entries in GenBank (an annotated collection of all publicly available DNA sequences) could be incorrectly annotated (incorrect connection of OTU and taxonomic name). [11] Therefore, our manually curated annotation method using four databases was an attempt to make correct fungal annotation by use of the pre-existing databases, since a conservative OTU approach decreased the rate of type I error findings.

Statistical considerations

The dataset consisted of sequential samples from the same mother-offspring pairs at different ages. In a longitudinal study with repeated measurements, the samples are assumed to be dependent upon each other. This entails that the presumption of independent samples is invalid, and thus a mixed model is required. Using mother-offspring pair as the random intercept in the models, and age, maternal fungal DNA concentration/presence, the mode of delivery and maternal probiotics use as covariates, we were able to do statistical calculations on the fungal quantity.

In Paper III, the faecal samples with undetectable levels of fungal abundance were included in the analysis. These were given the fungal levels of half the negative control (limit of detection/2). [149] The argument for performing this step was to include meaningful information on more participants into the statistical analysis so that the result would be more valid for the study participants. Thereby, the statistical analysis also consists of the participants without detectable fungal abundance. This method is well-known in biochemical markers, [149] although not so often applied in microbiological methods. Quantitative PCR is a

suitable method for DNA quantification, and we reckoned the statistical tests still to be valid after the inclusion of the undetected samples. In this analysis, effect estimates only showed minor differences (data not shown).

MAIN RESULTS DISCUSSION

Fungal abundance and diversity in offspring and mothers

Amongst the main findings of this project, we showed that gut fungi are detectable in most mothers and most of their offspring already at the age of 10 days. *S. cerevisiae* was the most abundant fungal species in mothers and 1- to 2-year-old offspring, whereas *D. hansenii* prevailed during the first months of life. There was also increased risk of fungal DNA presence in offspring if the mother had detectable fungi.

Our high detection rate of gut fungi in children coheres with similar studies, [37, 150] and different ages and methods (culturing vs. non-culturing, ITS1 vs. ITS2) could partly explain why the studies showed compositional disparities. Geographical differences and food intake could also be an explanatory factor.

We detected that *D. hansenii,* a ubiquitous *Saccharomycetaceae* species present in most cheeses, [151] were abundant in many neonates. *D. hansenii* has been detected by RNA-based methods in breast milk, (Boix-Amoros, 2017) has been cultured from human faeces and grows well in milky environments and possibly breast milk, since some strains are known to grow at 37 °C. [12, 152] This suggests that *D. hansenii* could be an autochthonous species of the early mycobiota.

In our selection, *S. cerevisiae* becomes a dominant species first at 1 year of age, at the time food containing *S. cerevisiae* (e.g., bread) is introduced. Whether this finding, at least partly, comes from a transient or long-term colonisation is unclear. However, being cultured in faecal samples and capable of causing opportunistic infections, [12, 153] S. cerevisiae could be an true commensal. The abundance of *S. cerevisiae* varies among studies, [5, 19, 25, 45] and similarly the low occurrence of *Candida* spp. in our study could depend on diet, genetics or fungal methods. In particular, culture-dependent techniques seem to obtain relatively more *Candida* spp. than culture-independent techniques [17, 150], which could partly explain the somewhat limited amounts of *Candida* spp. in our study.

We found that maternal fungal hosting made the offspring more inclined to host fungi. This effect was strongest at 10 days after birth. The increased chance of fungal hosting suggests

that these mother-offspring pairs share physiological fungal hosting abilities. Because fungi are ubiquitous in the environment, they may originate from the mother during birth, the mother's breast milk, [42] parental skin or anywhere else in the hospital or home environment with which the offspring come in contact. However, we did not observe any OTU abundance difference regarding vaginal or caesarean delivery. Thus, we find indications for the transfer of fungal hosting between mothers and offspring that appear to be independent of the mode of delivery and genetic predisposition seems a more likely explanation.

The fungal abundance varied by age, which supports the idea of physiological fungal succession in the early gut mycobiota. At 10 days, the gut fungi have just started to fight for their positions as ecological pioneer species start to colonise the gut, [108] and the reduction in fungal abundance could be due to an ecosystem that tunes in, where food, gut immunity and microbial interactions could play a role. The fungal decrease at 3 months coincides with high abundances of *Bifidobacterium* and *Lactobacillus* that could exhibit fungal antagonism. [9, 28] At two years of age, the gut mycobiota looks more like the adult samples, again indicating a succession of fungi.

Longitudinal growth and BMI development

Higher fungal abundance in faecal samples at 2 years correlated to taller children from 2-9 years. Assuming the range of fungal abundance of 6 units at 2 years (Figure 4A), this could represent a difference of about 3-4 cm at 6 years of age. This finding was in accordance with our hypothesis that a more abundant mycobiota could affect future height.

Growth stimulation by adding yeasts like *S. cerevisiae* into the feed has been shown in calves, piglets, and dairy cows, possibly through the growth hormone (GH) axis. [76, 154, 155] This was a prevalent species our samples by 2 years of age. The GH axis becomes the driving growth regulator from 1-2 years when entering the childhood growth phase, [69, 70] which could justify why the height growth association with fungal abundance becomes apparent from 2 years of age. As fungi can assist in digestion and extracting nutrients from unless indigestible foods, it is plausible that fungi may stimulate growth by providing extra energy, either directly or by promoting nutrient extraction from other parts of the microbiota. However, we did not find any association with fungal abundance and BMI-SDS in children at this age. Either way, this surmised effect probably represents normal growth physiology since the bulk of growth curves are within the normal distribution of longitudinal growth.

We did find, however, that children hosting higher abundances of fungi and bacteria and higher bacterial alpha diversity at 1 year had lower BMI in their first year of life, together with a tendency that higher fungal abundance at 3 months correlated with lower BMI at 0-1 years. Although this analysis could not show a causal direction, the finding at 3 months could indicate that higher fungal abundance could be seen before 1 year of age in those with lower infantile BMI. High bacterial diversity has been associated with childhood and adult leanness, [94] and having a high microbial diversity and abundance as food is introduced might be favourable for a lower BMI development.

The taxonomic analysis yielded no associations with BMI-SDS, similar to a meta-analysis, [94] although later investigations have shown several association with BMI and microbes. [79, 99-101] The lack of consistent findings could be due to sample variations, liberal statistical tests, and varying methods. Total microbial abundance could show another aspect for childhood growth than only microbial composition. More rigorous methods and statistical tools in this research field are required and will hopefully provide more robust analyses in the future.

Allergy-related diseases

Fungal abundance was associated with allergy-related diseases in our study, in consistence with two recent investigations from Ecuador and USA. [32, 111] Although the analysis between fungal abundance and allergy-related diseases was not constructed to prove causality, our analysis could imply that fungal abundance was present before the development of allergy-related diseases. Even though probiotic administration was not associated with fungal abundance or composition in our study, the probiotic administration could confer changes in the gut microbiota or the immunologic response that we could not detect with our methods. This rests as a potential uncertainty in Paper III.

Fungi are eukaryotes that are evolutionarily closer to humans than bacteria, sharing a great number of common biochemical pathways. Thus, they could impact human pathways through the production of human-like substances. Fungi are well-known producers of prostaglandins and leukotrienes. [13] The *Candida* yeast, and likely other fungi, can produce prostaglandins that may reduce gut macrophage activity against fungi and thereby improve fungal gut colonisation. [117] By orchestrating the immune system through self-produced priming cytokines, theoretically fungi could induce the naïve T helper (T_H) cells to pursue a T_H2 direction, [66] which could create both a fungus-friendly gut environment and a predisposition to allergy-related disease development. [118] Whilst this is a plausible biological explanation for

a causal association between gut mycobiota and later allergy-related diseases, our results cannot exclude that the association could also be driven by the mechanisms working in the opposite direction. Allergy-related diseases and their underlying immunopathology could themselves promote a fungus-friendly gut environment.

The absence of the lactic-acid bacteria *Enterococcus* spp. at 2 years is associated with eczema at 2 years. Enterococcus depletion has also been found allergic 8-year-old Swedish children. [112] Some enterococci are also used as probiotics, [156] harmonising with the finding that maternal probiotics reduce eczema by 49 % at 2 years. [129] *E. faecalis* could reduce T_H17 stimulation and ameliorate allergic airways disease in mice, (Pascal, Perez-Gordo et al. 2018) and could even be a partial fungal antagonist by decreasing virulence and hyphal morphogenesis of *Candida*. [157] Depletion of important taxa could therefore be of significance in allergy development.

Our PCoA plots suggested that 1-year-olds were prone to develop eczema if their bacterial microbiota compositions more closely resembled the composition seen in 2-year-olds, not explained by delivery, antibiotics administration, or breastfeeding. A timely succession of the microbiota could be important for the maturation of the immune system and prevention of allergy, in parallel with increased risk of allergy if there is retained breast milk-associated species and lower SCFA producers in school age. [112, 113]

Since FABP2 and LBP were not associated with allergy development or fungal abundance, the association between allergy-related diseases and fungal abundance appears not to be mediated through increased gut permeability and metabolic endotoxaemia. Although previous studies have shown increased gut permeability, [121, 122] this difference could be due to differing methods or participant selection.

SCFAs. Acetate, propionate, and butyrate are produced by anaerobic metabolism in bacteria. These are made from undigestible fibres, and they serve as a source of energy and signalling molecules. [113] A plethora of strain-specific metabolic pathways leads to these products. As the gastrointestinal system is increasingly depleted of oxygen, anaerobic fermentation remains the primary process. *Acetate* is the most abundant bacterial metabolite in the gut since most bacteria produce it, and it enters the human systemic circulation. In contrast, only certain bacteria produce *propionate,* namely Bacteroidetes species (*Bacteroides* spp., *Prevotella* spp.) and some Firmicutes species (*Roseburia* spp*., Eubacterium* spp.), and is used in the liver for gluconeogenesis. [113] *Butyrate* is a larger molecule that serves as an energy

source for the gut epithelial lining, and is produced by Firmicutes species like Ruminococcaceae (*F. prausnitzii*), Lachnospiraceae (*Roseburia* spp.), Erysipelotrichaceae and Clostridiaceae. [113]

Propionate and butyrate are shown to regulate appetite hormones and to also have anti-inflammatory properties by connecting to G-coupled receptors GPCR41 and GPCR43 on intestinal enteroendocrine cells and adipocytes inducing regulatory T cells. [125, 158] In mice, higher levels of SCFA are also associated with increased IGF-1, [77] and also, higher levels of SCFA at 1 year of age seem to protect against development of asthma [114] Propionate is a fungicide used in food industry and acetate is used as a conserving agent that decreases microbial growth. We cannot exclude that the higher fungal abundance in allergic children in our study is associated with lower SCFA levels found in other studies. In that way, these microbial metabolites could be important in both development of longitudinal growth, obesity, and allergy-related diseases. A further interesting path beyond this thesis would be to investigate if the mycobiota is affected by SCFA.

7 CONCLUSIONS

The emergence of the early microbiota as a field of research might give us new insight into how *the forgotten organ* plays in concert with the rest of the human body in early life. We have here described the establishment of the early gut mycobiota and its associations later in life with growth and development of allergy-related diseases. Additionally, we have shown some indications for mother-offspring transfer, and how childhood growth is associated to fungal DNA quantity. Together, this broadens the field of mycobiota into possibly having important physiological and pathophysiological consequences for the human host. As new methods emerge in this rapidly developing field, our findings need to be confirmed in larger studies with more precise methods.

Future perspectives

A reasonable direction to continue this work will be to explore whether there are mechanisms behind these associations with the gut fungal community in children. By finding the aetiological mechanisms behind diseases like growth and weight deviations and allergy-related diseases, potential targets for therapy could be revealed.

8 APPENDICES

FIGURES

A
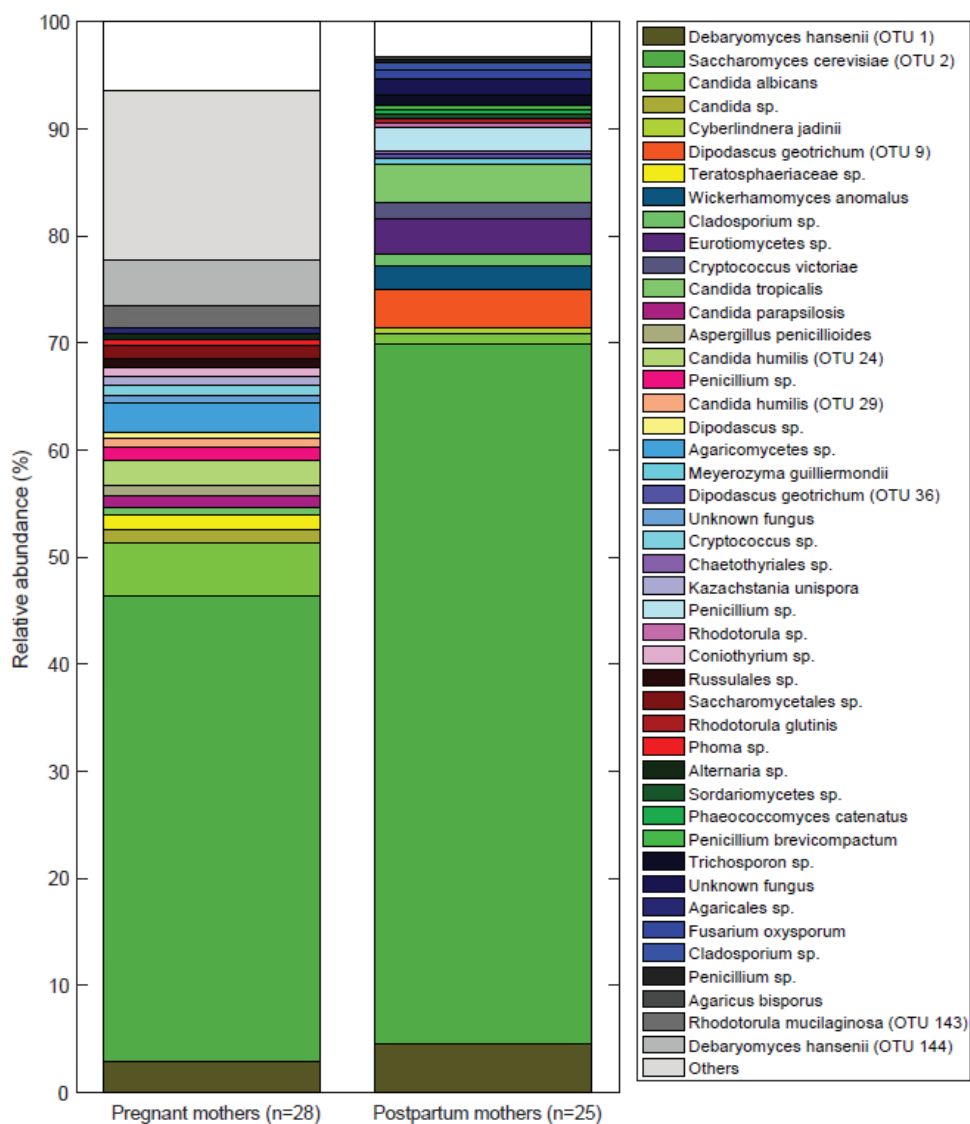
B
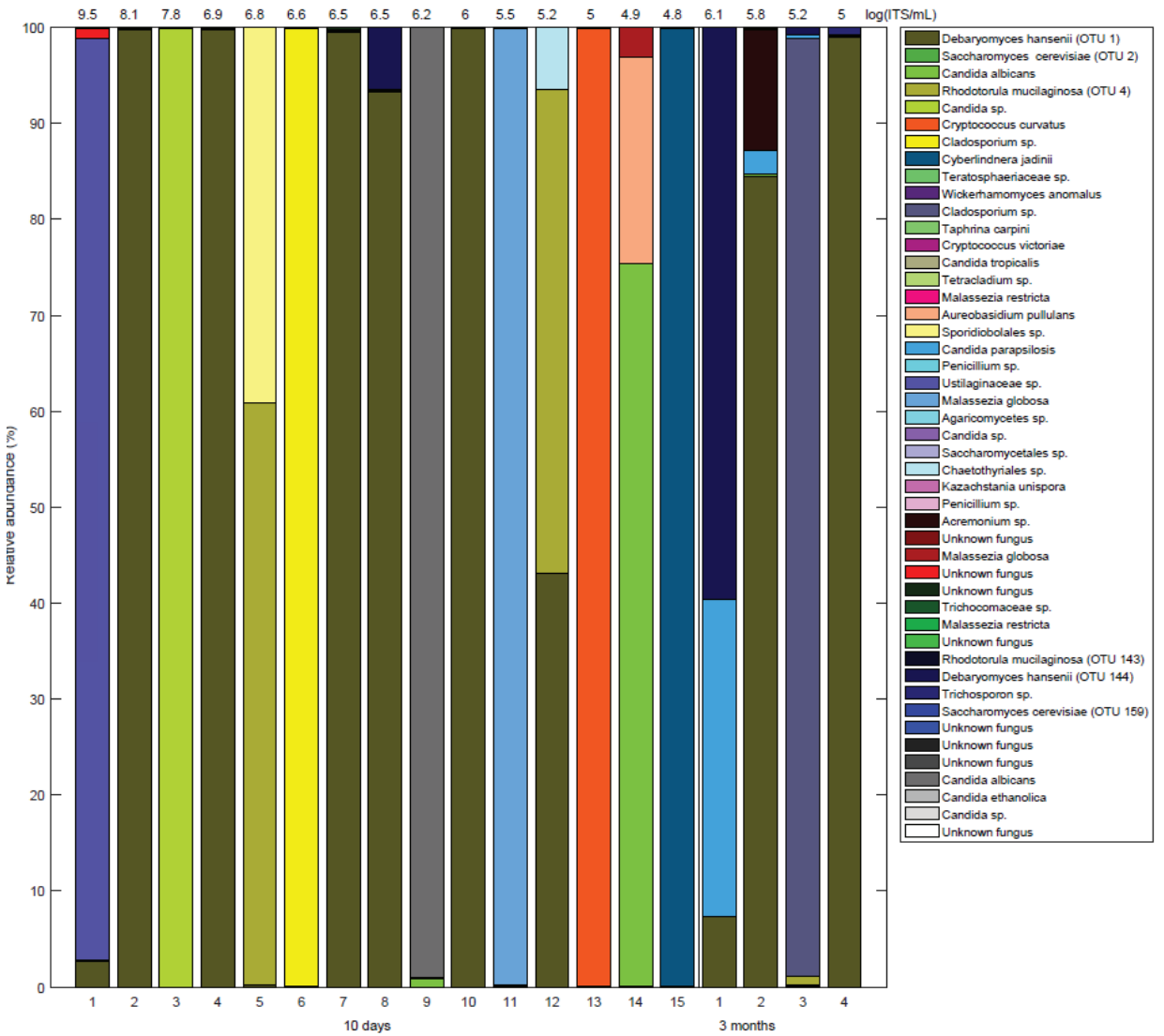
C
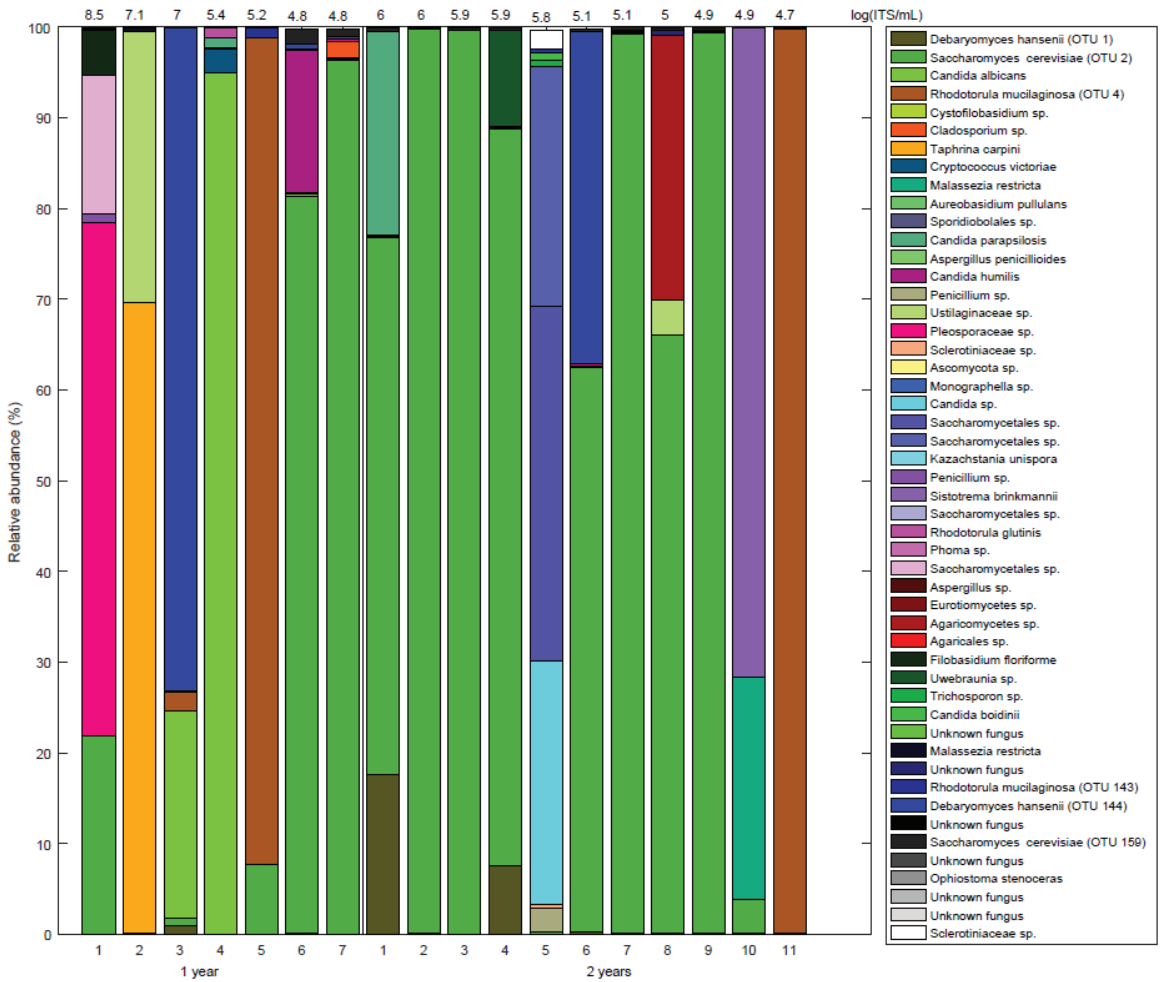
Figure S1. OTU abundances for all groups. Bar charts of the relatively most abundant OTUs in A) mothers, B) offspring from 10 days-3 months and C) offspring for 1-2 years. Each coloured box represents an OTU. The individual fungal ITS DNA concentration is on top of each bar. The figures are printed from Paper I, which is distributed under Creative Commons Licence.
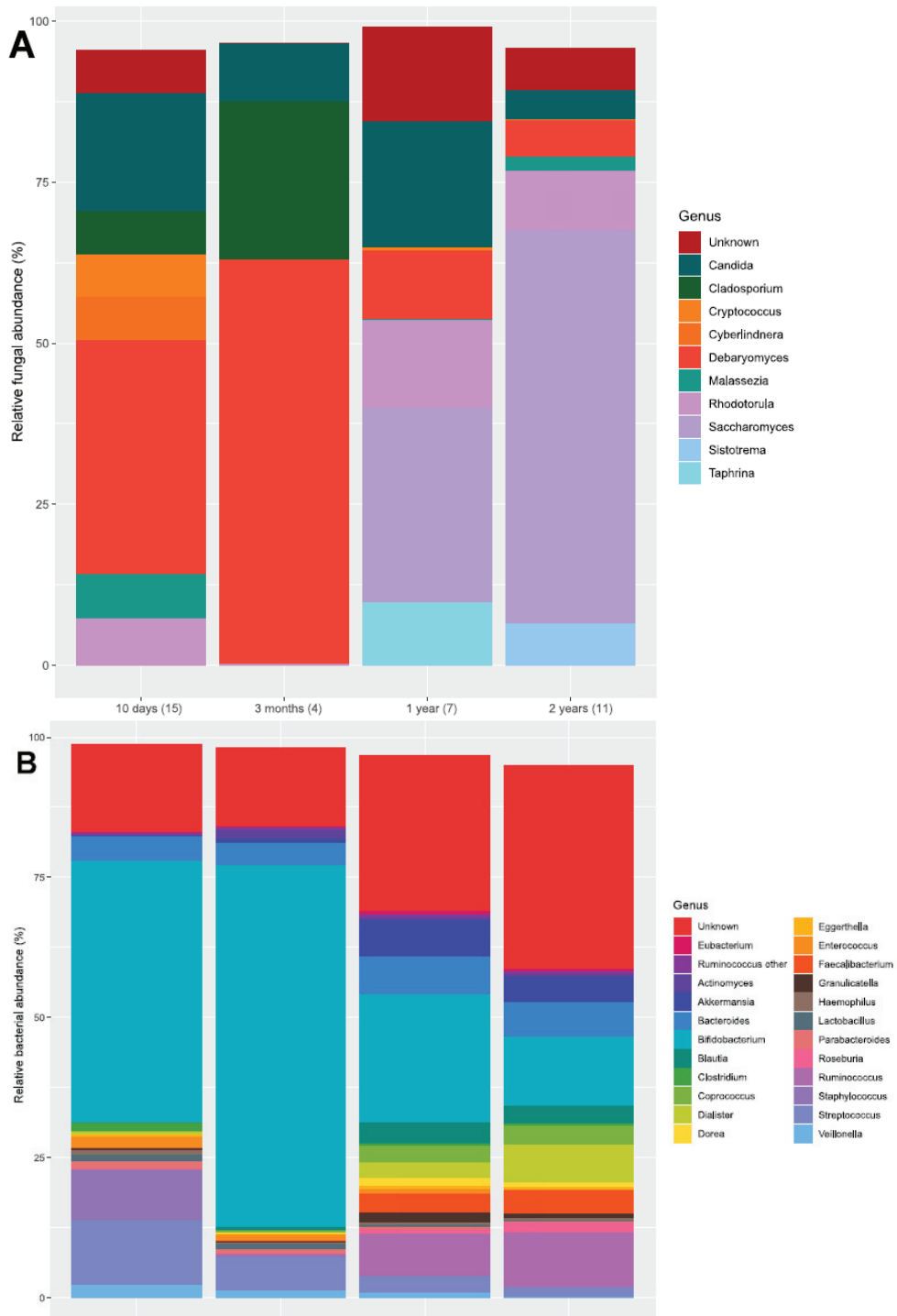
Figure S2. Bar charts of fungal and bacterial gut communities. A) Mean relative abundances for the fungal genera (>1 % abundant) for each age group. Each colour designates a genus. Number of samples for each bar is stated in brackets below the bar. B) Mean relative abundances for the bacterial genera for each age group. Each colour designates a genus. The number of samples is stated in brackets below the bar. The figures are printed from Paper II, which is distributed under Creative Commons Licence.

9 ERRATA

Paper I

During the ongoing work with this PhD thesis, we have become aware of an error in a minor sub-analysis in Paper I related to the probiotic short-term effect on pregnant mothers' mycobiota during pregnancy. We are now informed that most of the maternal faecal samples (week 36-38) were collected before the probiotic intervention started, and these samples should have been regarded as baseline samples in the analysis. This has resulted in incorrect interpretation of the result. Due to this unfortunate event, we have contacted the *Microbiome* editor and an erratum with the subsequent information will be published:

- Page 1, last sentence of the abstract: 'Probiotic consumption increased the gut mycobiota abundance in pregnant mothers (p =0.01).' *This sentence is incorrect.*
- Page 6, paragraph 'Probiotics and fungal DNA concentrations in the mothers and the offspring'. *This paragraph is incorrect.*
- Page 6, line 10 in the Discussion section: 'We also found that the maternal fungal DNA concentrations increased when the pregnant mothers drank probiotics.' *This sentence is incorrect.*
- Additional files Table S5 and Figure S4 and its captions. *This figure, table and captions are incorrect.*

Apart from this, we ensure that the analysis from Paper I remains correct. Fortunately, none of Paper I's 32 citing articles have cited this result.

10 REFERENCES

1.   Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell*. Jan 28 2016;164(3):337-40. doi:10.1016/j.cell.2016.01.013

2.   Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*. Mar 4 2010;464(7285):59-65. doi:10.1038/nature08821

3.   Rodriguez JM, Murphy K, Stanton C, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis*. 2015;26:26050. doi:10.3402/mehd.v26.26050

4.   Brussow H. Microbiota and the human nature: know thyself. *Environ Microbiol*. Jan 2015;17(1):10-5. doi:10.1111/1462-2920.12693

5.   Underhill DM, Iliev ID. The mycobiota: interactions between commensal fungi and the host immune system. *Nature reviews Immunology*. Jun 2014;14(6):405-16. doi:10.1038/nri3684

6.   Venter JC, Adams MD, Myers EW, et al. The Sequence of the Human Genome. *Science*. 2001;291(5507):1304-1351. doi:10.1126/science.1058040

7.   Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. 10.1038/nature06244. *Nature*. 10/18/print 2007;449(7164):804-810.

8.   Li J, Jia H, Cai X, et al. An integrated catalog of reference genes in the human gut microbiome. *Nature biotechnology*. Aug 2014;32(8):834-41. doi:10.1038/nbt.2942

9.   Huffnagle GB, Noverr MC. The emerging world of the fungal microbiome. *Trends in microbiology*. Jul 2013;21(7):334-41. doi:10.1016/j.tim.2013.04.002

10.   Richard ML, Sokol H. The gut mycobiota: insights into analysis, environmental interactions and role in gastrointestinal diseases. *Nature reviews Gastroenterology & hepatology*. Jun 2019;16(6):331-345. doi:10.1038/s41575-019-0121-2

11.   Tang J, Iliev ID, Brown J, Underhill DM, Funari VA. Mycobiome: Approaches to analysis of intestinal fungi. *Journal of immunological methods*. Jun 2015;421:112-21. doi:10.1016/j.jim.2015.04.004

12.   Suhr MJ, Hallen-Adams HE. The human gut mycobiome: pitfalls and potentials--a mycologist's perspective. *Mycologia*. Nov-Dec 2015;107(6):1057-73. doi:10.3852/15-147

13.   Noverr MC, Toews GB, Huffnagle GB. Production of prostaglandins and leukotrienes by pathogenic fungi. *Infect Immun*. Jan 2002;70(1):400-2. doi:10.1128/iai.70.1.400-402.2002

14.   Fiers WD, Gao IH, Iliev ID. Gut mycobiota under scrutiny: fungal symbionts or environmental transients? *Curr Opin Microbiol*. Aug 2019;50:79-86. doi:10.1016/j.mib.2019.09.010

15.   Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? *The Yale journal of biology and medicine*. Mar 2017;90(1):135-145.

16.   Anderson HW. *The yeast-like Fungi of the human intestinal tract*. 1917:49, 1 p., 1 l.

17.   Gouba N, Drancourt M. Digestive tract mycobiota: a source of infection. *Medecine et maladies infectieuses*. Jan-Feb 2015;45(1-2):9-16. doi:10.1016/j.medmal.2015.01.007

18.   Hamad I, Raoult D, Bittar F. Repertory of eukaryotes (eukaryome) in the human gastrointestinal tract: taxonomy and detection methods. *Parasite Immunol*. Jan 2016;38(1):12-36. doi:10.1111/pim.12284

19.   Nash AK, Auchtung TA, Wong MC, et al. The gut mycobiome of the Human Microbiome Project healthy cohort. *Microbiome*. Nov 25 2017;5(1):153. doi:10.1186/s40168-017-0373-4

20.   Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PloS one*. 2013;8doi:10.1371/journal.pone.0066019

21.   Gouba N, Raoult D, Drancourt M. Eukaryote culturomics of the gut reveals new species. *PloS one*. 2014;9(9):e106994. doi:10.1371/journal.pone.0106994

22.   Borges FM, de Paula TO, Sarmiento MRA, et al. Fungal Diversity of Human Gut Microbiota Among Eutrophic, Overweight, and Obese Individuals Based on Aerobic Culture-Dependent Approach. *Current microbiology*. Jun 2018;75(6):726-735. doi:10.1007/s00284-018-1438-8

23.   Hoffmann C, Dollive S, Grunberg S, et al. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PloS one*. 2013;8(6):e66019. doi:10.1371/journal.pone.0066019

24.   Cuskin F, Lowe EC, Temple MJ, et al. Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism. *Nature*. Jan 8 2015;517(7533):165-9. doi:10.1038/nature13995

25.   Cui L, Morris A, Ghedin E. The human mycobiome in health and disease. *Genome medicine*. 2013;5(7):63. doi:10.1186/gm467

26.   Rizzetto L, De Filippo C, Cavalieri D. Richness and diversity of mammalian fungal communities shape innate and adaptive immunity in health and disease. *Eur J Immunol*. Nov 2014;44(11):3166-81. doi:10.1002/eji.201344403

27.   Doron I, Leonardi I, Li XV, et al. Human gut mycobiota tune immunity via CARD9-dependent induction of anti-fungal IgG antibodies. *Cell*. Feb 18 2021;184(4):1017-1031 e14. doi:10.1016/j.cell.2021.01.016

28.   Backhed F, Roswall J, Peng Y, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell host & microbe*. May 13 2015;17(5):690-703. doi:10.1016/j.chom.2015.04.004

29.   Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature*. Jun 14 2012;486(7402):222-7. doi:10.1038/nature11053

30.   Magurran AE. *Measuring biological diversity*. Blackwell Pub.; 2004:viii, 256 p.

31.   Parfrey LW, Walters WA, Lauber CL, et al. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. *Frontiers in microbiology*. 2014;5:298. doi:10.3389/fmicb.2014.00298

32.   Fujimura KE, Sitarik AR, Havstad S, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. *Nature medicine*. Oct 2016;22(10):1187-1191. doi:10.1038/nm.4176

33.   Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Science translational medicine*. May 21 2014;6(237):237ra65. doi:10.1126/scitranslmed.3008599

34.   Jimenez E, Fernandez L, Marin ML, et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Current microbiology*. Oct 2005;51(4):270-4. doi:10.1007/s00284-005-0020-3

35.   Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Scientific reports*. Mar 22 2016;6:23129. doi:10.1038/srep23129

36.   Koenig JE, Spor A, Scalfone N, et al. Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America*. Mar 15 2011;108 Suppl 1:4578-85. doi:10.1073/pnas.1000081107

37.   Ward TL, Dominguez-Bello MG, Heisel T, Al-Ghalith G, Knights D, Gale CA. Development of the Human Mycobiome over the First Month of Life and across Body Sites. *mSystems*. May-Jun 2018;3(3)doi:10.1128/mSystems.00140-17

38.   LaTuga MS, Ellis JC, Cotton CM, et al. Beyond bacteria: a study of the enteric microbial consortium in extremely low birth weight infants. *PloS one*. 2011;6(12):e27858. doi:10.1371/journal.pone.0027858

39.   James SA, Phillips S, Telatin A, et al. Preterm Infants Harbour a Rapidly Changing Mycobiota That Includes Candida Pathobionts. *J Fungi (Basel)*. Nov 9 2020;6(4)doi:10.3390/jof6040273

40.   Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU, Ryan RM. Vertical and horizontal transmission of Candida albicans in very low birth weight infants using DNA fingerprinting techniques. *Pediatr Infect Dis J*. Mar 2008;27(3):231-5. doi:10.1097/INF.0b013e31815bb69d

41.   Ward TL, Knights D, Gale CA. Infant fungal communities: current knowledge and research opportunities. *BMC Med*. Feb 13 2017;15(1):30. doi:10.1186/s12916-017-0802-z

42.   Boix-Amoros A, Martinez-Costa C, Querol A, Collado MC, Mira A. Multiple Approaches Detect the Presence of Fungi in Human Breastmilk Samples from Healthy Mothers. *Scientific reports*. Oct 12 2017;7(1):13016. doi:10.1038/s41598-017-13270-x

43.   Simpson MR, Brede G, Johansen J, et al. Human Breast Milk miRNA, Maternal Probiotic Supplementation and Atopic Dermatitis in Offspring. *PloS one*. 2015;10(12):e0143496. doi:10.1371/journal.pone.0143496

44.   Jost T, Lacroix C, Braegger C, Chassard C. Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. *Nutr Rev*. Jul 2015;73(7):426-37. doi:10.1093/nutrit/nuu016

45.   Strati F, Paola M, Stefanini I, Albanese D, Rizzetto L, Lionetti P. Age and gender affect the composition of fungal population of the human gastrointestinal tract. *Frontiers in microbiology*. 2016;7doi:10.3389/fmicb.2016.01227

46.   Bergstrom A, Skov TH, Bahl MI, et al. Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. *Applied and environmental microbiology*. May 2014;80(9):2889-900. doi:10.1128/AEM.00342-14

47.   Avershina E, Lundgard K, Sekelja M, et al. Transition from infant- to adult-like gut microbiota. *Environ Microbiol*. Feb 23 2016;doi:10.1111/1462-2920.13248

48.   Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clinical microbiology reviews*. Oct 2007;20(4):593-621. doi:10.1128/cmr.00008-07

49.   Byrne CS, Chambers ES, Morrison DJ, Frost G. The role of short chain fatty acids in appetite regulation and energy homeostasis. *Int J Obes (Lond)*. May 14 2015;doi:10.1038/ijo.2015.84

50.   Avershina E, Storro O, Oien T, et al. Bifidobacterial succession and correlation networks in a large unselected cohort of mothers and their children. *Applied and environmental microbiology*. Jan 2013;79(2):497-507. doi:10.1128/AEM.02359-12

51.   Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. *Nature*. May 12 2011;473(7346):174-80. doi:10.1038/nature09944

52.   Gorvitovskaia A, Holmes SP, Huse SM. Interpreting Prevotella and Bacteroides as biomarkers of diet and lifestyle. journal article. *Microbiome*. April 12 2016;4(1):15. doi:10.1186/s40168-016-0160-7

53.   Jakobsson HE, Abrahamsson TR, Jenmalm MC, et al. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut*. Apr 2014;63(4):559-66. doi:10.1136/gutjnl-2012-303249

54.   Fernandes KE, Carter DA. The Antifungal Activity of Lactoferrin and Its Derived Peptides: Mechanisms of Action and Synergy with Drugs against Fungal Pathogens. *Frontiers in microbiology*. 2017;8:2. doi:10.3389/fmicb.2017.00002

55.   Gifford JL, Hunter HN, Vogel HJ. Lactoferricin. journal article. *Cellular and Molecular Life Sciences*. November 02 2005;62(22):2588. doi:10.1007/s00018-005-5373-z

56.   Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. *Frontiers in immunology*. 2014;5:427. doi:10.3389/fimmu.2014.00427

57.   Mukherjee PK, Sendid B, Hoarau G, Colombel JF, Poulain D, Ghannoum MA. Mycobiota in gastrointestinal diseases. *Nature reviews Gastroenterology & hepatology*. Nov 11 2014;doi:10.1038/nrgastro.2014.188

58.   Gammelsrud KW, Sandven P, Hoiby EA, Sandvik L, Brandtzaeg P, Gaustad P. Colonization by Candida in children with cancer, children with cystic fibrosis, and healthy controls. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. Dec 2011;17(12):1875-81. doi:10.1111/j.1469-0691.2011.03528.x

59.   Lankelma JM, Nieuwdorp M, de Vos WM, Wiersinga WJ. The gut microbiota in internal medicine: implications for health and disease. *The Netherlands journal of medicine*. Feb 2015;73(2):61-8.

60.   Franzosa EA, Sirota-Madi A, Avila-Pacheco J, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nature microbiology*. Feb 2019;4(2):293-305. doi:10.1038/s41564-018-0306-4

61.   Walker AW, Lawley TD. Therapeutic modulation of intestinal dysbiosis. *Pharmacol Res*. Mar 2013;69(1):75-86. doi:10.1016/j.phrs.2012.09.008

62.   Mar Rodriguez M, Perez D, Javier Chaves F, et al. Obesity changes the human gut mycobiome. *Scientific reports*. 2015;5:14600. doi:10.1038/srep14600

63.   Wensveen FM, Valentić S, Šestan M, Turk Wensveen T, Polić B. The "Big Bang" in obese fat: Events initiating obesity-induced adipose tissue inflammation. *European Journal of Immunology*. 2015;45(9):2446-2456. doi:10.1002/eji.201545502

64.   Sokol H, Leducq V, Aschard H, et al. Fungal microbiota dysbiosis in IBD. *Gut*. Feb 3 2016;doi:10.1136/gutjnl-2015-310746

65.   Ott SJ, Kuhbacher T, Musfeldt M, et al. Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scand J Gastroenterol*. 2008;43(7):831-41. doi:10.1080/00365520801935434

66.   van Tilburg Bernardes E, Gutierrez MW, Arrieta M-C. The Fungal Microbiome and Asthma. Review. *Frontiers in Cellular and Infection Microbiology*. 2020-November-26 2020;10(736)doi:10.3389/fcimb.2020.583418

67.   Honkanen J, Vuorela A, Muthas D, et al. Fungal Dysbiosis and Intestinal Inflammation in Children With Beta-Cell Autoimmunity. Original Research. *Frontiers in immunology*. 2020-March-19 2020;11(468)doi:10.3389/fimmu.2020.00468

68.   Kliegman RL. *Nelson Textbook of Pediatrics. Growth, development and behavior* vol 1. Elsevier; 2016.

69.   Murray PG, Clayton PE. Endocrine Control of Growth. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*. 2013;163(2):76-85. doi:10.1002/ajmg.c.31357

70.   Karlberg J. A Biologically-Oriented Mathematical Model (ICP) for Human Growth. 1989;78(s350):70-94. doi:doi:10.1111/j.1651-2227.1989.tb11199.x

71.   Koletzko B, Chourdakis M, Grote V, et al. Regulation of early human growth: impact on long-term health. *Annals of nutrition & metabolism*. 2014;65(2-3):101-9. doi:10.1159/000365873

72.   Juliusson PB, Roelants M, Nordal E, et al. Growth references for 0-19 year-old Norwegian children for length/height, weight, body mass index and head circumference. *Ann Hum Biol*. May 2013;40(3):220-7. doi:10.3109/03014460.2012.759276

73.   Schwarzer M. Gut microbiota: puppeteer of the host juvenile growth. *Curr Opin Clin Nutr Metab Care*. May 2018;21(3):179-183. doi:10.1097/mco.0000000000000463

74.   Robertson RC, Manges AR, Finlay BB, Prendergast AJ. The Human Microbiome and Child Growth - First 1000 Days and Beyond. *Trends in microbiology*. Feb 2019;27(2):131-147. doi:10.1016/j.tim.2018.09.008

75.   Cromwell G. WHY AND HOW ANTIBIOTICS ARE USED IN SWINE PRODUCTION. *Animal Biotechnology*. 2002/07/01 2002;13(1):7-27. doi:10.1081/ABIO-120005767

76.   Chaucheyras-Durand F, Walker ND, Bach A. Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. *Animal Feed Science and Technology*. 2008/08/14/ 2008;145(1):5-26. doi:http://dx.doi.org/10.1016/j.anifeedsci.2007.04.019

77.   Yan J, Herzog JW, Tsang K, et al. Gut microbiota induce IGF-1 and promote bone formation and growth. *Proceedings of the National Academy of Sciences of the United States of America*. Nov 22 2016;113(47):E7554-E7563. doi:10.1073/pnas.1607235113

78.   Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. Jun 19 2014;510(7505):417-21. doi:10.1038/nature13421

79.   Korpela K, Zijlmans MA, Kuitunen M, et al. Childhood BMI in relation to microbiota in infancy and lifetime antibiotic use. *Microbiome*. Mar 03 2017;5(1):26. doi:10.1186/s40168-017-0245-y

80.   Vatanen T, Plichta DR, Somani J, et al. Genomic variation and strain-specific functional adaptation in the human gut microbiome during early life. *Nature microbiology*. Mar 2019;4(3):470-479. doi:10.1038/s41564-018-0321-5

81.   Saari A, Virta LJ, Sankilampi U, Dunkel L, Saxen H. Antibiotic exposure in infancy and risk of being overweight in the first 24 months of life. *Pediatrics*. Apr 2015;135(4):617-26. doi:10.1542/peds.2014-3407

82.   Yang YJ, Sheu BS, Yang HB, Lu CC, Chuang CC. Eradication of Helicobacter pylori increases childhood growth and serum acylated ghrelin levels. *World J Gastroenterol*. Jun 7 2012;18(21):2674-81. doi:10.3748/wjg.v18.i21.2674

83.   Costalos C, Skouteri V, Gounaris A, et al. Enteral feeding of premature infants with Saccharomyces boulardii. *Early human development*. Nov 2003;74(2):89-96.

84.   Xu L, Wang Y, Wang Y, et al. A double-blinded randomized trial on growth and feeding tolerance with Saccharomyces boulardii CNCM I-745 in formula-fed preterm infants. *Jornal de pediatria*. May-Jun 2016;92(3):296-301. doi:10.1016/j.jped.2015.08.013

85.   Cole TJ, Lobstein T. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. *Pediatric obesity*. Aug 2012;7(4):284-94. doi:10.1111/j.2047-6310.2012.00064.x

86.   Juliusson PB, Hjelmesaeth J, Bjerknes R, Roelants M. New curves for body mass index among children and adolescents. *Tidsskr Nor Laegeforen*. Oct 3 2017;137(18)Nye kurver for kroppsmasseindeks blant barn og unge. doi:10.4045/tidsskr.17.0570

87.   Giles LC, Whitrow MJ, Davies MJ, Davies CE, Rumbold AR, Moore VM. Growth trajectories in early childhood, their relationship with antenatal and postnatal factors, and development of obesity by age 9 years: results from an Australian birth cohort study. *Int J Obes (Lond)*. Jul 2015;39(7):1049-56. doi:10.1038/ijo.2015.42

88.   Lobstein T, Jackson-Leach R, Moodie ML, et al. Child and adolescent obesity: part of a bigger picture. *Lancet*. Jun 20 2015;385(9986):2510-20. doi:10.1016/s0140-6736(14)61746-3

89.   WHO. Obesity and Overweight Fact Sheet. Accessed 28.02.2021, 2021. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight

90.   Wijnhoven TM, van Raaij JM, Spinelli A, et al. WHO European Childhood Obesity Surveillance Initiative: body mass index and level of overweight among 6-9-year-old children from school year 2007/2008 to school year 2009/2010. *BMC public health*. 2014;14:806. doi:10.1186/1471-2458-14-806

91.   Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. Dec 21 2006;444(7122):1027-31. doi:10.1038/nature05414

92.   Ridaura VK, Faith JJ, Rey FE, et al. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. *Science*. Sep 6 2013;341(6150):1079-U49. doi:DOI 10.1126/science.1241214

93.   Luoto R, Collado MC, Salminen S, Isolauri E. Reshaping the gut microbiota at an early age: functional impact on obesity risk? *Annals of nutrition & metabolism*. 2013;63 Suppl 2:17-26. doi:10.1159/000354896

94.   Sze MA, Schloss PD. Looking for a Signal in the Noise: Revisiting Obesity and the Microbiome. *mBio*. Aug 23 2016;7(4)doi:10.1128/mBio.01018-16

95.   Turnbaugh PJ, Backhed F, Fulton L, Gordon JI. Marked alterations in the distal gut microbiome linked to diet-induced obesity. *Cell host & microbe*. 2008;3(4):213-223. doi:10.1016/j.chom.2008.02.015

96.   Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature*. Jan 22 2009;457(7228):480-4. doi:10.1038/nature07540

97.   Angelakis E, Armougom F, Million M, Raoult D. The relationship between gut microbiota and weight gain in humans. *Future microbiology*. Jan 2012;7(1):91-109. doi:10.2217/fmb.11.142

98.   Dogra S, Sakwinska O, Soh SE, et al. Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. *mBio*. Feb 03 2015;6(1)doi:10.1128/mBio.02419-14

99.   Forbes JD, Azad MB, Vehling L, et al. Association of exposure to formula in the hospital and subsequent infant feeding practices with gut microbiota and risk of overweight in the first year of life. *JAMA pediatrics*. 2018;172(7):e181161. doi:10.1001/jamapediatrics.2018.1161

100.   Stanislawski MA, Dabelea D, Wagner BD, et al. Gut Microbiota in the First 2 Years of Life and the Association with Body Mass Index at Age 12 in a Norwegian Birth Cohort. *mBio*. Oct 23 2018;9(5)doi:10.1128/mBio.01751-18

101.   Tun HM, Bridgman SL, Chari R, et al. Roles of Birth Mode and Infant Gut Microbiota in Intergenerational Transmission of Overweight and Obesity From Mother to Offspring. *JAMA pediatrics*. Apr 1 2018;172(4):368-377. doi:10.1001/jamapediatrics.2017.5535

102.   Yang L, Fu J, Zhou Y. Research Progress in Atopic March. *Frontiers in immunology*. 2020;11:1907. doi:10.3389/fimmu.2020.01907

103.   Asher MI, Montefort S, Bjorksten B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet*. Aug 26 2006;368(9537):733-43. doi:10.1016/S0140-6736(06)69283-0

104.   Mallol J, Crane J, von Mutius E, Odhiambo J, Keil U, Stewart A. The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three: a global synthesis. *Allergologia et immunopathologia*. Mar-Apr 2013;41(2):73-85. doi:10.1016/j.aller.2012.03.001

105.   Bjerre RD, Bandier J, Skov L, Engstrand L, Johansen JD. The role of the skin microbiome in atopic dermatitis: a systematic review. *British Journal of Dermatology*. 2017;177(5):1272-1278. doi:10.1111/bjd.15390

106.   Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? *Lancet*. Sep 12 2015;386(9998):1075-85. doi:10.1016/s0140-6736(15)00156-7

107.   Ege MJ, Mayer M, Normand AC, et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med*. Feb 24 2011;364(8):701-9. doi:10.1056/NEJMoa1007302

108.   Fujimura KE, Lynch SV. Microbiota in allergy and asthma and the emerging relationship with the gut microbiome. *Cell host & microbe*. May 13 2015;17(5):592-602. doi:10.1016/j.chom.2015.04.007

109.   Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. Jun 2014;44(6):842-50. doi:10.1111/cea.12253

110.   Bisgaard H, Li N, Bonnelykke K, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol*. Sep 2011;128(3):646-52 e1-5. doi:10.1016/j.jaci.2011.04.060

111.   Arrieta MC, Arevalo A, Stiemsma L, et al. Associations between infant fungal and bacterial dysbiosis and childhood atopic wheeze in a nonindustrialized setting. *J Allergy Clin Immunol*. Aug 2018;142(2):424-434 e10. doi:10.1016/j.jaci.2017.08.041

112.   Simonyte Sjodin K, Hammarstrom ML, Ryden P, et al. Temporal and long-term gut microbiota variation in allergic disease: A prospective study from infancy to school age. *Allergy*. Jan 2019;74(1):176-185. doi:10.1111/all.13485

113.   Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol*. Jan 2017;19(1):29-41. doi:10.1111/1462-2920.13589

114.   Roduit C, Frei R, Ferstl R, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. *Allergy*. Apr 2019;74(4):799-809. doi:10.1111/all.13660

115.   Skalski JH, Limon JJ, Sharma P, et al. Expansion of commensal fungus Wallemia mellicola in the gastrointestinal mycobiota enhances the severity of allergic airway disease in mice. *PLoS pathogens*. Sep 2018;14(9):e1007260. doi:10.1371/journal.ppat.1007260

116.   Keller NP. Fungal secondary metabolism: regulation, function and drug discovery. *Nature reviews Microbiology*. Mar 2019;17(3):167-180. doi:10.1038/s41579-018-0121-1

117.   Tan TG, Lim YS, Tan A, Leong R, Pavelka N. Fungal Symbionts Produce Prostaglandin E2 to Promote Their Intestinal Colonization. *Front Cell Infect Microbiol*. 2019;9:359. doi:10.3389/fcimb.2019.00359

118.   Matsuoka T, Hirata M, Tanaka H, et al. Prostaglandin D2 as a mediator of allergic asthma. *Science*. Mar 17 2000;287(5460):2013-7. doi:10.1126/science.287.5460.2013

119.   Kim YG, Udayanga KG, Totsuka N, Weinberg JB, Nunez G, Shibuya A. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE(2). *Cell host & microbe*. Jan 15 2014;15(1):95-102. doi:10.1016/j.chom.2013.12.010

120.   Grozer Z, Toth A, Toth R, et al. Candida parapsilosis produces prostaglandins from exogenous arachidonic acid and OLE2 is not required for their synthesis. *Virulence*. 2015;6(1):85-92. doi:10.4161/21505594.2014.988097

121.   Benard A, Desreumeaux P, Huglo D, Hoorelbeke A, Tonnel AB, Wallaert B. Increased intestinal permeability in bronchial asthma. *J Allergy Clin Immunol*. Jun 1996;97(6):1173-8. doi:10.1016/s0091-6749(96)70181-1

122.   Hijazi Z, Molla AM, Al-Habashi H, Muawad WM, Molla AM, Sharma PN. Intestinal permeability is increased in bronchial asthma. *Arch Dis Child*. Mar 2004;89(3):227-9. doi:10.1136/adc.2003.027680

123.   Maruvada P, Leone V, Kaplan LM, Chang EB. The Human Microbiome and Obesity: Moving beyond Associations. *Cell host & microbe*. Nov 8 2017;22(5):589-599. doi:10.1016/j.chom.2017.10.005

124.   Bischoff SC, Barbara G, Buurman W, et al. Intestinal permeability – a new target for disease prevention and therapy. *BMC gastroenterology*. 2014/11/18 2014;14(1):189. doi:10.1186/s12876-014-0189-7

125.   Guida S, Venema K. Gut microbiota and obesity: Involvement of the adipose tissue. *Journal of Functional Foods*. 4// 2015;14(0):407-423. doi:http://dx.doi.org/10.1016/j.jff.2015.02.014

126.   Kheirandish-Gozal L, Peris E, Wang Y, et al. Lipopolysaccharide-Binding Protein Plasma Levels in Children: Effects of Obstructive Sleep Apnea and Obesity. *The Journal of Clinical Endocrinology & Metabolism*. 2014;99(2):656-663. doi:10.1210/jc.2013-3327

127.   Bischoff SC, Barbara G, Buurman W, et al. Intestinal permeability--a new target for disease prevention and therapy. *BMC gastroenterology*. Nov 18 2014;14:189. doi:10.1186/s12876-014-0189-7

128.   Guthmann F, Börchers T, Wolfrum C, Wustrack T, Bartholomäus S, Spener F. Plasma concentration of intestinal- and liver-FABP in neonates suffering from necrotizing enterocolitis and in healthy preterm neonates. *Mol Cell Biochem*. 2002;239(1-2):227-234.

129.   Dotterud CK, Storro O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. *The British journal of dermatology*. Sep 2010;163(3):616-23. doi:10.1111/j.1365-2133.2010.09889.x

130.   Dotterud CK, Avershina E, Sekelja M, et al. Does Maternal Perinatal Probiotic Supplementation Alter the Intestinal Microbiota of Mother and Child? *J Pediatr Gastroenterol Nutr*. Aug 2015;61(2):200-7. doi:10.1097/mpg.0000000000000781

131.   Williams HC, Burney PG, Pembroke AC, Hay RJ. Validation of the U.K. diagnostic criteria for atopic dermatitis in a population setting. U.K. Diagnostic Criteria for Atopic Dermatitis Working Party. *The British journal of dermatology*. Jul 1996;135(1):12-7.

132.   Williams HC, Burney PG, Pembroke AC, Hay RJ. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. *The British journal of dermatology*. Sep 1994;131(3):406-16. doi:10.1111/j.1365-2133.1994.tb08532.x

133.   Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnology and bioengineering*. Mar 20 2005;89(6):670-9. doi:10.1002/bit.20347

134.   Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Brief Communication. *Nat Meth*. 10//print 2013;10(10):996-998. doi:10.1038/nmeth.2604

http://www.nature.com/nmeth/journal/v10/n10/abs/nmeth.2604.html#supplementary-information

135.   Hibbett DS, Taylor JW. Fungal systematics: is a new age of enlightenment at hand? *Nature reviews Microbiology*. Feb 2013;11(2):129-33. doi:10.1038/nrmicro2963

136.   Lin H, Peddada SD. Analysis of microbial compositions: a review of normalization and differential abundance analysis. *NPJ Biofilms Microbiomes*. Dec 2 2020;6(1):60. doi:10.1038/s41522-020-00160-w

137.   Mandal S, Van Treuren W, White RA, Eggesbo M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis*. 2015;26:27663. doi:10.3402/mehd.v26.27663

138.   Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biology*. 2011/06/24 2011;12(6):R60. doi:10.1186/gb-2011-12-6-r60

139.   Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* 13.05.2001 2001;4(1)(4)

140.   McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*. 2013;8(4):e61217. doi:10.1371/journal.pone.0061217

141.   Juliusson PB, Eide GE, Roelants M, Waaler PE, Hauspie R, Bjerknes R. Overweight and obesity in Norwegian children: prevalence and socio-demographic risk factors. *Acta Paediatr*. Jun 2010;99(6):900-5. doi:10.1111/j.1651-2227.2010.01730.x

142.   Gong T, Lundholm C, Rejno G, Mood C, Langstrom N, Almqvist C. Parental socioeconomic status, childhood asthma and medication use--a population-based study. *PloS one*. 2014;9(9):e106579. doi:10.1371/journal.pone.0106579

143.   Backe B, Heggestad T, Lie T. [The epidemic of Caesarean section: has it reached Norway?]. *Tidsskr Nor Laegeforen*. May 29 2003;123(11):1522-4. Har keisersnittsepidemien nadd Norge?

144.   Fiedorova K, Radvansky M, Nemcova E, et al. The Impact of DNA Extraction Methods on Stool Bacterial and Fungal Microbiota Community Recovery. *Frontiers in microbiology*. 2019;10:821. doi:10.3389/fmicb.2019.00821

145.   Lavrinienko A, Jernfors T, Koskimaki JJ, Pirttila AM, Watts PC. Does Intraspecific Variation in rDNA Copy Number Affect Analysis of Microbial Communities? *Trends in microbiology*. Jan 2021;29(1):19-27. doi:10.1016/j.tim.2020.05.019

146.   Edwards JE, Forster RJ, Callaghan TM, et al. PCR and Omics Based Techniques to Study the Diversity, Ecology and Biology of Anaerobic Fungi: Insights, Challenges and Opportunities. *Frontiers in microbiology*. 2017;8:1657. doi:10.3389/fmicb.2017.01657

147.   Taylor DL, Walters WA, Lennon NJ, et al. Accurate Estimation of Fungal Diversity and Abundance through Improved Lineage-Specific Primers Optimized for Illumina Amplicon Sequencing. *Applied and environmental microbiology*. Dec 15 2016;82(24):7217-7226. doi:10.1128/aem.02576-16

148.   Lucking R, Aime MC, Robbertse B, et al. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? *IMA Fungus*. 2020;11:14. doi:10.1186/s43008-020-00033-z

149.   Shoari N, Dube JS. Toward improved analysis of concentration data: Embracing nondetects. *Environ Toxicol Chem*. Mar 2018;37(3):643-656. doi:10.1002/etc.4046

150.   Strati F, Di Paola M, Stefanini I, et al. Age and Gender Affect the Composition of Fungal Population of the Human Gastrointestinal Tract. *Frontiers in microbiology*. 2016;7:1227. doi:10.3389/fmicb.2016.01227

151.   Banjara N, Suhr MJ, Hallen-Adams HE. Diversity of yeast and mold species from a variety of cheese types. *Current microbiology*. Jun 2015;70(6):792-800. doi:10.1007/s00284-015-0790-1

152.   Breuer U, Harms H. Debaryomyces hansenii--an extremophilic yeast with biotechnological potential. *Yeast (Chichester, England)*. Apr 30 2006;23(6):415-37. doi:10.1002/yea.1374

153.   Vaughan-Martini A, Martini A. Chapter 61 - Saccharomyces Meyen ex Reess (1870) A2 - Kurtzman, Cletus P. In: Fell JW, Boekhout T, eds. *The Yeasts (Fifth Edition)*. Elsevier; 2011:733-746.

154.   Jiang Z, Wei S, Wang Z, et al. Effects of different forms of yeast Saccharomyces cerevisiae on growth performance, intestinal development, and systemic immunity in early-weaned piglets. journal article. *Journal of Animal Science and Biotechnology*. November 14 2015;6(1):47. doi:10.1186/s40104-015-0046-8

155.   Nasiri AH, Towhidi A, Shakeri M, Zhandi M, Dehghan-Banadaky M, Colazo MG. Effects of live yeast dietary supplementation on hormonal profile, ovarian follicular dynamics, and reproductive performance in dairy cows exposed to high ambient temperature. *Theriogenology*. Dec 2018;122:41-46. doi:10.1016/j.theriogenology.2018.08.013

156.   Hanchi H, Mottawea W, Sebei K, Hammami R. The Genus Enterococcus: Between Probiotic Potential and Safety Concerns-An Update. *Frontiers in microbiology*. 2018;9:1791. doi:10.3389/fmicb.2018.01791

157.   Cruz MR, Graham CE, Gagliano BC, Lorenz MC, Garsin DA. Enterococcus faecalis inhibits hyphal morphogenesis and virulence of Candida albicans. *Infection and immunity*. 2013;81(1):189-200. doi:10.1128/IAI.00914-12

158.   Smith PM, Howitt MR, Panikov N, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science*. Aug 2 2013;341(6145):569-73. doi:10.1126/science.1241165

PAPER I

Microbiome

Early gut mycobiota and mother-offspring transfer

CrossMark

Kasper Schei[1*] , Ekaterina Avershina[2], Torbjørn Øien[3], Knut Rudi[2], Turid Follestad[3], Saideh Salamati[4†] and Rønnaug Astri Ødegård[1,4†]

Abstract

Background: The fungi in the gastrointestinal tract, the *gut mycobiota*, are now recognised as a significant part of the gut microbiota, and they may be important to human health. In contrast to the adult gut mycobiota, the establishment of the early gut mycobiota has never been described, and there is little knowledge about the fungal transfer from mother to offspring.

Methods: In a prospective cohort, we followed 298 pairs of healthy mothers and offspring from 36 weeks of gestation until 2 years of age (1516 samples) and explored the gut mycobiota in maternal and offspring samples. Half of the pregnant mothers were randomised into drinking probiotic milk during and after pregnancy. The probiotic bacteria included *Lactobacillus rhamnosus* GG (LGG), *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* La-5. We quantified the fungal abundance of all the samples using qPCR of the fungal internal transcribed spacer (ITS)1 segment, and we sequenced the 18S rRNA gene ITS1 region of 90 high-quantity samples using the MiSeq platform (Illumina).

Results: The gut mycobiota was detected in most of the mothers and the majority of the offspring. The offspring showed increased odds of having detectable faecal fungal DNA if the mother had detectable fungal DNA as well (OR = 1.54, $p = 0.04$). The fungal alpha diversity in the offspring gut increased from its lowest at 10 days after birth, which was the earliest sampling point. The fungal diversity and fungal species showed a succession towards the maternal mycobiota as the child aged, with *Debaryomyces hansenii* being the most abundant species during breast-feeding and *Saccharomyces cerevisiae* as the most abundant after weaning. Probiotic consumption increased the gut mycobiota abundance in pregnant mothers ($p = 0.01$).

Conclusion: This study provides the first insight into the early fungal establishment and the succession of fungal species in the gut mycobiota. The results support the idea that the fungal host phenotype is transferred from mother to offspring.

Trial registration: Clinicaltrials.gov NCT00159523

Keywords: Gut microbiota, Mycobiota, Fungi, Newborn, Infant, Infant health, Probiotics

Background

The fungi that populate the gastrointestinal tract (gut mycobiota) have recently been recognised as a substantial part of the gut microbiota and can be important for human health [1]. The adult gut mycobiota, which probably comprise approximately 13% of the gut microbial volume, consists of a species selection from approximately 140 different fungal genera [2, 3], with the most abundant ones being *Candida*, *Saccharomyces* and *Cladosporium* spp. [1].

Gut microbiota has been extensively studied over the last two decades. The Human Microbiome Project, or HMP [4], and the Metagenomics of the Human Intestinal Tract (MetaHIT) [5] have contributed greatly to our knowledge of the human microbial community structure, although no comprehensive and uniformly processed database can represent the human gut

* Correspondence: kasperschei@gmail.com
†Equal contributors
[1]Department of Laboratory Medicine, Children's and Women's Health, Faculty of Medicine and Health Sciences, NTNU – Norwegian University of Science and Technology, Postboks 8905, 7491 Trondheim, Norway
Full list of author information is available at the end of the article

microbiome [6]. Fungal communities are far less studied. However, a positive association between the archaeon *Methanobrevibacter* and *Candida* with relative abundance differences in *Prevotella* might exist. Similarly, the relative abundance differences in *Bacteriodes* are associated with the archaeon *Nitrososphaera*, and they are negatively correlated with gut fungi [7].

The human gut mycobiota confers several physiological effects to the human body. These fungi consume nutrients and may facilitate nutrient extraction and assist in digestion through enzyme and vitamin production [7, 8]. The gut mycobiota is also essential as a form of antigen exposures to train the immune system and its responses. Through activating the fungus-specific pathogen-recognition receptors (PRRs) and adjacent mechanisms, defences against harmful pathogens and likewise a tolerance towards helpful commensals are formed [1, 9, 10].

However, for some humans, fungi can have unfavourable impacts, and the term *fungal dysbiosis* describes a state of unbalanced mycobiota associated with disease [11]. This phenomenon is most extensively studied in immunocompromised patients who regularly contract opportunistic commensal fungal infections [1] and in patients with obesity and inflammatory bowel disease (IBD) [12–15]. Obesity and metabolic disorders have been associated with the increased presence and abundance of *Saccharomycetes* spp., *Dipodascaceae* spp. and *Tremellomycetes* spp. [12]. Obesity and metabolic syndrome are pro-inflammatory states, and *Tremellomycetes* spp. are associated with higher inflammation levels. Accordingly, a lower abundance of the ascomycotic *Eurotiomycetes* spp. and particularly less of the zygomycotic *Mucor* spp. might actually protect against an unhealthy metabolic profile [12, 13]. By contrast, IBD patients host lower concentrations of *Saccharomyces cerevisiae* and more *Candida albicans* than healthy subjects do. In this disorder, an increased Basidiomycota-Ascomycota ratio is also observed, along with increased fungal diversity and richness [14, 15]. Interestingly, many IBD and obesity patients also produce anti-*S. cerevisiae* antibodies [1, 16, 17], although they host different abundance levels of these gut fungi. Taken together, these findings imply that the gut mycobiota could be aetiologically important in human diseases.

An understanding of the role of the gut mycobiota is emerging in relation to physiological as well as pathophysiological processes, but there is little knowledge of how the mycobiome is shaped from early life. High abundances of the genera *Penicillium*, *Aspergillus* and *Candida* (species-non-specific) were found in 10 Italian children who were each sampled once from 0 to 2 years of age [18]. Additionally, some common species have been studied. *C. albicans* and *Malassezia* spp. are partly transferred vertically from mothers to their offspring [19–21], supporting the theory that fungi colonise the neonatal gut through the birth canal. Culturing has also shown that the *Candida* spp. prevalence in neonates is 23%, and it more than doubles to 50% within 4 months [20]. The paradigm of the sterile intrauterine environment is now shifting, and several studies confirm the prenatal presence of commensal bacterial taxa in the placenta and amniotic fluid and the possible transmission of these bacteria to the foetus long before birth [22–26]. Corresponding knowledge on fungi is scarce. Early life microbiomes can also be affected by maternal exposure during pregnancy, ranging from high-fat diets to probiotics [27, 28]. Generally, we know that the mycobiome may be shaped by bacteria-fungus interactions [29, 30], as well as by the diet, by probiotics and by antibiotic administration (as shown in mice) [7, 31–33]. However, to our knowledge, the settling of early mycobiota with respect to the quantity, diversity and association with the maternal mycobiome has not been described before nor has the probiotic impact on the mycobiota been investigated.

In this prospective cohort, we describe the gut mycobiota in 298 pairs of healthy pregnant women and offspring from birth to 2 years of age. We report major shifts in the fungal abundance and diversity within these populations, and it supports the idea of a succession of mycobiotic hosting from mother to child and over the first 2 years of life.

Methods
Material
We selected 298 mother-offspring pairs with at least one pair of faecal samples from mothers and offspring who participated in the Probiotics in the Prevention of Allergy among Children in Trondheim study (ProPACT) (Table 1). The ProPACT study is a population-based, randomised, placebo-controlled and double-blinded trial on probiotics from Trondheim, Norway, and it has been described in detail elsewhere [34, 35]. Briefly, the pregnant women who attended the regular Norwegian Antenatal Care Programme were asked to participate by completing questionnaires on their health and risk factors and by collecting faecal samples from themselves and their offspring. The health questionnaire details, including antibiotic administration, were collected at 36 weeks of gestation and 6 weeks, 1 year and 2 years after birth. Although we do not have details on the antibiotic administration in the offspring before 10 days of life, no offspring that were delivered by caesarean section received antibiotics during labour, but one developed septicaemia afterwards and was treated accordingly. The probiotic milk administration was double-blinded and randomly provided to one half of

Table 1 Maternal and offspring characteristics

Maternal age at delivery (years (SD))	29.6 (± 3.9)
Caesarean sections	12.8%
Probiotic users	49.4%
Antibiotic therapy during pregnancy	7.2%
Male offspring	46.4%
Gestational age (weeks (SD))	40.4 (± 1.5)
Birth weight (kg (SD))	3.6 (± 0.4)
Birth length (cm (SD))	50.7 (± 3.3)
Breast-fed at 3 months	97.1%
Formula-fed at 3 months	6.5%
Proportion of children receiving antibiotic treatment within	
- 6 weeks	6.3%
- 1 year	17.2%
- 2 years	44.4%

the pregnant population from 36 weeks of gestation until 3 months after birth. The probiotic milk contained 5×10^{10} colony-forming units (CFUs) of *Lactobacillus rhamnosus* GG (LGG), 5×10^{10} CFUs of *Bifidobacterium animalis* subsp. *lactis* Bb-12 and 5×10^{10} CFUs of *Lactobacillus acidophilus* La-5 per day. The remaining half received placebos in the form of heat-treated fermented skimmed milk with no probiotic bacteria.

A total of 1516 faecal samples were collected (Table 2). Maternal samples were collected at 35–38 gestational weeks of pregnancy and 3 months postpartum. Offspring faeces were obtained at 10 days, 3 months, 1 year and 2 years, and they were sampled from the diapers. Faecal samples were stored in a Cary-Blair transport medium, immediately frozen to − 18 °C at home, and collected and held in a frozen state until their permanent storage at − 80 °C before further analyses.

Quantification

We used a protocol for bacterial DNA extraction that involved mechanical and chemical cell lysis. The stool samples were homogenised by bead beating with acid-washed glass beads (Sigma). We isolated the DNA with an LGC mag nucleic extraction maxi kit (LGC Genomics, Middlesex, UK) together with a KingFisher FLEX magnetic particle processor (ThermoScientific, Waltham, MA) according to the manufacturer's recommendations,

including a negative control as contamination control. Fungal internal transcribed spacer 1 (ITS1) amplicons were constructed using the primer pairs ITS1F (CTTG GTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTC TTCATCGATGC), according to Tang et al. [36]. The fungal ITS quantities in 1516 samples were assessed with a Light-Cycler qPCR (Roche) of 50 cycles, using thermocycles comprising 95 °C in 15 min, then (95 °C in 30 s, 56 °C in 30 s, 72 °C for 45 s) × 50. For each qPCR plate, we included positive and negative controls (*S. cerevisiae* and sterile water, respectively). The qPCR cycle threshold (CT) value cut-off for fungal detection was set to either within the value of the negative control or to 45 cycles, because DNA quantification beyond 45 cycles can produce misleading results.

Sequencing of the 18S rRNA gene ITS1 region

Since many of the samples had low ITS DNA quantities, we chose to sequence the 18S ribosomal RNA (rRNA) gene ITS1 region of only those samples with sufficiently high ITS DNA quantities. We used a CT value of less than 35 cycles as the cut-off for sequencing. For the sequencing preparation, we measured the ITS DNA concentrations of the 125 selected ITS DNA samples (in addition to four positive and four negative controls) with FLx 800 cse (Cambrex), and they were normalised with a Biomek 3000 (Beckman Coulter) and prepared for amplicon sequencing using Illumina MiSeq v3 600-cycle chemistry, according to the producer's instructions. Four positive and four negative controls were also included to the library. The library was quantified by using a Droplet Digital PCR (ddPCR, BioRad) and then diluted to a concentration recommended for sequencing. We then performed gene paired-read sequencing of the 18S rRNA gene ITS1 region on a MiSeq platform (Illumina). Resulting sequencing reads were first filtered out based on the quality score (minimum average q-score 25) and the barcode (no mismatches in the barcode were allowed). Remaining sequences were then pair-end joined and further filtered through UPARSE algorithm (max expected error (maxEE) value set to 0.25). The sequencing of the 18S rRNA gene ITS1 region produced a total of 3,722,830 reads. The median number of reads per sample was 16,355 reads and the mean was 27,991 reads, ranging from 3 to 119,463 reads per sample. We then used 6000 reads per sample as a cut-off for the rarefaction to ensure even representation of each sample in

Table 2 DNA quantification and 18 rRNA gene ITS1 region sequencing of faecal samples

	Pregnant	Postpartum	10 days	3 months	1 year	2 years	Total
All faecal samples (count)	248	253	274	246	247	248	1516
Detected fungal ITS1	221 (89%)	220 (87%)	153 (54%)	148 (60%)	163 (66%)	189 (76%)	1094 (72%)
Sequenced samples	47 (19%)	27 (11%)	28 (10%)	4 (2%)	7 (3%)	12 (5%)	125 (8%)
Passed rarefaction and taxonomic classification	28 (11%)	25 (10%)	15 (6%)	4 (2%)	7 (3%)	11 (4%)	90 (6%)

the dataset. The final dataset comprised 100 samples with more than 6000 sequences per sample and a total of 214 operational taxonomic units (OTUs). Ten of the samples were later discarded due to incorrect inclusion criteria, resulting in the inclusion of 90 samples in the analysis (Table 2).

We used the QIIME (Quantitative Insights into Microbial Ecology) pipeline for quality filtering and diversity estimation, whereas the UPARSE algorithm was used for OTU clustering [37]. In applying the rarefaction cut-off at 6000 reads per sample, we ensured minimal losses in the number of samples whilst maintaining the diversity (Additional file 1: Table S1 and Additional file 2: Figure S1).

The alpha diversity refers to the fungal diversity within each sample, and it was calculated by using Simpson's reciprocal index, which describes how many OTUs prevail in each sample [38]. The beta diversity expresses the difference between the samples in terms of the number and abundance of OTUs within an age group, and it was calculated with the Bray-Curtis dissimilarity index.

Since there is no well-established quality annotation database designed for mycobiotic taxonomy assignment at present, and because fungi are often subject to misclassification, we used a conservative concordance system for the taxonomic annotation. We compared the OTU sequences with the four databases as follows: GenBank (NCBI, ≥ 97% identity and E value $< 10^{-50}$); the Warcup Fungal ITS and UNITE Fungal ITS (User-friendly Nordic ITS Ectomycorrhiza Database with a bootstrapping threshold of 80%) through the Ribosomal Database Project (RDP) Classifier (https://rdp.cme.msu.edu/classifier/classifier.jsp); and the Targeted Host-Associated Fungi ITS Database (THF) [36], which was especially curated for the gut mycobiome (see Additional file 3 and Additional file 4). A concordance of at least three of these databases at the lowest taxonomic level or two databases with a justifiable was determined as sufficient qualification for the final assignment of each OTU. We followed the recent taxonomic reclassification for the fungi by manually curating the classification of OTU representative sequences with Index Fungorum as the reference [39].

Statistical methods

A standard curve was made to convert the qPCR CT values into fungal ITS copy concentrations in the faecal samples. The averages of three dilutions of the positive control for each qPCR plate of known fungal concentrations of *S. cerevisiae* were used for the calculation (see Additional file 5). The fungal ITS copy concentrations were logarithmically expressed to obtain a normal distribution. The fungal DNA data from the offspring samples were analysed using a linear mixed model for the fungal DNA concentration and a mixed logistic regression for
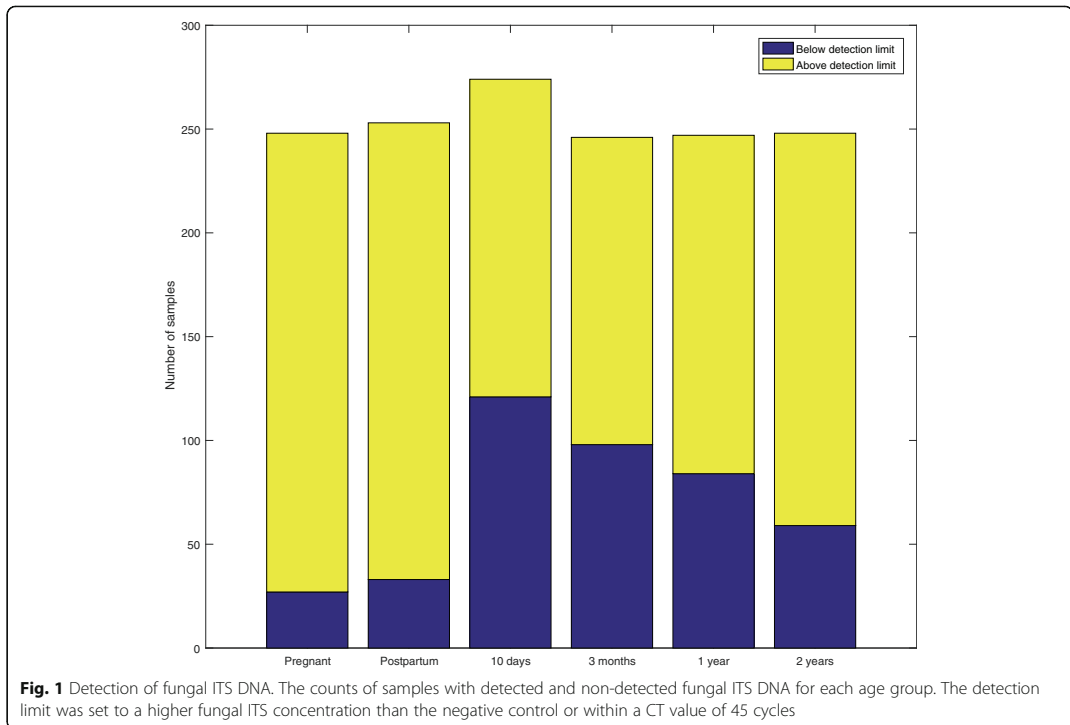
the presence of fungal DNA. The models included a random intercept for mother-offspring pair, and age, maternal fungal DNA concentration/presence, the mode of delivery and maternal probiotics use were used as covariates. The effect of antibiotic use was studied in a separate model because we lacked information on the antibiotic use within 10 days of life. The interaction terms were investigated and included in the final analyses if significant. For independent data, multiple linear regression analyses were performed to test the associations between the fungal DNA abundance and clinical characteristics. The diversities and OTU abundances between the groups were analysed with non-parametric Mann-Whitney U tests and Kruskal-Wallis tests. By this, we disregard the potential within-subjects correlations; nevertheless, a non-parametric test for repeated measurements, i.e. Friedman's test was not applicable due to the low numbers of observations in some groups. We defined the statistical significance as $p < 0.05$ and corrected the non-parametric analyses for multiple testing by controlling the false discovery rate through the Benjamini-Hochberg procedure. The statistical analyses were conducted using MATLAB 2016a (The Math-Works, Inc.), STATA 14 (StataCorp) and SPSS Statistics version 23.0 (IBM).

Results

Fungal DNA concentration and diversity

In total, 88% of the mothers and 56–76% of the offspring had detectable gut fungi (Fig. 1). The total fungal abundance was quantified by the amount of fungal ITS DNA copies in the sample. The samples from the pregnant women had the highest fungal DNA concentrations of all the groups (3.85 log(copies/mL) and 95% CI 3.62–4.07), which were significantly higher than those of the postpartum mothers (3.05 and 2.82–3.26, $p < 0.001$) (Fig. 2, Additional file 1: Table S2). Among the offspring samples, there was a tendency to uncover the highest fungal DNA concentration at 10 days (2.81 and 2.55–3.08), which then fell to the lowest levels at 1 year (2.19 and 1.94–2.45) before an increase at 2 years.

Among all the groups, the alpha diversity was lowest in the 10-day samples (1.21, $p < 0.05$, Simpson's reciprocal index) (Fig. 3a) and tended to be higher in the pregnant women (2.16, ns). This finding indicates that only 1–3 fungal species prevailed in mothers as well as offspring. The alpha diversity showed a consistent tendency to increase from birth to 2 years, but it did not reach significance due to the low number of samples in each group. A similar distribution was observed for the mean number of OTUs in each age group (Fig. 3b). In contrast to the alpha diversity, the beta diversity was highest at 10 days after birth (0.97, median Bray-Curtis dissimilarity index) and showed a spread between 0.6

Fig. 1 Detection of fungal ITS DNA. The counts of samples with detected and non-detected fungal ITS DNA for each age group. The detection limit was set to a higher fungal ITS concentration than the negative control or within a CT value of 45 cycles

and 1.0 in the other maternal and offspring samples (Fig. 3c). In summary, the mothers tended to have both higher alpha and beta diversity in pregnancy than postpartum. In the offspring, the alpha diversity seemed to increase steadily from birth, whereas the beta diversity was highest in 10-day-old offspring.

The PCoA plot at 6000 reads distinguished the 10-day–3-month-old offspring (breast-fed and/or formula-fed) from the other samples. Conversely, the samples from the 1–2-year-old offspring (fed a diet more similar to their mothers) converged towards the maternal pattern (Additional file 6: Figure S2).

OTU taxonomic classification

In applying our stringent annotation method, 140 out of 245 OTUs were annotated at least up to fungal phylum, and 101 OTUs were classified by genus (Additional file 4).

Twelve OTUs differed significantly in their abundance between age groups, whilst they made up > 1% of the total relative abundance (Additional file 7: Figure S3). *S. cerevisiae* was most abundant in mothers and in offspring from 1 year of age onwards, whereas it was detected in very low quantities in offspring at 10 days and 3 months after birth. *Debaryomyces hansenii* exhibited its greatest abundance in offspring at 10 days and
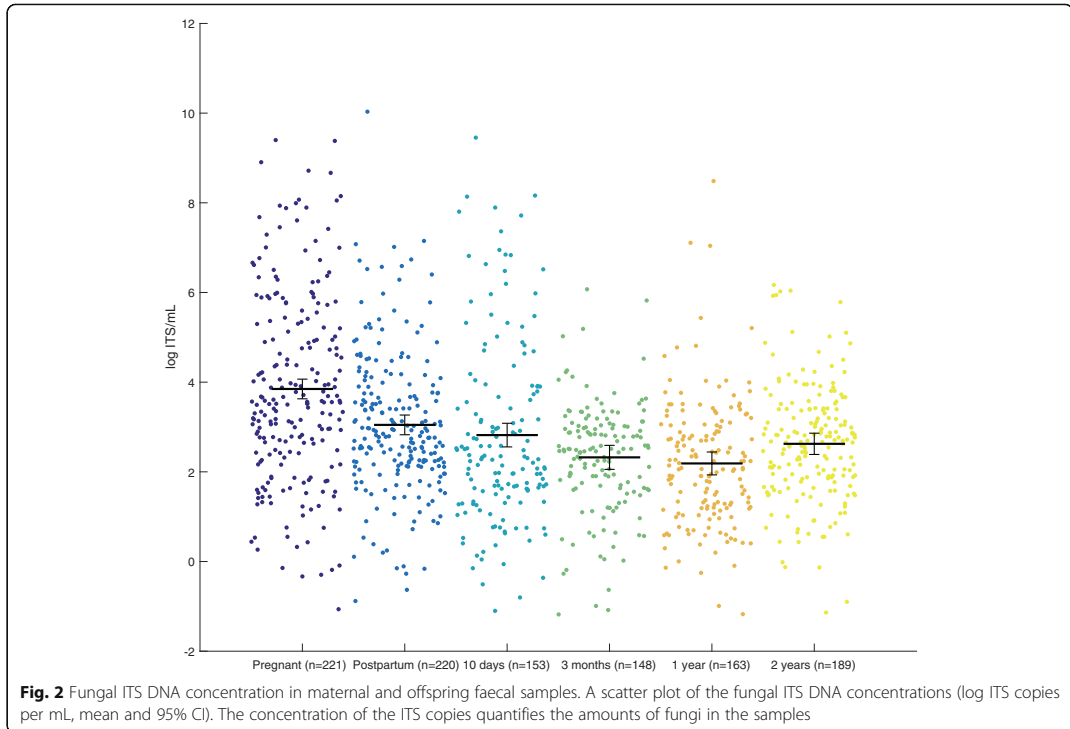
3 months (Fig. 4a–c). The 10-day, 1-year and 2-year samples were richer in *Rhodotorula mucilaginosa*, whereas the 3-month samples showed a greater presence of *Candida parapsilosis* and a *Cladosporium* sp.

Ascomycota spp. comprised 86.4% of the fungi in all the age groups, with no significant difference between the age groups ($p = 0.74$). In total, 88.6% of the fungal community consisted of yeast species with no significant difference between the age groups ($p = 0.60$).

Transfer of fungi from the mothers to the offspring

The odds of detecting fungal DNA in the offspring samples increased if the mothers also had detectable fungal DNA (odds ratio (OR) = 1.54 (95% CI 1.01–2.34, $p = 0.04$)) compared to mothers with no detectable fungi upon mixed logistic regression (Additional file 1: Table S3). Investigating the interactions, this effect was strongest 10 days after birth (OR = 3.7 (1.24–11.0), $p = 0.019$). In particular, we observed no effects of mode of delivery nor did we see any effects of maternal probiotic use or offspring antibiotic use. The intraclass coefficient (ICC) was < 0.01, which indicates that the repeated measurements were largely unrelated.

We found no significant associations between the offspring fungal DNA concentrations and the maternal

Fig. 2 Fungal ITS DNA concentration in maternal and offspring faecal samples. A scatter plot of the fungal ITS DNA concentrations (log ITS copies per mL, mean and 95% CI). The concentration of the ITS copies quantifies the amounts of fungi in the samples

fungal DNA concentrations, probiotics, mode of delivery or the offspring antibiotic use (Additional file 1: Table S4). The ICC was < 0.01, which shows that the repeated measurements were largely unrelated.

By sequencing the 18S rRNA gene ITS1 regions of merely high-quantity samples, 5 mother-offspring pairs remained. In these pairs, there were 11 overlapping species, with *D. hansenii* and *S. cerevisiae* being the most frequently overlapping ones (Fig. 5). These two species were also retained between pregnant and postpartum mother pairs (data not shown). Several other species were also present in the mother-offspring overlap, and these mostly belonged to the *Saccharomycetaceae* family, including *Candida* spp., in addition to *R. mucilaginosa*, *Malassezia* spp. and *Cladosporium* spp.

Probiotics and fungal DNA concentrations in the mothers and the offspring

The pregnant mothers who were randomised to receive probiotics had significantly increased fungal DNA concentrations compared to the controls ($p < 0.01$, Additional file 1: Table S5). Adjusting for a history of antibiotic treatment did not change the effect estimator. One *S. cerevisiae* strain (OTU 159) tended to be underrepresented in the probiotic-receiving pregnant women ($p = 0.07$); however,

the distributions of the other *S. cerevisiae* strain (OTU 2) remained the same (Additional file 8: Figure S4).

Discussion

In this study, we showed that the gut fungi are detectable in most mothers and the majority of their offspring already at the age of 10 days. *S. cerevisiae* was the most abundant fungal species in mothers and 1- to 2-year-old offspring, whereas *D. hansenii* prevailed during the first months of life. Furthermore, there was an increased risk of fungal DNA presence in offspring if the mother had detectable fungi. We also found that the maternal fungal DNA concentrations increased when the pregnant mothers drank probiotics.

Almost 90% of the mothers and 60–80% of the offspring had detectable gut fungi, which highlights fungi as an inherent part of the gut microbiota. These proportions cohere with the findings of an Italian cross-sectional fungal cultivation study on children and adults [18].

Many of the fungal species that we detected in the offspring have previously been detected in the adult gut (see Additional file 5). Strikingly, *D. hansenii*, an ubiquitous *Saccharomycetaceae* species that is often used in the food industry as a cheese yeast [40], dominates the offspring gut mycobiota during the breast-feeding
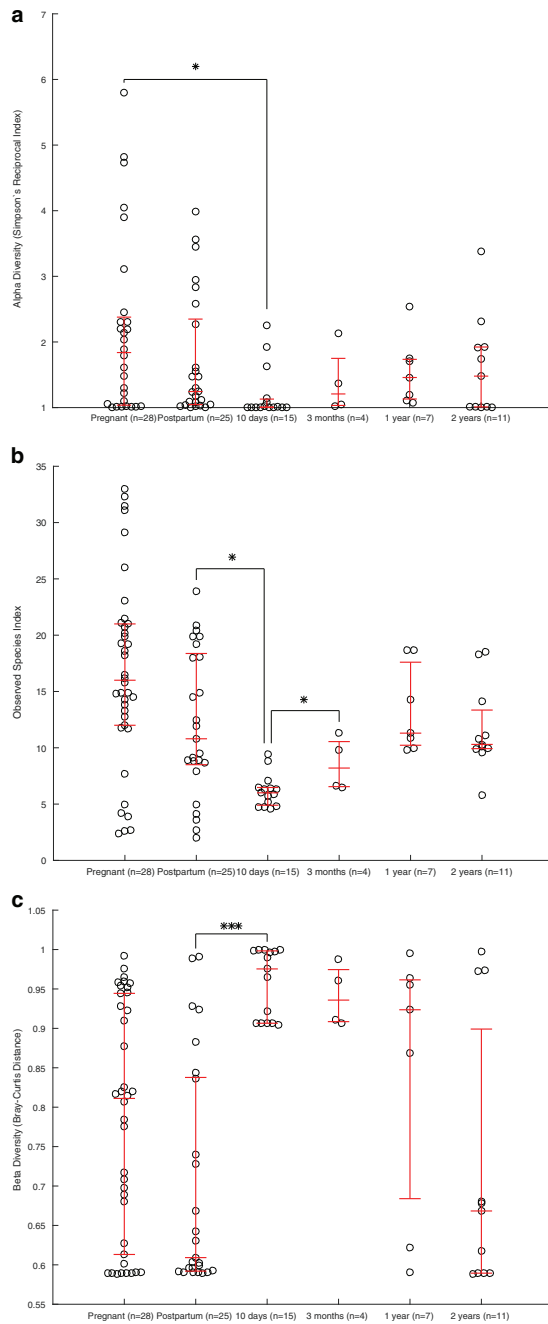
Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Alpha and beta diversity for the faecal samples. A scatter plot of the diversities; *red whiskers* designate the median and interquartile ranges. **a** Alpha diversity as Simpson's reciprocal index. The Simpson reciprocal index describes how many OTUs prevail in each sample [36]. **b** The observed species index describes the sample richness, i.e. how many OTUs are detected in each sample. **c** The beta diversity as Bray-Curtis Distance describes the between-sample diversity from 0 to 1
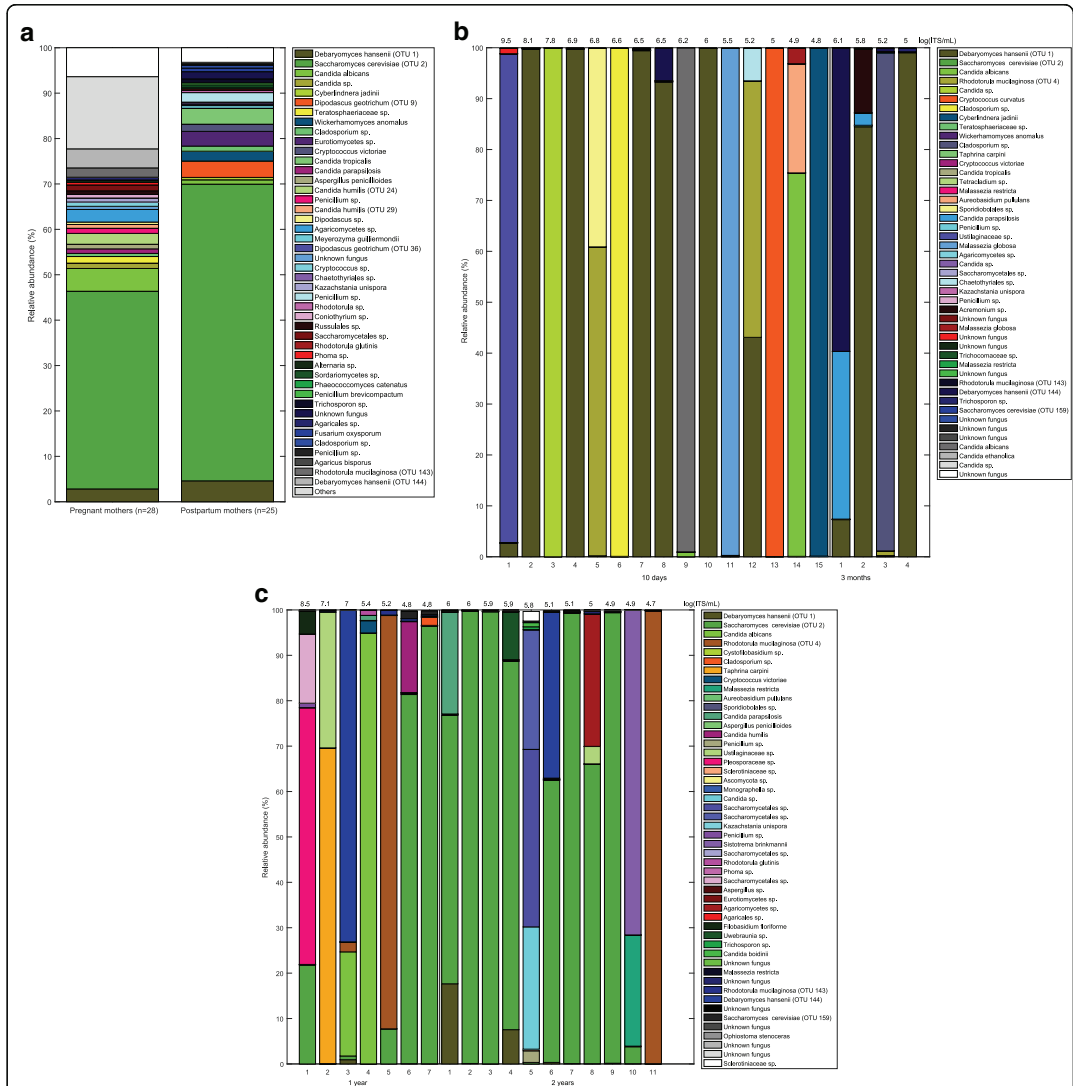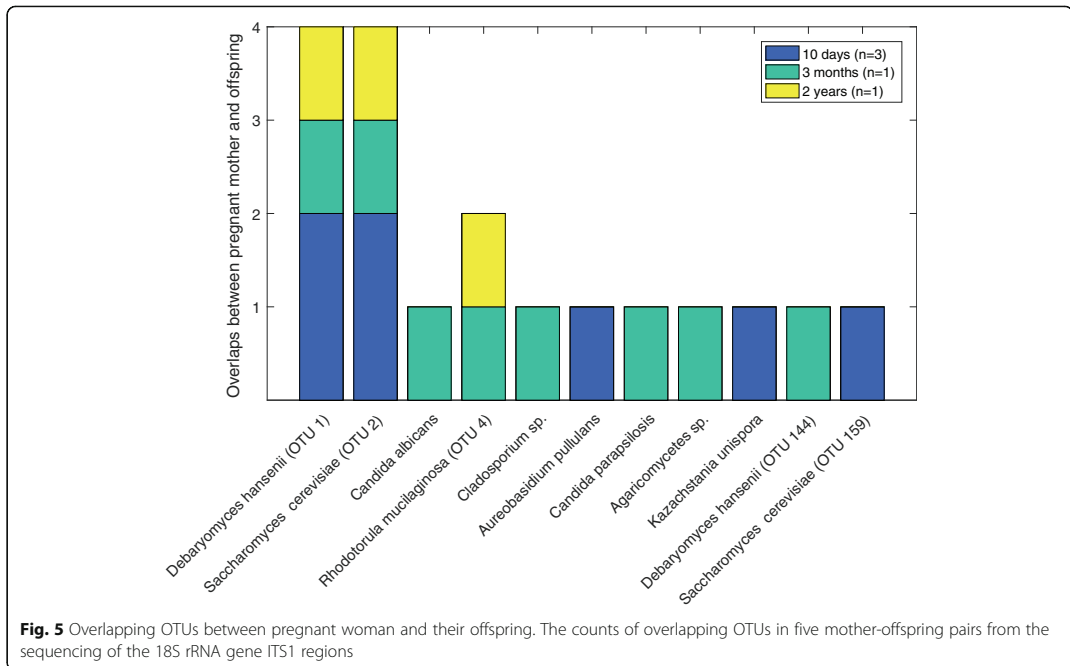

Fig. 4 a–c OTU abundances for all groups. Bar charts of the relatively most abundant OTUs in **a** mothers, **b** offspring from 10 days to 3 months and **c** offspring for 1–2 years. Each *coloured box* represents an OTU. The individual fungal ITS DNA concentration is on *top* of each bar

Fig. 5 Overlapping OTUs between pregnant woman and their offspring. The counts of overlapping OTUs in five mother-offspring pairs from the sequencing of the 18S rRNA gene ITS1 regions

period. *D. hansenii* can stem from breast milk because this fluid was the only dietary component at 3 months, and similar yeasts (*C. albicans*) have been found in breast milk before [41]. *D. hansenii* has also been found on the facial skin of children [42]. *D. hansenii* has previously been cultured from human faeces and has been shown to grow well in milky environments like cheese and possibly breast milk, since some strains are known to grow at 37 °C [20, 40]. All these findings suggest that *D. hansenii* could be an autochthonous species of the early mycobiota.

We found *S. cerevisiae* in the newborn offspring, which has not been shown before. Intriguingly, *S. cerevisiae* first surges and then becomes a dominant species at 1 year of age, after the introduction of food containing *S. cerevisiae* (e.g. bread) into the diet. This finding suggests both a birth-related and a dietary means of colonisation. *S. cerevisiae* is present in most diets and has been found and cultured from faecal samples. However, it is also capable of causing opportunistic infections [20, 43], implying that it is an autochthonous species. We observed that *S. cerevisiae* was substantially more abundant in offspring and adult gut mycobiota than previously described [1, 9], and there was a relatively low occurrence of *Candida* spp.; this variation may be due to different regional diets, host genetics or fungal detection methods. Some studies have shown that by using culture-

dependent techniques, one would obtain relatively more *Candida* spp. than when using culture-independent techniques [2, 18], which could partly explain the rather limited amounts of *Candida* spp. in our study.

Some of the identified fungi should be regarded as transients that do not colonise the gut. The OTUs *Agaricomycetes/Agaricales* sp. are likely from edible and non-colonising fungi, e.g. the button mushroom *Agaricus bisporus*, and similarly, *Ustilaginaceae* spp. are well-known plant pathogens. These fungi are generally of lower quantities and are often known not to live in anaerobic and body temperature environments; they are thus most likely transients from food. Not all OTUs were annotated to the species level because the fungal databases still are under development. However, our strict classification improved the chance of an accurate classification.

We found that maternal fungal hosting makes the offspring more inclined to host fungi. This effect was strongest at 10 days after birth. The increased chance of fungal hosting suggests that these mother-offspring pairs share physiological fungal hosting abilities. Because fungi are ubiquitous in the environment, they may originate from the mother during birth, the mother's breast milk, parental skin or anywhere else in the hospital or home environment with which the offspring come in contact. However, we did not observe any OTU abundance

difference regarding vaginal or caesarean delivery. Thus, we find indications for the transfer of fungal hosting between mothers and offspring that appear to be independent of the mode of delivery.

The overlapping OTUs in the mother and offspring guts were mostly *Saccharomycetaceae* spp. This fungal family seems to have adapted well to the human gut environment and may be the species that are fittest to survive the transfer into the newborn fungal host environment. In addition to the gut mycobiota, *Saccharomycetaceae* spp. are also the most abundant species in healthy human mouth mycobiota [44].

The fungal abundance varied by age, which supports the idea that physiological fungal succession occurs in the early gut mycobiota. We have shown that the gut mycobiota is establishing already at 10 days after birth, albeit at a lower abundance and diversity than what is detected in their mothers' guts. At 10 days, the gut fungi have just started to fight for their positions in a seemingly first-come first-serve model; the reduction in fungal abundance is probably determined by feeding, gut immunity and their interactions. The decrease at 3 months may be due to a previously described temporarily high abundances of *Bifidobacterium* spp. and *Lactobacillus* spp. that exhibit fungal antagonism [30, 45]. Upon approaching 2 years of age, the gut mycobiota consists of fungi specific for the adult mycobiota, as observed in our study.

Interestingly, pregnant mothers that received probiotics showed a higher abundance of gut fungi. This finding could indicate that the probiotic bacteria used in our study promote the symbiotic growth of gut fungi, like other lactic acid bacteria that are known to grow mutually with yeasts [46].

Due to a strict ITS quantity cut-off, a smaller proportion of the samples was sequenced. It cannot be excluded that the lower-abundance samples have different compositions. Nevertheless, in this unselected study population of mothers and offspring, it is reasonable to surmise that this selection could reflect the gut mycobiota of a healthy gut. The study design gave us limited control of the faecal sampling, but all the mothers were well informed about how to collect and quickly freeze the samples to avoid contamination and improve preservation. In the future, a lysis protocol optimised for fungal DNA extraction would be preferable, but this approach would require the fungal extraction analysis of a representative selection of human-associated fungi that is not yet available.

Conclusion

Our findings provide the first insight into the gut mycobiota that is established in offspring and into the transfer of fungal hosting from mother to child. This study covers a large, unselected population cohort of mothers and offspring, and it broadens the field of gut mycobiota as a new research area. The ways in which the early mycobiota can affect a child's normal physiology with respect to growth, immunity and metabolism remain to be elucidated.

Additional files

Additional file 1: Table S1. Rarefaction table of sequenced samples. **Table S2.** ITS DNA concentration for all age groups. **Table S3.** Detectable fungal DNA in offspring according to maternal characteristics and offspring age. **Table S4.** Fungal DNA concentration in offspring according to maternal presence and offspring age. **Table S5.** Association between fungal DNA concentration in pregnant mothers and maternal use of antibiotics and probiotics. (XLSX 16 kb)

Additional file 2: Figure S1. Number of samples vs. rarefaction cut-off. To compare the samples, a rarefaction is performed to obtain the same number of sequences in each sample. By increasing the rarefaction cut-off, the number of observed species increases with the sacrifice in the number of included samples. Using 6000 sequences as the rarefaction cut-off is a reasonable trade-off. (PDF 6 kb)

Additional file 3: Concordance of fungal databases and final assignment. (XLSX 101 kb)

Additional file 4: Table of fungal species. (XLSX 18 kb)

Additional file 5: Concentration and standard curve calculations. (DOCX 32 kb)

Additional file 6: Figure S2. Principal coordinates analysis (PCoA). A PCoA plot at 6000 reads per sample. (PDF 8 kb)

Additional file 7: Figure S3. Significantly different OTUs between groups. A significantly different abundance of OTUs between groups in terms of relative abundance, as tested by Kruskal-Wallis test. Each bar represents an OTU, for which the relative abundances of all the groups are added. Only species > 1% of the relative abundance in at least one age group are included in the analysis. (PDF 5 kb)

Additional file 8: Figure S4. OTU abundances for probiotics in pregnant mothers. The OTU abundances in pregnant mothers with and without probiotics. Each coloured box represents an OTU. (PDF 5 kb)

Authors' contributions
All the authors contributed to the research question. TØ conducted the sampling. KS performed the laboratory work and the statistical analyses, and KS wrote the manuscript draft. All the authors revised and approved the final article.

Ethics approval and consent to participate
This study was approved by the Regional Committee for Medical and Health Research Ethics for Central Norway (ref: 120-2000 and 2014/1796/REK midt) and the Norwegian Data Inspectorate (ref: 2003/953-3 KBE/-). At least one of each child's parent was informed and signed an informed consent form.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Department of Laboratory Medicine, Children's and Women's Health, Faculty of Medicine and Health Sciences, NTNU – Norwegian University of Science and Technology, Postboks 8905, 7491 Trondheim, Norway. [2]Department of Chemistry, Biotechnology and Food Science, NMBU – Norway University of Life Sciences, Ås, Norway. [3]Department of Public Health and Nursing, Faculty of Medicine and Health Science, NTNU – Norwegian University of Science and Technology, Trondheim, Norway. [4]ObeCe – Regional Centre for Obesity Research and Innovation, St. Olav's University Hospital, Trondheim, Norway.

References

1. Underhill DM, Iliev ID. The mycobiota: interactions between commensal fungi and the host immune system. Nat Rev Immunol. 2014;14:405–16.
2. Gouba N, Drancourt M. Digestive tract mycobiota: a source of infection. Medecine et maladies infectieuses. 2015;45:9–16.
3. Hamad I, Raoult D, Bittar F. Repertory of eukaryotes (eukaryome) in the human gastrointestinal tract: taxonomy and detection methods. Parasite Immunol. 2016;38:12–36.
4. Huse SM, Ye Y, Zhou Y, Fodor AA. A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS One. 2012;7:e34242.
5. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464:59–65.
6. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol. 2014;32: 834–41.
7. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, et al. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. PLoS One. 2013;8:e66019.
8. Cuskin F, Lowe EC, Temple MJ, Zhu Y, Cameron EA, Pudlo NA, et al. Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism. Nature. 2015;517:165–9.
9. Cui L, Morris A, Ghedin E. The human mycobiome in health and disease. Genome medicine. 2013;5:63.
10. Rizzetto L, De Filippo C, Cavalieri D. Richness and diversity of mammalian fungal communities shape innate and adaptive immunity in health and disease. Eur J Immunol. 2014;44:3166–81.
11. Walker AW, Lawley TD. Therapeutic modulation of intestinal dysbiosis. Pharmacol Res. 2013;69:75–86.
12. Mar Rodriguez M, Perez D, Javier Chaves F, Esteve E, Marin-Garcia P, Xifra G, et al. Obesity changes the human gut mycobiome. Sci Rep. 2015;5:14600.
13. Wensveen FM, Valentić S, Šestan M, Turk Wensveen T, Polić B. The "big bang" in obese fat: events initiating obesity-induced adipose tissue inflammation. Eur J Immunol. 2015;45:2446–56.
14. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in IBD. Gut. 2017;66:1039-48.
15. Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, et al. Fungi and inflammatory bowel diseases: alterations of composition and diversity. Scand J Gastroenterol. 2008;43:831–41.
16. Salamati S, Martins C, Kulseng B. Baker's yeast (Saccharomyces cerevisiae) antigen in obese and normal weight subjects. Clinical obesity. 2015;5:42–7.
17. Rinaldi M, Perricone R, Blank M, Perricone C, Shoenfeld Y. Anti-Saccharomyces cerevisiae autoantibodies in autoimmune diseases: from bread baking to autoimmunity. Clin Rev Allergy Immunol. 2013;45:152–61.
18. Strati F, Di Paola M, Stefanini I, Albanese D, Rizzetto L, Lionetti P, et al. Age and gender affect the composition of fungal population of the human gastrointestinal tract. Front Microbiol. 2016;7:1227.
19. Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU, Ryan RM. Vertical and horizontal transmission of Candida albicans in very low birth weight infants using DNA fingerprinting techniques. Pediatr Infect Dis J. 2008;27:231–5.
20. Suhr MJ, Hallen-Adams HE. The human gut mycobiome: pitfalls and potentials—a mycologist's perspective. Mycologia. 2015;107:1057–73.
21. Ward TL, Knights D, Gale CA. Infant fungal communities: current knowledge and research opportunities. BMC Med. 2017;15:30.
22. Funkhouser LJ, Bordenstein SR. Mom knows best: the universality of maternal microbial transmission. PLoS Biol. 2013;11:e1001631.
23. Stinson LF, Payne MS, Keelan JA. Planting the seed: origins, composition, and postnatal health significance of the fetal gastrointestinal microbiota. Crit Rev Microbiol. 2017;43:352–69.
24. Prince AL, Chu DM, Seferovic MD, Antony KM, Ma J, Aagaard KM. The perinatal microbiome and pregnancy: moving beyond the vaginal microbiome. Cold Spring Harbor Perspect Med. 2015;5
25. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Sci Transl Med. 2014;6:237ra65.
26. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep. 2016;6:23129.
27. Soderborg TK, Borengasser SJ, Barbour LA, Friedman JE. Microbial transmission from mothers with obesity or diabetes to infants: an innovative opportunity to interrupt a vicious cycle. Diabetologia. 2016;59:895–906.
28. Ma J, Prince AL, Bader D, Hu M, Ganu R, Baquero K, et al. High-fat maternal diet during pregnancy persistently alters the offspring microbiome in a primate model. Nat Commun. 2014;5:3889.
29. Mason KL, Erb Downward JR, Mason KD, Falkowski NR, Eaton KA, Kao JY, et al. Candida albicans and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. Infect Immun. 2012;80:3371–80.
30. Huffnagle GB, Noverr MC. The emerging world of the fungal microbiome. Trends Microbiol. 2013;21:334–41.
31. Dollive S, Chen YY, Grunberg S, Bittinger K, Hoffmann C, Vandivier L, et al. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. PLoS One. 2013;8:e71806.
32. Matsubara VH, Bandara HM, Mayer MP, Samaranayake LP. Probiotics as antifungals in mucosal candidiasis. Clin Infect Dis. 2016;62:1143–53.
33. Kumar S, Bansal A, Chakrabarti A, Singhi S. Evaluation of efficacy of probiotics in prevention of candida colonization in a PICU—a randomized controlled trial. Crit Care Med. 2013;41:565–72.
34. Dotterud CK, Storro O, Johnsen R, Oien T. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. Br J Dermatol. 2010;163:616–23.
35. Dotterud CK, Avershina E, Sekelja M, Simpson MR, Rudi K, Storro O, et al. Does maternal perinatal probiotic supplementation alter the intestinal microbiota of mother and child? J Pediatr Gastroenterol Nutr. 2015;61:200–7.
36. Tang J, Iliev ID, Brown J, Underhill DM, Funari VA. Mycobiome: approaches to analysis of intestinal fungi. J Immunol Methods. 2015;421:112–21.
37. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Meth. 2013;10:996–8.
38. Magurran AE. Measuring biological diversity. Maldan: Blackwell Pub; 2004.
39. Hibbett DS, Taylor JW. Fungal systematics: is a new age of enlightenment at hand? Nat Rev Microbiol. 2013;11:129–33.
40. Breuer U, Harms H. Debaryomyces hansenii—an extremophilic yeast with biotechnological potential. Yeast (Chichester, England). 2006;23:415–37.
41. Mutschlechner W, Karall D, Hartmann C, Streiter B, Baumgartner-Sigl S, Orth-Höller D, et al. Mammary candidiasis: molecular-based detection of Candida species in human milk samples. Eur J Clin Microbiol Infect Dis. 2016;1–5.

42. Arzumanyan VG, Magarshak OO, Semenov BF. Yeast fungi in patients with allergic diseases: species variety and sensitivity to antifungal drugs. Bull Exp Biol Med. 2000;129:601–4.
43. Vaughan-Martini A, Martini A. Chapter 61 - Saccharomyces Meyen ex Reess (1870) A2 - Kurtzman, Cletus P. In: Fell JW, Boekhout T, editors. The yeasts. Fifth ed. London: Elsevier; 2011. p. 733–46.
44. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010;6:e1000713.
45. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe. 2015;17:690–703.
46. Furukawa S, Watanabe T, Toyama H, Morinaga Y. Significance of microbial symbiotic coexistence in traditional fermentation. J Biosci Bioeng. 2013;116:533–9.

Paper II

Check for updates

Early Gut Fungal and Bacterial Microbiota and Childhood Growth

Kasper Schei [1*], Melanie Rae Simpson [2,3], Ekaterina Avershina [4†], Knut Rudi [4], Torbjørn Øien [2], Pétur Benedikt Júlíusson [5,6], David Underhill [1,7], Saideh Salamati [8] and Rønnaug Astri Ødegård [1,8]

[1] Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, NTNU – Norwegian University of Science and Technology, Trondheim, Norway, [2] Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, NTNU – Norwegian University of Science and Technology, Trondheim, Norway, [3] Clinic of Laboratory Medicine, St. Olavs Hospital, Trondheim, Norway, [4] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway, [5] Department of Health Registries, Norwegian Institute of Public Health, Bergen, Norway, [6] Department of Clinical Science, Faculty of Medicine, University of Bergen, Bergen, Norway, [7] Division of Immunology Research, Cedars-Sinai, Los Angeles, CA, United States, [8] Regional Centre of Obesity Research and Innovation (ObeCe), Trondheim University Hospital, Trondheim, Norway

Introduction: Childhood growth is a sensitive marker of health. Animal studies show increased height and weight velocity in the presence of fungal as well as antibiotic supplement in feed. Human studies on early gut microbiota and anthropometrics have mainly focused on bacteria only and overweight, with diverging results. We thus aimed to investigate the associations between childhood growth [height and body mass index (BMI)] and early fungal and bacterial gut microbiota.

Methods: In a population-based cohort, a subset of 278 pregnant mothers was randomized to drink milk with or without probiotic bacteria during and after pregnancy. We obtained fecal samples in offspring at four time points between 0 and 2 years and anthropometric measurements 0 and 9 years. By quantitative PCR and 16S/ITS rRNA gene sequencing, children's gut microbiota abundance and diversity were analyzed against height standard deviation score (SDS) and BMI-SDS and presented as effect estimate (β) of linear mixed models.

Results: From 278 included children (149 girls), 1,015 fecal samples were collected. Maternal probiotic administration did not affect childhood growth, and the groups were pooled. Fungal abundance at 2 years was positively associated with height-SDS at 2–9 years ($\beta = 0.11$ height-SDS; 95% CI, 0.00, 0.22) but not with BMI-SDS. Also, higher fungal abundance at 1 year was associated with a lower BMI-SDS at 0–1 year ($\beta = -0.09$ BMI-SDS; 95% CI, -0.18, -0.00), and both bacterial abundance and bacterial alpha diversity at 1 year were associated with lower BMI-SDS at 0–1 year ($\beta = -0.13$ BMI-SDS; 95% CI, -0.22, -0.04; and $\beta = -0.19$ BMI-SDS; 95% CI, -0.39, -0.00, respectively).

Conclusions: In this prospective cohort following 0–9-year-old children, we observed that higher gut fungal abundances at 2 years were associated with taller children between 2 and 9 years. Also, higher gut fungal and bacterial abundances and higher gut bacterial diversity at 1 year were associated with lower BMI in the first year of life. The results may indicate interactions between early gut fungal microbiota and the human growth-regulating physiology, previously not reported.

Clinical Trial Registration: Clinicaltrials.gov, NCT00159523.

Keywords: gut microbiota, mycobiota, fungi, childhood growth, height velocity

INTRODUCTION

Childhood growth constitutes a prominent and important sign of bodily development, and thus this sensitive health marker is assured by growth control programs worldwide (1). Human growth comprises four overlapping phases including foetal, infancy, childhood, and pubertal growth. Each growth phase is driven by certain endocrine processes, as well as being influenced by genetic, nutritional, and environmental factors (1, 2). Recent investigations suggest that the gut microbiota could be a possible growth regulator too (3, 4).

The gut microbiota refers to the microbial community within our gastrointestinal tract, housing symbiotic microbes like bacteria and fungi. The fungal proportion of the microbiota is denoted mycobiota. Early gut microbiota patterns have been associated with childhood obesity with various findings (5–8), e.g., at 3 months the relative abundances of Firmicutes and Lachnospiraceae were positively and for *Bifidobacterium* spp. negatively associated with early overweight and obesity. Associations with height velocity have been poorly explored, but in pre-school children, height velocity has been found associated with certain Firmicutes spp. at 3 months and higher gut bacterial diversity at 0–3 years (4, 7, 9, 10). Since the 1950s, antibiotics have been widely used as growth promotors in livestock production (11). While early human antibiotic use may predispose for later childhood obesity (12), its possible effect on height velocity is less elucidated. A *Helicobacter pylori* eradication study with 1 week administering broad-spectrum antibiotics in 6–10-year-old children showed increased height standard deviation scores (SDS) by 20% in the intervention group compared with the control within 1 year, even when *H. pylori* was not eradicated (13). The same antibiotics-height association was observed in a large Finnish infancy cohort (12).

When the European Union banned the use of antimicrobials as growth promotors in animal production, the search for non-antimicrobial growth promotors in animals led to a wide-spread use of yeast and its cell wall products as new growth promotors (14). In the early human gut, the most abundant yeast genera are *Debaryomyces*, *Candida*, and *Saccharomyces*, with a development toward higher diversity of species (alpha diversity) and more *Saccharomyces cerevisiae* as the children age (15). In two randomized-controlled trials in which preterm neonates (28–32 and 30–37 weeks of gestational age, respectively) were supplemented with a probiotic *S. cerevisiae* strain (*Saccharomyces boulardii*), probiotic groups experienced greater weight gain than the control group (length was not measured in one study and increased non-significantly in the other) (16, 17). This indicates that the early mycobiota could promote early human growth. All the same, the possible role for mycobiota as a human growth promotor remains unexplored.

Abbreviations: Bb-12, *Bifidobacterium animalis* subsp. *lactis* Bb-12; BMI, body mass index; CFU, colony-forming unit; CI, confidence interval; CT, Cycle threshold; ITS, internal transcribed spacer; GH, growth hormone; La-5, *Lactobacillus acidophilus* La-5; LGG, *Lactobacillus rhamnosus* GG; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction; QIIME, Quantitative Insights into Microbial Ecology; rrn, ribosomal RNA operons; SDS, standard deviation score; sp./spp., species (singular/plural).

The objective of the current study was therefore to study associations between early gut fungal and bacterial microbiota and childhood height-SDS and BMI-SDS in a longitudinal cohort of healthy children up to the age of 9 years.

MATERIALS AND METHODS

Materials

The aim of the current study was to investigate the association between early gut microbiota and childhood growth. The stool samples analyzed in this study were collected during a randomized trial of probiotics (ProPACT) (18). In total, 415 mothers were randomized to drink probiotic or placebo milk from inclusion to 3 months post partum. The probiotic milk contained 5×10^{10} colony-forming units (CFUs) of each of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb-12 and 5×10^9 CFUs of *Lactobacillus acidophilus* La-5, whereas the placebo milk was sterile and contained no probiotic bacteria. This maternal probiotic administration led to an increased presence and abundance of LGG in the infants' gut microbiotas at 10 days and 3 months, but no significant difference at 1 and 2 years, as previously shown (18). Apart from this, there were no other statistically significant differences in the microbiota composition or diversity between the groups (18). Since we considered these differences to be minimal, the two arms were pooled in the analysis of the present study.

In total, 278 of 415 participating children supplied 1,015 fecal samples at 10 days, 3 months, 1 and 2 years after birth (**Table 1**). The stool samples were collected from the diaper and transferred to a tube with 10 ml Cary-Blair transport medium (∼20 times dilution) before immediate freezing at −18°C at home. The parents were instructed to collect one big spoon of fecal matter with an enclosed spoon as sampling equipment. After transport to the laboratory, the samples were stored at −80°C before further analyses. Self-reported questionnaires about the health and environment of the child were collected in pregnancy, 6 weeks after birth, at 1 and 2 years, with information on mode of delivery, breast-feeding length, antibiotic administration to mother and offspring, and gestational age.

Ethics Approval and Consent to Participate

The parents signed an informed consent at inclusion and were once more informed when the anthropometry data were drawn, with the ability to withdraw, which two participants did. The study protocol was approved by the Regional Ethical Committee of Central Norway (2014/1796; Trial registration at Clinicaltrials.gov NCT00159523, registered 08.09.2005).

Methods

Anthropometric Measurements

Height and weight were measured at routine follow-ups at public health centers. Height was measured supine <2 years and standing thereafter with a stadiometer, and weight was measured with a digital weight, according to Norwegian guidelines. Anthropometrics were collected and converted to SDS (z-scores) based on a large Norwegian child population reference (19). BMI-SDS constitutes a more explanatory and

TABLE 1 | rRNA gene quantification and 16S/ITS rRNA gene region sequencing of fecal samples.

	10 Days	3 Months	1 Year	2 Years	Total
All fecal samples (count)	274	246	247	248	1,015
Detected bacterial DNA (16S rRNA gene region)	266 (97%)	243 (99%)	247 (100%)	243 (98%)	999 (98%)
Sequenced 16S rRNA V3–V4 gene region amplicons (after rarefaction)[a]	178 (65%)	193 (78%)	216 (87%)	170 (69%)	757 (75%)
Detected fungal DNA (ITS rRNA gene region)	153 (54%)	148 (60%)	163 (66%)	189 (76%)	653 (64%)
Sequenced ITS gene region amplicons (after rarefaction)[a]	15 (6%)	4 (2%)	7 (3%)	11 (4%)	37 (4%)

[a]Samples were sequenced if the qPCR cycle threshold was <35 cycles to provide trustworthy results in the sequencing procedure. Few samples were excluded due to rarefaction.

TABLE 2 | Maternal and offspring characteristics.

Participant characteristics	ProPACT participants with fecal samples ($n = 278$)	ProPACT participants without fecal samples ($n = 136$)	P-value[a]
Maternal age at delivery [mean (SD), years]	30.0 (4.3)	29.3 (4.8)	0.03
Cesarean sections [No. (%)]	35 (12.6)	_[b]	_[b]
Allocated to probiotics [No. (%)]	141 (50.5)	63 (43.6)	0.42
Maternal higher education [No. (%)]	217 (77.8)	79 (58.5)	< 0.01
Female offspring [No. (%)]	149 (53.4)	57 (54.3)	0.88
Gestational age [mean (SD), weeks]	40.3 (1.57)	40.2 (1.68)	0.47
Birth weight [mean (SD), g]	3,633 (485)	3,617 (446)	0.78
Birth length mean (SD; cm)	50.5 (1.94)	51.5 (6.09)	0.18
Breastfed after 3 months [No. (%[c])]	224 (97.4)	39 (97.5)	0.97
Formula fed after 3 months [No. (%[c])]	98 (36.3)	18 (40.0)	0.63
Breastfed beyond 1 year [No. (%[c])]	73 (28.1)	9 (29.0)	0.99
CHILDREN RECEIVING ANTIBIOTIC TREATMENT WITHIN [NO. (%[c])]			
6 weeks	6 (2.5)	1 (1.5)	0.61
1 year	36 (13.9)	12 (14.3)	0.93
2 years	117 (41.9)	22 (25.9)	< 0.01
Pregnant mothers receiving antibiotics [No. (%[c])]	16 (6.5)	2 (2.9)	0.09
Overweight (BMI-SDS ≥1) at 7–9 years [No. (%[c])]	44 (18.2)	_[b]	_[b]
Obesity (BMI-SDS ≥2) at 7–9 years [No. (%[c])]	6 (2.5)	_[b]	_[b]

[a]P-values calculated using χ^2 test for binary variables and t-test for continuous variables.
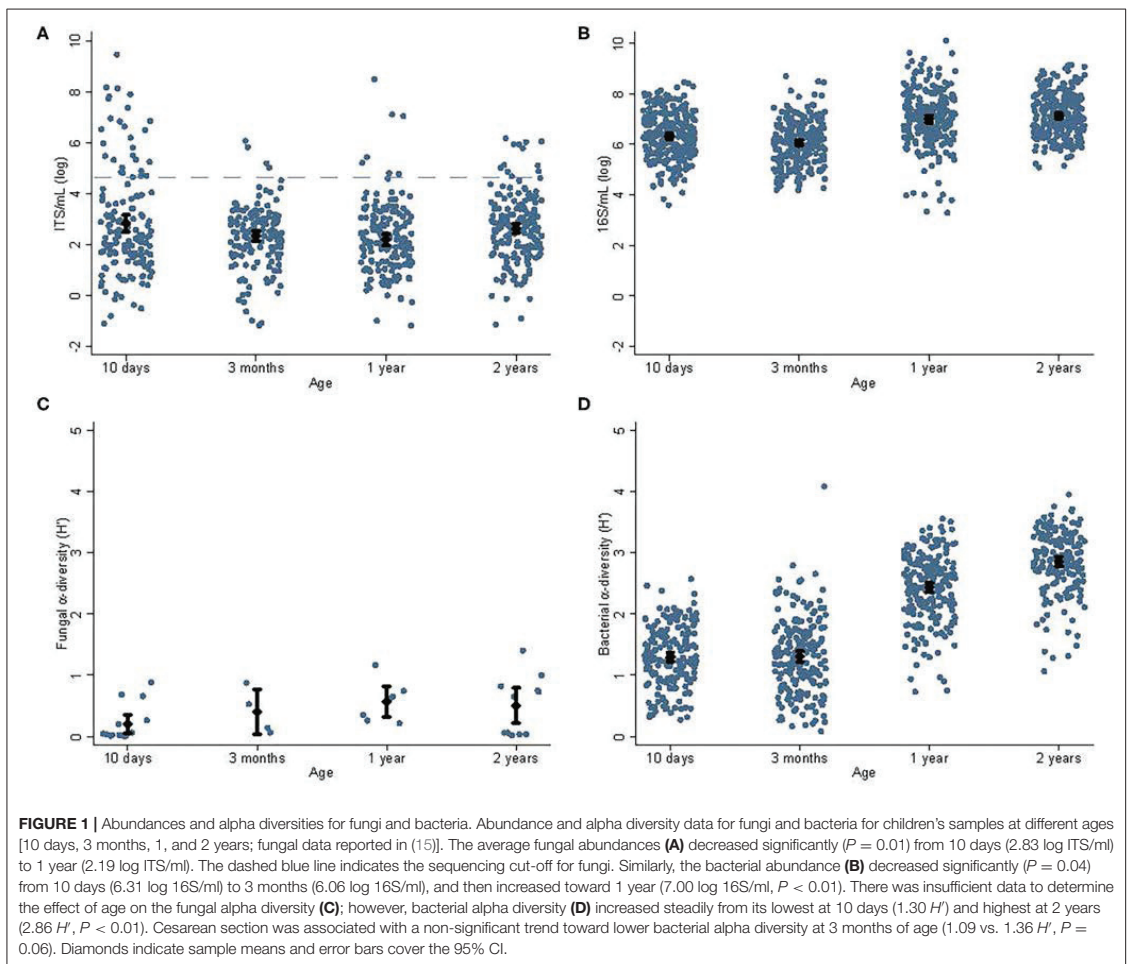[b]Not available.
[c]Percentage of total respondents of the present questionnaire.

precise way to describe children's weight development since BMI-SDS is adjusted for age and sex. Likewise, height-SDS better presents the height growth and indicates along which height percentile curve the child grows. To identify data errors and outliers, we identified height-SDS and weight SDS values ≤3 and >3, as well as measurements where height decreased in two consecutive measurements. These growth curves were evaluated, and datapoints were removed when one could assume that the measurements were incorrectly recorded. To ensure good-quality anthropometric data before analysis, all individual growth curves were modeled for inspection.

Microbiota Analyses

The microbiota analyses are thoroughly explained in the **Supplementary Material**. Briefly, stool samples were homogenized before DNA was extracted using a bacterial protocol (20) as no fungal protocols for fecal DNA extraction were validated. However, although different extraction kits

may produce differing total amounts of DNA, the relative proportions of various DNA abundances seem to largely correspond within the assays (21). We used bacteria-targeted primers (V3–V4 part of 16S rRNA gene) (22) and fungi-targeted primers (ITS1 part of 18S rRNA gene) (23) for quantification by quantitative PCR (qPCR). The qPCR cut-off value was set to the negative control if fungal abundance was lower than negative control, or excluded from analysis if cycle threshold value (CT value) at ≥45. CT values were converted to fungal and bacterial DNA concentrations using standard curves (**Supplementary Material**). Fungal quantification of the rRNA 18S/ITS1 gene region has been performed previously in bovine rumen studies (24), and recently, strongly correlated abundance estimations have been obtained using the ITS region (25). These qPCR quantifications of the microbial rRNA genes [16S (V3–V4) for bacteria and ITS1 for fungi] were therefore used as abundance markers in this study. The majority of bacterial samples were sequenced (**Table 1**). Ensuring high-quality sequencing, only

FIGURE 1 | Abundances and alpha diversities for fungi and bacteria. Abundance and alpha diversity data for fungi and bacteria for children's samples at different ages [10 days, 3 months, 1, and 2 years; fungal data reported in (15)]. The average fungal abundances **(A)** decreased significantly ($P = 0.01$) from 10 days (2.83 log ITS/ml) to 1 year (2.19 log ITS/ml). The dashed blue line indicates the sequencing cut-off for fungi. Similarly, the bacterial abundance **(B)** decreased significantly ($P = 0.04$) from 10 days (6.31 log 16S/ml) to 3 months (6.06 log 16S/ml), and then increased toward 1 year (7.00 log 16S/ml, $P < 0.01$). There was insufficient data to determine the effect of age on the fungal alpha diversity **(C)**; however, bacterial alpha diversity **(D)** increased steadily from its lowest at 10 days (1.30 H') and highest at 2 years (2.86 H', $P < 0.01$). Cesarean section was associated with a non-significant trend toward lower bacterial alpha diversity at 3 months of age (1.09 vs. 1.36 H', $P = 0.06$). Diamonds indicate sample means and error bars cover the 95% CI.
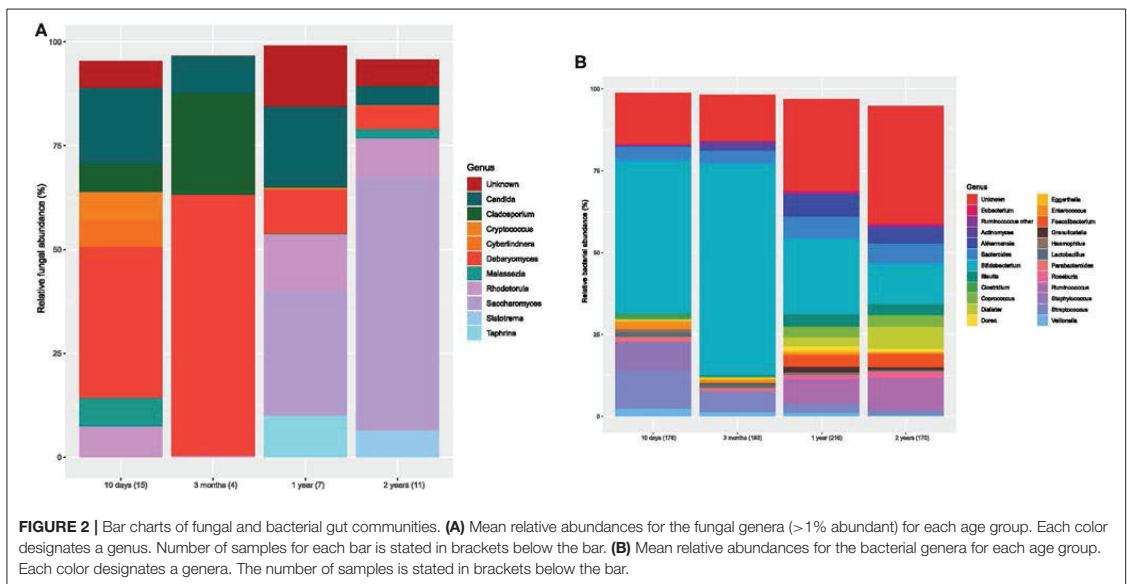
fungal samples <35 CT were sequenced, hence only 37 fungal samples underwent sequencing. The 16S and ITS1 rRNA gene regions were sequenced with Illumina MiSeq and thereafter processed with the Quantitative Insights into Microbial Ecology pipeline and UPARSE for operational taxonomic unit (OTU) clustering, described previously (15, 20). Rarefaction cut-offs of 2,000 bacterial reads/sample and 6,000 fungal reads/sample were used to ensure even representation while retaining most samples. Taxonomic annotation of the OTUs were done against the Greengenes database v13.8 for bacteria, and using a self-curated concordance system for fungi, as there are no well-established methods for fungal annotation (15).

Statistics

The influence of fungal and bacterial abundances and bacterial diversity on height-SDS and BMI-SDS was estimated using linear mixed models, accounting for repeated anthropometric measurements with individuals as random intercept and age as fixed slope and random slope in a maximum likelihood model. The distributions of bacterial and fungal abundances were right skewed and therefore log transformed. Abundances and diversity were tested against breastfeeding, length of breastfeeding, and delivery mode with linear mixed models. The models were tested for interaction between the abundance/diversity and age, which did not change the estimates and was therefore not included in the final model. The analyses were also controlled for use of antibiotics within 2 years without substantial effect on the associations; thus, unadjusted analyses are reported. Probiotic supplementation and antibiotic use were not associated with growth and are therefore not included. However, statistically significant associations were stratified into probiotic and placebo groups to ensure that the effect estimators for growth were consistent and to look for possible confounding by probiotics. The growth data were divided into three age groups for analysis:

FIGURE 2 | Bar charts of fungal and bacterial gut communities. **(A)** Mean relative abundances for the fungal genera (>1% abundant) for each age group. Each color designates a genus. Number of samples for each bar is stated in brackets below the bar. **(B)** Mean relative abundances for the bacterial genera for each age group. Each color designates a genera. The number of samples is stated in brackets below the bar.

0–1, 1–2, and 2–9 years. These analyses were computed in StataMP15 (StataCorp) and remained uncorrected due to their exploratory nature. Alpha diversity was measured in Shannon-index (H'), representing the individual microbial diversity and computed using PAST (26). Fungal diversity was only used as material description and not in the final analyses due to a low sample size. Fungal detection in samples were tested against antibiotic administration in children and length of breastfeeding and showed no significant differences. Correlation of consecutive samples was evaluated with Pearson's pair-wise correlation. The microbial calculations and heatmaps were conducted in R using PhyloSeq (27). The OTU analyses were conducted with ANCOM in R (28), with zero-prevalence cut-off at 0.9, corrected for multiple comparisons by the Benjamini-Hochberg procedure and dichotomised into high and low SDS for height and weight at 0 SDS. The significance level was set to $\alpha = 0.05$.

RESULTS

Study Population
From the 415 mother-child pairs in the ProPACT study, we included 278 participants with at least one childhood fecal sample and clinical follow-up data (67%). Included participants' health characteristics are compared with those without fecal samples (**Table 2**), showing that included mothers were 8 months older and more educated, and their offspring had received more antibiotics between the first and second years of life.

Fungal and Bacterial Abundances and Diversities
The fungal and bacterial abundances and alpha diversities at different ages are shown in **Figures 1**, **2**. The fungal data have been reported previously (15) but not in relation to bacterial data.
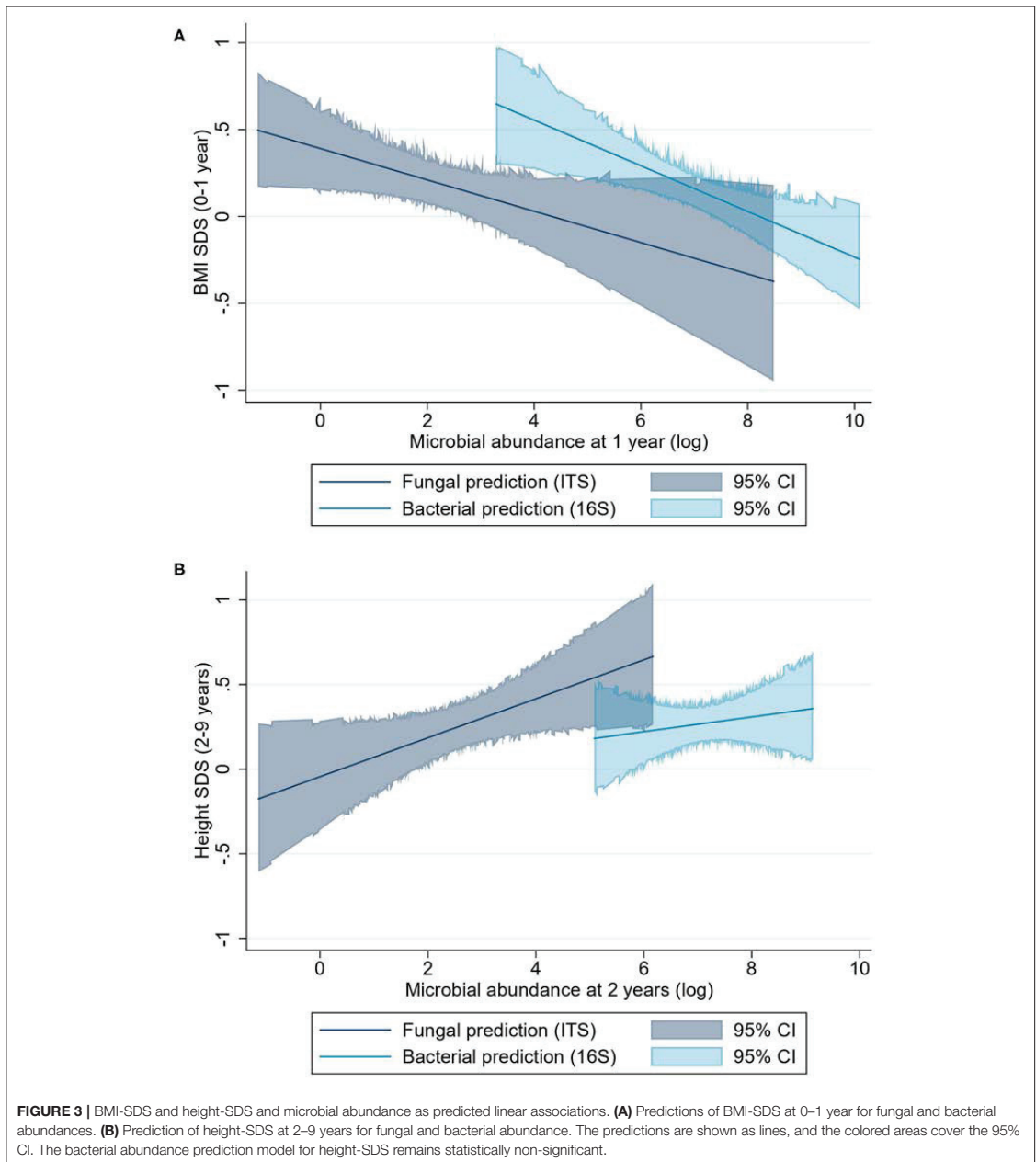
There was no association between mode of delivery and fungal abundance, bacterial abundance, or bacterial alpha diversity in fecal samples collected from children; nor did antibiotic treatment within 6 weeks, 1, or 2 years of age correlate with fungal abundance, bacterial abundance, or bacterial alpha diversity (not shown). Duration of breastfeeding was not associated with fungal abundance, bacterial abundances, or bacterial alpha diversity in the mixed model analysis including all age groups. In a subgroup analysis, breastfeeding longer than 1 year was associated with lower bacterial diversity at 1 year -0.23 H' (95% CI, -0.06 to -0.39), $P = 0.007$) but not at 2 years.

Microbiota and Childhood Growth (Height-SDS and BMI-SDS)
About 13 (median; IQR, 12–16) data points for both weight and height per child were included in the analysis.

Zero- to One-Year Growth
The linear mixed regression model suggested that higher fungal abundance at 1 year was associated with a lower BMI-SDS from 0 to 1 year ($\beta = -0.09$ BMI-SDS; 95% CI, -0.18 to -0.00; $P = 0.04$) (**Figure 3A**). However, visualization of the relationship between fungal abundance quartiles and height-SDS indicates that this relationship may not be linear (**Figure 4C**). There was a trend that a higher fungal abundance in the 3-month sample also was associated to lower BMI-SDS at 0–1 year, but this did not reach statistical significance ($\beta = -0.10$ BMI-SDS; 95% CI, -0.20 to 0.00; $P = 0.06$). Bacterial abundance and bacterial alpha diversity at 1 year were also associated with lower BMI-SDS at 0–1 year ($\beta = -0.13$ BMI-SDS; 95% CI, -0.22 to -0.04; $P = 0.004$; and $\beta = -0.19$ BMI-SDS; 95% CI, -0.39 to -0.00; $P = 0.047$, respectively) (**Figures 3A**, **4**, **Supplementary Figure 1**).
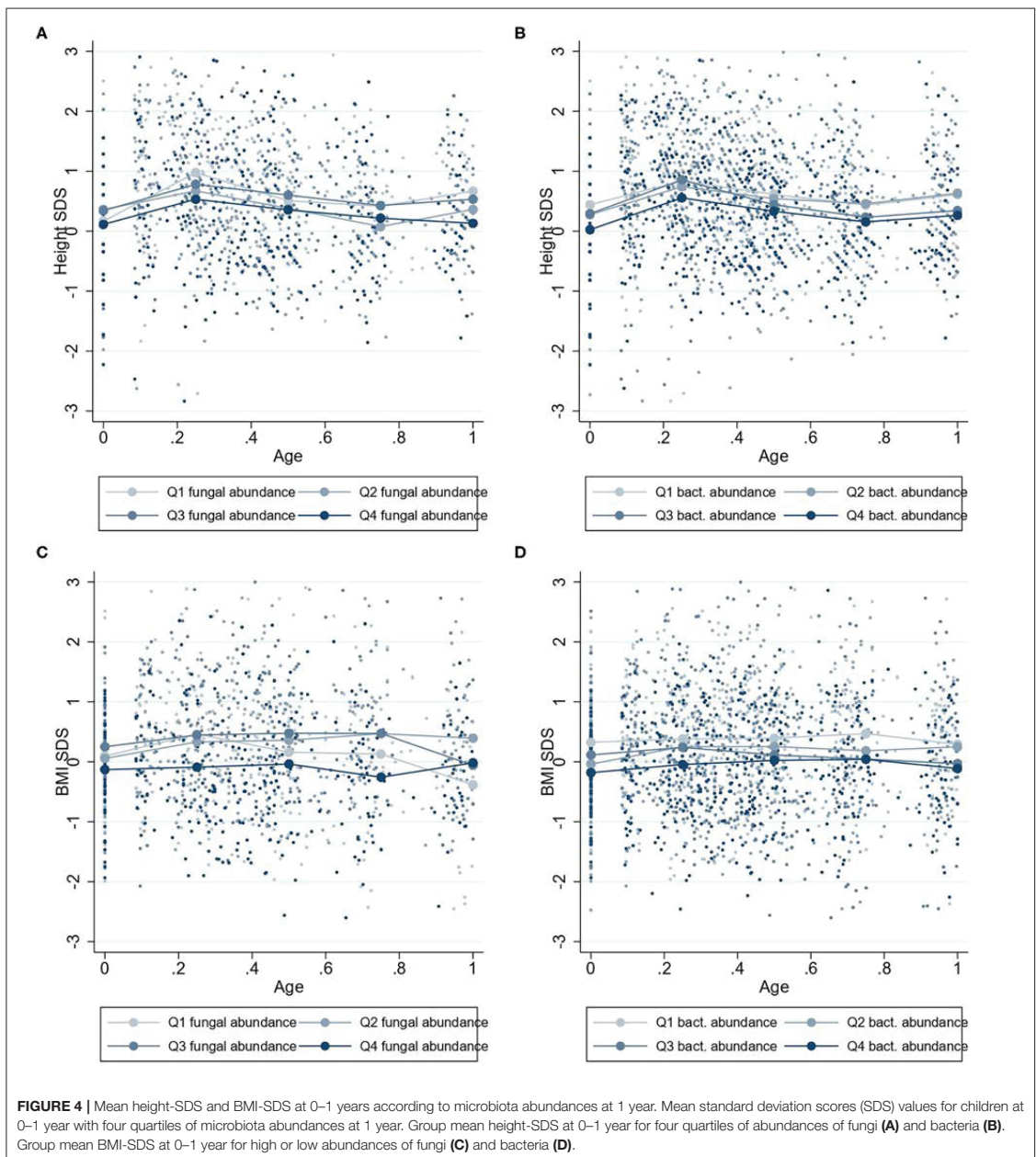
FIGURE 3 | BMI-SDS and height-SDS and microbial abundance as predicted linear associations. **(A)** Predictions of BMI-SDS at 0–1 year for fungal and bacterial abundances. **(B)** Prediction of height-SDS at 2–9 years for fungal and bacterial abundance. The predictions are shown as lines, and the colored areas cover the 95% CI. The bacterial abundance prediction model for height-SDS remains statistically non-significant.

One- to Two-Year Growth

There were no statistically or clinically significant associations between fungal or bacterial abundances or bacterial diversity and height-SDS or BMI-SDS from 1 to 2 years (data not shown).

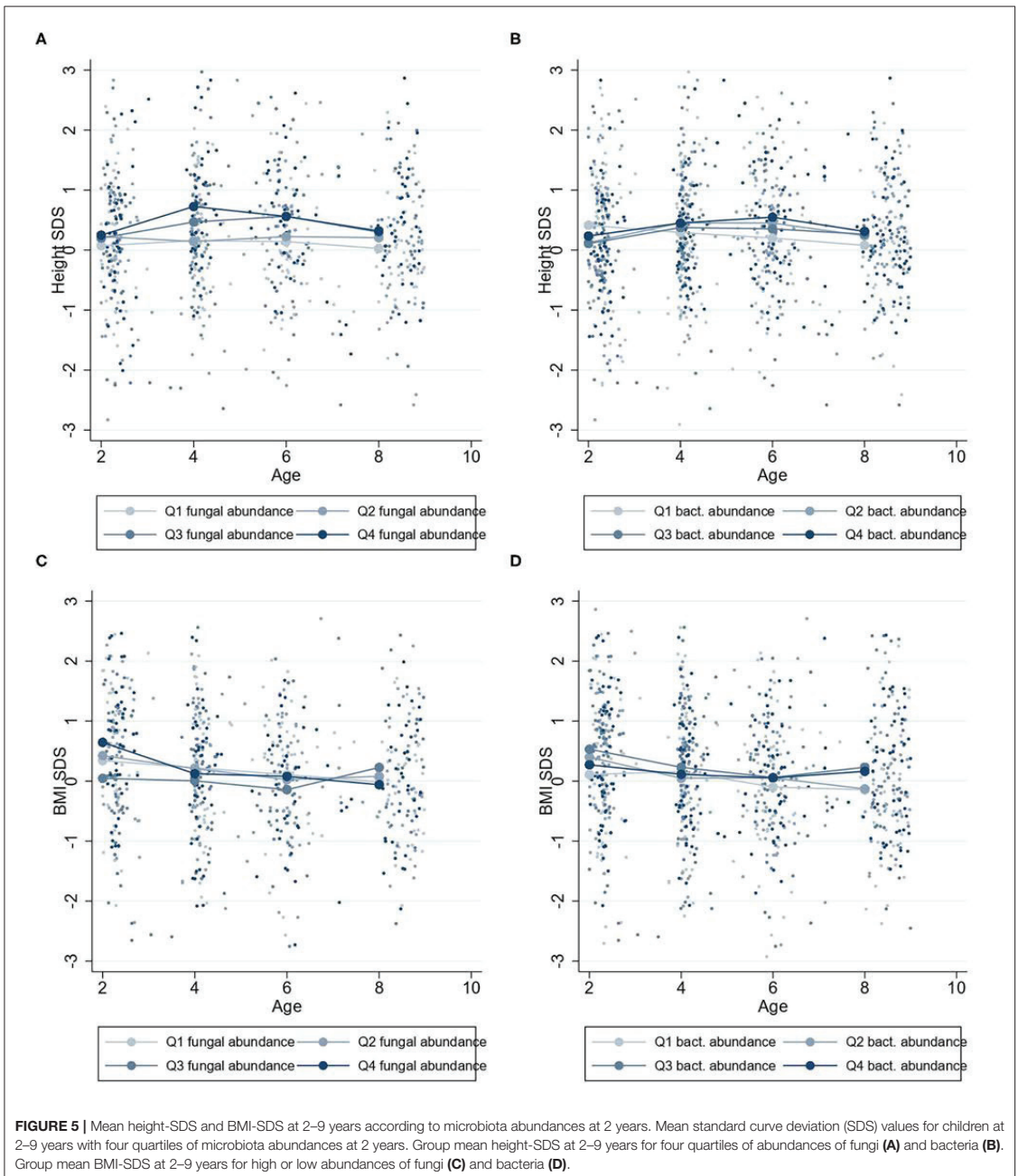### Two- to Nine-Year Growth

Higher fungal abundance at 2 years was positively associated with height-SDS at 2–9 years (β = 0.11 height-SDS; 95% CI, 0.00–0.22; P = 0.04) (**Figure 3B**), and by visualization, the mean height-SDS was greater for each quartile of fungal

FIGURE 4 | Mean height-SDS and BMI-SDS at 0–1 years according to microbiota abundances at 1 year. Mean standard deviation scores (SDS) values for children at 0–1 year with four quartiles of microbiota abundances at 1 year. Group mean height-SDS at 0–1 year for four quartiles of abundances of fungi **(A)** and bacteria **(B)**. Group mean BMI-SDS at 0–1 year for high or low abundances of fungi **(C)** and bacteria **(D)**.

abundance at all time points (**Figure 5**). There was no association with fungal abundance at 2 years and BMI-SDS at 2–9 years. Also, there was no association between bacterial abundance or bacterial alpha diversity and height-SDS or BMI-SDS (**Figure 3**, **Supplementary Figure 1**).

Microbial Taxa

Neither height-SDS nor BMI-SDS appeared to be associated with compositions of microbial communities (**Supplementary Figure 2**). For longitudinal ANCOM models analysing individual taxa, no individual taxa were associated with

FIGURE 5 | Mean height-SDS and BMI-SDS at 2–9 years according to microbiota abundances at 2 years. Mean standard curve deviation (SDS) values for children at 2–9 years with four quartiles of microbiota abundances at 2 years. Group mean height-SDS at 2–9 years for four quartiles of abundances of fungi **(A)** and bacteria **(B)**. Group mean BMI-SDS at 2–9 years for high or low abundances of fungi **(C)** and bacteria **(D)**.

anthropometry. For ANCOM models including fecal samples at 2 years and anthropometry from 2 to 9 years, there was a negative association between relative abundance of *Bifidobacterium*

longum and height-SDS (**Supplementary Figure 3**). No other microbial taxa differed significantly with height-SDS or BMI-SDS, indicating that the taxa abundances stay

relatively stable with increased total abundance (at least for bacteria).

DISCUSSION

In this prospective population study, we found that greater abundances of gut mycobiota at 2 years were associated with increased height in children at 2–9 years. Furthermore, greater fungal and bacterial abundance and greater bacterial diversity at 1 year of age were associated with lower BMI-SDS in children in the first year of life. These new findings may suggest a link between the gut microbiota and childhood growth.

A greater fungal abundance in the 2-year fecal samples was associated with taller children from 2 to 9 years and was supported by an increasing trend in height-SDS in the quartile analysis (**Figure 5A**). Assuming the range of fungal abundance of 6 units at 2 years (**Figure 1**), this would represent a difference of about 3–4 cm at 6 years of age. This finding was in accordance with our hypothesis that a more abundant mycobiota could affect future height. Growth stimulation by adding *S. cerevisiae* into the feed has been shown in piglets and dairy cows, possibly through the growth hormone (GH) axis (29, 30). *S. cerevisiae* is one of many fungi found in the human gut mycobiota, with increasing abundance toward 2 years of age (15). The GH axis becomes the driving growth regulator from 1 to 2 years when entering the childhood growth phase (1, 2), which may justify why the association between fungal abundance and height growth becomes apparent from 2 years of age.

Children hosting higher abundances of fungi and bacteria and higher bacterial alpha diversity at 1 year had lower BMI in their first year of life, in this cohort of healthy well-nourished Norwegian children with BMI-SDS normally distributed around zero. Assuming the same range of microbial abundance of 6 units at 1 year (**Figure 1**), this would represent a BMI difference of about 1 BMI unit at 1 year of age. We also observed a tendency that higher fungal abundance at 3 months correlated with lower BMI-SDS at 0–1 years. Thus, the relation between BMI-SDS and microbial abundance and bacterial diversity depicts a process happening after the first months of life. Our data do not prove a causal direction in the analysis of microbial abundances/diversity and infantile BMI. However, the indication at 3 months could suggest that at least fungal abundance increases at least within a few months after birth in those with lower infantile BMI-SDS. The gut microbial abundance and diversity normally increase from birth to 1 year (31), and having a considerably high microbial diversity and abundance as food is introduced might be favorable for a lower BMI development. High bacterial diversity has been associated with childhood and adult leanness (32), in accordance with our finding.

Interestingly, the taxonomic analysis yielded no associations with BMI-SDS, using established and conservative methods. This is in contrast with several recent investigations that showed divergent associations with BMI and microbes (5–8). The lack of consistent findings could be due to sample variations, liberal statistical tests, or varying methods. By investigating microbial total abundance, we observed links to both height velocity and lower BMI. The absolute abundances appear thus to reveal more than the microbial composition concerning growth. More rigorous methods and statistical tools in this research field are required (and are under development) and will hopefully provide more robust analyses in the future.

We found no associations between antibiotic usage and growth. This contrasts other human studies showing increased childhood longitudinal growth after broad-spectrum antibiotics treatments (12, 13). The livestock growth promotors are low doses of broad-spectrum antibiotics continually, whereas the children in our cohort received short-time treatments of narrow-spectrum antibiotics. Thus, the different treatment lengths and varying antimicrobial spectrums may explain the differing findings.

This large population-based cohort of healthy Norwegian children has a 9-year-long follow-up that enabled us to explore associations between childhood growth and gut microbiota. A conservative OTU approach decreased the rate of type I error findings, and the bacterial analysis is robust. We managed to quantify fungal DNA abundances in most samples, although the lack of well-established fungal DNA extraction protocols validated for stools might have reduced the extraction rate of fungal DNA. Underlining the difficulty of fungal analyses, low fungal amounts in general and a bacterially focused DNA extraction made us unable to describe the total fungal diversity as only 37 samples were sequenced for fungi, although 64% of samples were quantified to measure microbial abundances. Furthermore, the parents collected the fecal samples, which could represent a random sampling misclassification. There are no databases for the number of repeats of fungal ribosomal RNA operons (*rrn*) for every fungal species detected, which could impair the quantification precision. Also, as for all DNA-based microbiome sequencing studies, the proportion of inactive transient microbes remains unknown. Therefore, these findings should be replicated, preferably with fungal-specific extraction kits. However, this is the first study to show an association between childhood growth and early gut mycobiota abundance, introducing a novel research area on how early gut mycobiota may impact human health and might possibly serve as a growth promotion target.

CONCLUSION

In a 9-year follow-up of healthy well-nourished children, increased gut fungal abundance appears to be more strongly associated with childhood anthropometrics (increased height velocity and reduced BMI) than bacterial abundance and diversity (reduced BMI only). Analysing gut fungi remains challenging; nevertheless, the findings call for more research on how the mycobiota could affect human growth physiology.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The datasets of the current study are not publicly available due to legislation of the Norwegian Authorities on the sharing of personal yet non-identifiable data. The datasets

are available upon reasonable request and are stored at our university. Requests to access these datasets should be directed to Torbjørn Øien, torbjorn.oien@ntnu.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethical Committee of Central Norway (2014/1796). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KS was involved in the microbiome data generation, did the statistical analysis, interpretation, and drafted the initial manuscript. MS and PJ contributed to the statistical analysis and interpretation and reviewed and revised the manuscript. EA and KR were involved in the microbiome data generation and reviewed and revised the manuscript. TØ designed the study, enrolled the participants, coordinated and supervised the data collection, and reviewed and revised the manuscript. DU and SS were involved in the conceptualisation and design of the study and reviewed and revised the manuscript. RØ supervised the study, conceptualized and designed the study, interpreted the data, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.572538/full#supplementary-material

REFERENCES

1. Murray PG, Clayton PE. Endocrine control of growth. *Am J Med Genet Part C.* (2013) 163:76–85. doi: 10.1002/ajmg.c.31357
2. Karlberg J. A biologically-oriented mathematical model (ICP) for human growth. (1989) 78:70–94. doi: 10.1111/j.1651-2227.1989.tb11199.x
3. Schwarzer M. Gut microbiota: puppeteer of the host juvenile growth. *Curr Opin Clin Nutr Metab Care.* (2018) 21:179–83. doi: 10.1097/MCO.0000000000000463
4. Robertson RC, Manges AR, Finlay BB, Prendergast AJ. The human microbiome and child growth - first 1000 days and beyond. *Trends Microbiol.* (2019) 27:131–47. doi: 10.1016/j.tim.2018.09.008
5. Forbes JD, Azad MB, Vehling L, Tun HM, Konya TB, Guttman DS, et al. Association of exposure to formula in the hospital and subsequent infant feeding practices with gut microbiota and risk of overweight in the first year of life. *JAMA Pediatr.* (2018) 172:e181161. doi: 10.1001/jamapediatrics.2018.1161
6. Stanislawski MA, Dabelea D, Wagner BD, Iszatt N, Dahl C, Sontag MK, et al. Gut microbiota in the first 2 years of life and the association with body mass index at age 12 in a norwegian birth cohort. *mBio.* (2018) 9:e01751–18. doi: 10.1128/mBio.01751-18
7. Korpela K, Zijlmans MA, Kuitunen M, Kukkonen K, Savilahti E, Salonen A, et al. Childhood BMI in relation to microbiota in infancy and lifetime antibiotic use. *Microbiome.* (2017) 5:26. doi: 10.1186/s40168-017-0245-y
8. Tun HM, Bridgman SL, Chari R, Field CJ, Guttman DS, Becker AB, et al. Roles of birth mode and infant gut microbiota in intergenerational transmission of overweight and obesity from mother to offspring. *JAMA Pediatr.* (2018) 172:368–77. doi: 10.1001/jamapediatrics.2017.5535
9. Subramanian S, Huq S, Yatsunenko T, Haque R, Mahfuz M, Alam MA, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature.* (2014) 510:417–21. doi: 10.1038/nature13421
10. Vatanen T, Plichta DR, Somani J, Munch PC, Arthur TD, Hall AB, et al. Genomic variation and strain-specific functional adaptation in the human gut microbiome during early life. *Nat Microbiol.* (2019) 4:470–9. doi: 10.1038/s41564-018-0321-5
11. Cromwell G. Why and how antibiotics are used in swine production. *Anim Biotechnol.* (2002) 13:7–27. doi: 10.1081/ABIO-120005767
12. Saari A, Virta LJ, Sankilampi U, Dunkel L, Saxen H. Antibiotic exposure in infancy and risk of being overweight in the first 24 months of life. *Pediatrics.* (2015) 135:617–26. doi: 10.1542/peds.2014-3407
13. Yang YJ, Sheu BS, Yang HB, Lu CC, Chuang CC. Eradication of *Helicobacter pylori* increases childhood growth and serum acylated ghrelin levels. *World J Gastroenterol.* (2012) 18:2674–81. doi: 10.3748/wjg.v18.i21.2674
14. Chaucheyras-Durand F, Walker ND, Bach A. Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. *Anim Feed Sci Technol.* (2008) 145:5–26. doi: 10.1016/j.anifeedsci.2007.04.019
15. Schei K, Avershina E, Øien T, Rudi K, Follestad T, Salamati S, et al. Early gut mycobiota and mother-offspring transfer. *Microbiome.* (2017) 5:107. doi: 10.1186/s40168-017-0319-x
16. Costalos C, Skouteri V, Gounaris A, Sevastiadou S, Triandafilidou A, Ekonomidou C, et al. Enteral feeding of premature infants with *Saccharomyces boulardii. Early Hum Dev.* (2003) 74:89–96. doi: 10.1016/S0378-3782(03)00090-2
17. Xu L, Wang Y, Wang Y, Fu J, Sun M, Mao Z, et al. A double-blinded randomized trial on growth and feeding tolerance with *Saccharomyces boulardii* CNCM I-745 in formula-fed preterm infants. *J Pediatr.* (2016) 92:296–301. doi: 10.1016/j.jped.2015.08.013
18. Dotterud CK, Avershina E, Sekelja M, Simpson MR, Rudi K, Storro O, et al. Does maternal perinatal probiotic supplementation alter the intestinal microbiota of mother and child? *J Pediatr Gastroenterol Nutr.* (2015) 61:200–7. doi: 10.1097/MPG.0000000000000781
19. Juliusson PB, Roelants M, Nordal E, Furevik L, Eide GE, Moster D, et al. Growth references for 0-19 year-old Norwegian children for length/height, weight, body mass index and head circumference. *Ann Hum Biol.* (2013) 40:220–7. doi: 10.3109/03014460.2012.759276

20. Avershina E, Lundgard K, Sekelja M, Dotterud C, Storro O, Oien T, et al. Transition from infant- to adult-like gut microbiota. *Environ Microbiol.* (2016) 18:2226–36. doi: 10.1111/1462-2920.13248

21. Fiedorova K, Radvansky M, Nemcova E, Grombirikova H, Bosak J, Cernochova M, et al. The impact of DNA extraction methods on stool bacterial and fungal microbiota community recovery. *Front Microbiol.* (2019) 10:821. doi: 10.3389/fmicb.2019.00821

22. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng.* (2005) 89:670–9. doi: 10.1002/bit.20347

23. Tang J, Iliev ID, Brown J, Underhill DM, Funari VA. Mycobiome: approaches to analysis of intestinal fungi. *J Immunol Methods.* (2015) 421:112–21. doi: 10.1016/j.jim.2015.04.004

24. Edwards JE, Forster RJ, Callaghan TM, Dollhofer V, Dagar SS, Cheng Y, et al. PCR and omics based techniques to study the diversity, ecology and biology of anaerobic fungi: insights, challenges and opportunities. *Front Microbiol.* (2017) 8:1657. doi: 10.3389/fmicb.2017.01657

25. Taylor DL, Walters WA, Lennon NJ, Bochicchio J, Krohn A, Caporaso JG, et al. Accurate estimation of fungal diversity and abundance through improved lineage-specific primers optimized for Illumina amplicon sequencing. *Appl Environ Microbiol.* (2016) 82:7217–26. doi: 10.1128/AEM.02576-16

26. Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. *Palaeontol Electr.* (2001) 4:9. Available online at: http://palaeo-electronica.org/2001_1/past/issue1_01.htm

27. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217

28. Mandal S, Van Treuren W, White RA, Eggesbo M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis.* (2015) 26:27663. doi: 10.3402/mehd.v26.27663

29. Jiang Z, Wei S, Wang Z, Zhu C, Hu S, Zheng C, et al. Effects of different forms of yeast *Saccharomyces cerevisiae* on growth performance, intestinal development, and systemic immunity in early-weaned piglets. *J Anim Sci Biotechnol.* (2015) 6:47. doi: 10.1186/s40104-015-0046-8

30. Nasiri AH, Towhidi A, Shakeri M, Zhandi M, Dehghan-Banadaky M, Colazo MG. Effects of live yeast dietary supplementation on hormonal profile, ovarian follicular dynamics, and reproductive performance in dairy cows exposed to high ambient temperature. *Theriogenology.* (2018) 122:41–6. doi: 10.1016/j.theriogenology.2018.08.013

31. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe.* (2015) 17:690–703. doi: 10.1016/j.chom.2015.05.012

32. Sze MA, Schloss PD. Looking for a signal in the noise: revisiting obesity and the microbiome. *mBio.* (2016) 7:e01018-16. doi: 10.1128/mBio.01018-16

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1 # Allergy-related diseases and early gut fungal and bacterial microbiota in children

3 **Short title: Allergy and gut myco- and microbiota in children**
4 **Journal: Clinical and Translational Allergy**

5 **Kasper Schei*[1], Melanie Rae Simpson[2,3], Torbjørn Øien[2], Saideh Salamati[4], Knut Rudi[5],**
6 **Rønnaug Astri Ødegård[1,4].**

7 [1]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, NTNU –
8 Norwegian University of Science and Technology, Trondheim, Norway;
9 [2]Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, NTNU –
10 Norwegian University of Science and Technology, Trondheim, Norway;
11 [3]Clinic of Laboratory Medicine, St. Olav's Hospital, Trondheim, Norway.
12 [4]Regional Centre of Obesity Research and Innovation (ObeCe), St. Olav's Hospital, Trondheim,
13 Norway;
14 [5]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås,
15 Norway;

16 *** Correspondence:**
17 Kasper Schei
18 kasperschei@gmail.com

22 **Language:** British English

This paper is awaiting publication and is not included in NTNU Open

NTNU
Norwegian University of
Science and Technology