The intestinal mycobiota of zebrafish – community profiling and exploration of the impact of yeast exposure early in life

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Preface

This dissertation is submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research that was carried out over a period of 4 years from 06.06.2014 to 10.08.2018. This project was funded by “Bioteknologi – en framtidsrettet næring” (FR-274/16) from the Nordland County Council, with additional support from “NorMur” (13011) by the Nord University. Prabhugouda Siriyappagouder is supported by an International Fellowship from the Indian Council of Agricultural Research (ICAR), India.

The project team consisted of the following members:

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Jorge M. O. Fernandes, Professor, FBA, Nord University: main supervisor

Kiron Viswanath, Professor, FBA, Nord University: co-supervisor

Prabhugouda Siriyappagouder

Bodø, 13th August, 2018

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List of abbreviations

16S rRNA	-	16S ribosomal RNA
bp	-	base pairs
CFU	-	Colony-forming units
CLRs	-	C-type lectin receptors
CR	-	Conventionally-raised
GF	-	Germ-free
GI	-	Gastrointestinal
ITS	-	Internal transcribed spacer
NGS	-	Next-generation sequencing
OTUs	-	Operational taxonomic units
PAMPs	-	Pathogen-associated molecular patterns
PCR	-	Polymerase chain reaction
PRRs	-	Pattern recognition receptors
QIIME	-	Quantitative insights into microbial ecology
rRNA	-	Ribosomal RNA
TLRs	-	Toll-like receptors

Abstract

The complex microbial assembly of bacteria, fungi and viruses that inhabit the gastrointestinal tract of vertebrates has a remarkable influence on the host homeostasis and diseases. Most of the previous studies have focused only on the bacterial community but there is increasing interest in its fungal component (mycobiota), since intestinal mycobiota can also influence host physiology, metabolism and immunity. Fungal communities associated with fish and their importance are poorly understood. The aim of this thesis was to characterize the fungal communities found in the intestine of zebrafish and to understand the impact of commensal yeast on the bacterial communities and transcriptomic responses of host. Three connected studies were performed: 1. Molecular profiling of fungal communities in the intestine of wild-caught and laboratory-reared zebrafish; 2. Determining the influence of fish-derived yeast (either *Pseudozyma* or *Debaryomyces*) on the intestinal bacterial composition and diversity in zebrafish larvae; 3. Evaluation of the impact of host-associated yeast on the zebrafish larvae transcriptome.

Molecular profiling showed that zebrafish mycobiota consists of many fungal phylotypes, belonging mainly to phyla Ascomycota and Basidiomycota. Our results also indicated that wild-caught zebrafish have a distinct and more diverse fungal community than their laboratory–reared counterparts. Yeast exposure leads to marked alterations in the abundance of bacterial communities and these changes could be related to shifts in the relative abundance of core taxa and elevation of certain beneficial bacteria. Transcriptomic analysis indicated that exposure to yeast at early developmental stages influences the host transcriptome by modulating the expression of genes involved in metabolic and immune-related processes. Taken together, these studies shed light into the diverse fungal consortium in gastrointestinal tract of zebrafish and revealed the potential ability of fish-derived yeast to modulate the bacterial community and transcriptomic responses of host after transient exposure during early larval stages.

1. Introduction

Microbiota is an assemblage of commensal microorganisms that include pathogens and mutualists present in and on multicellular organisms (Marchesi and Ravel. 2015). The host and their microbiota can be considered as holobiont, since they form an ecological unit (van de Guchte et al. 2018). Microbiota are found in different areas of the body—including the skin, gastrointestinal (GI), urogenital, and respiratory tracts. However, the composition of the microbiota in different body sites varies significantly (Ursell et al. 2012). The GI tract of vertebrates, including fish, represents the largest interface between the host, their microbiota and the environment (Thursby and Juge. 2017, Wang et al. 2017). Over the past decades, studies related to GI microbiota have been at the forefront of biological research because of the involvement of microbes in a range of physiological processes, including metabolic, and immunological functions (Brestoff and Artis. 2013). Intestinal microbiota offers many benefits to the host, such as the synthesis of essential vitamins and enzymes, energy harvest by hydrolyzing complex molecules, stimulation of the immune defense and protection of host from infections (Brestoff and Artis. 2013). The microbiota composition is unique to each individual, and its establishment and composition are influenced by various factors, e.g., diet, geographical location, and genome and health status of the host (Rodríguez et al. 2015a). For instance, the microbiota composition is determined by the host genotype, since monozygotic twins have more similar microbiota than dizygotic twins, and family members share similar microbial communities with their relatives than unrelated individuals (Goodrich et al. 2014, Turnbaugh et al. 2009).

The GI tract is a complex environment hosting a variety of microbes, mainly bacteria, archaea, viruses and eukaryotes, collectively referred to as ‘gut microbiota’ (Marchesi et al. 2016). Most studies to date focused solely on bacterial communities and their involvement in host physiological functions. Recently, there is increasing evidence pointing to the importance of other microbial members, including fungi, and their influence on a variety of host biological processes and association with diseases (Huseyin et al. 2017a, Huseyin et al. 2017b). The alteration of diversity and composition of fungal

community is associated with obesity, diabetes, hepatitis and inflammatory bowel diseases. Sokol et al (2017) observed an increased ratio of certain fungal phylum in inflammatory bowel disease patients compared to healthy subjects. An elevated level of fungal diversity in mucosa is also associated with an increase in prevalence of intestinal inflammation in patients with Crohn's disease (Li et al. 2014). GI fungi may also be involved in the gut-brain axis, a bidirectional communication between the GI tract and the brain, through immune and non-immune mediated crosstalk systems (Enaud et al. 2018). For example, chronic fungal infection increases the risk of Alzheimer's disease by secretion of amyloidogenic fungal proteins (amyloid) that alters the aggregation of the Aβ protein in the brain (Alonso et al. 2014).

1.1. Mycobiota

Compared to bacteria, the fungal community (mycobiota), are considerably less represented in the GI tract. The term "mycobiota" is derived from the Greek words *myco* (fungus) and *bios* (life) and is used to refer to a fungal community in a defined environment (Underhill and Iliev. 2014). Fungi is a kingdom comprised of diverse eukaryotic organisms—yeasts, molds, and mushrooms, which appear in various forms and shapes. It is currently classified into 5 different phyla (Figure 1) based on their mode of sexual reproduction and molecular data (Blackwell et al. 2012). Fungi are ubiquitous but only some of them, particularly yeasts, are most commonly associated with human and animals, including fish. Yeasts are microscopic fungi that reproduce by budding and/ or fission, and belong to the phyla Ascomycota and Basidiomycota (Kurtzman and Fell. 1998). Yeasts that inhabit the GI tract as commensal mutualists or opportunistic pathogens are estimated to make up approximately 0.03 to 2% of the microbes in the intestinal tract (Rodríguez et al. 2015b). Nevertheless, they are involved in various host physiological processes, including metabolic functions, regulation of the immune responses and shaping the microbial community structure (Seed 2015). Most of our knowledge about fungal community structure and functions is derived mainly from studies on mammals, since this is a relatively new research area (Iliev et al. 2012, Qiu et al. 2015, Foster et al. 2013, Hamad et al. 2012, Hoffmann et al. 2013).

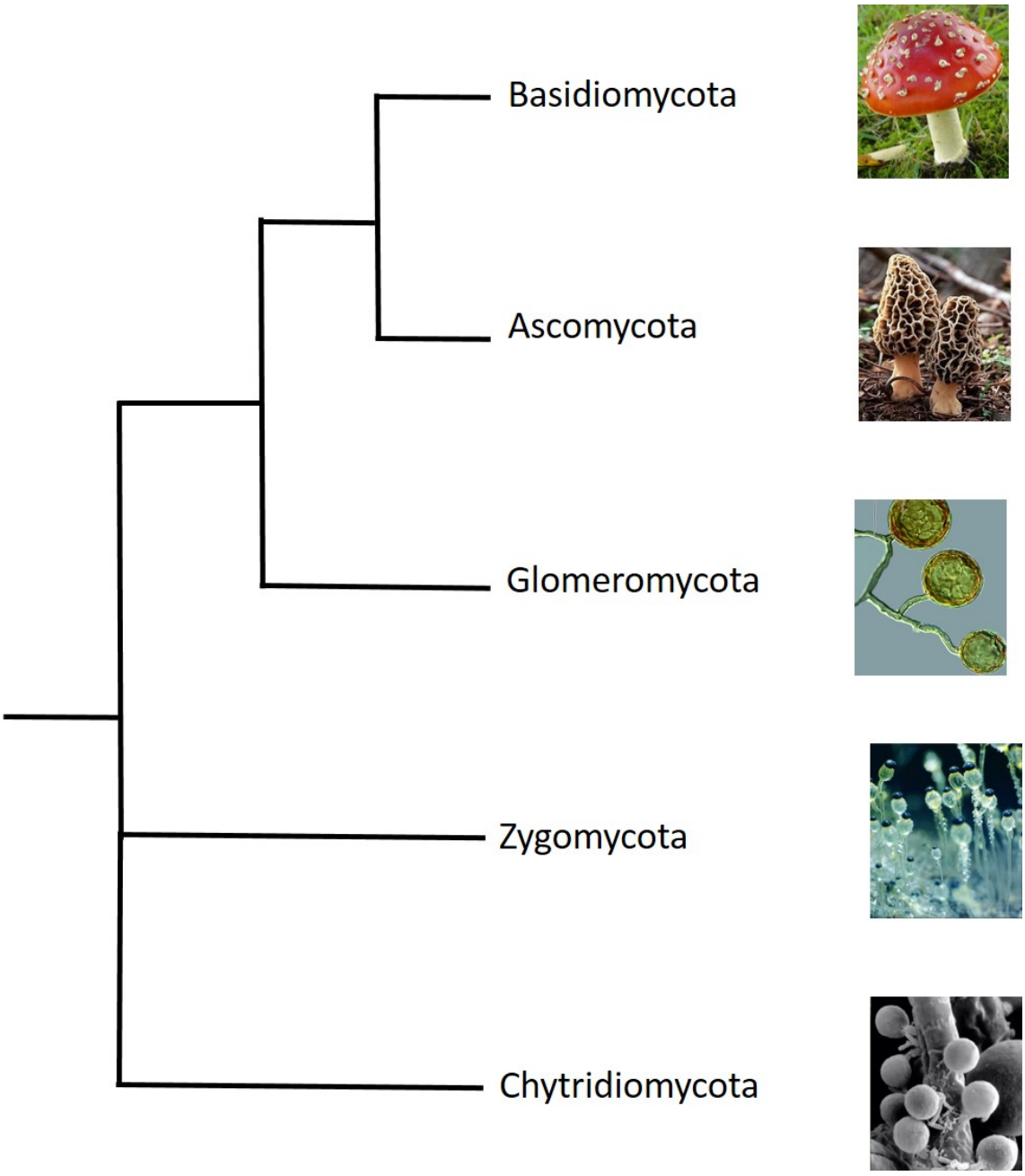

Figure 1. Schematic representation of the Fungi phylogeny. The Kingdom Fungi is subdivided into 5 different phyla (only traditional phyla are represented) based on their mode of sexual reproduction and molecular data. Branch lengths are not proportional to evolutionary distance. The figure is modified from Blackwell et al. (2012), Moore (2013) and Hibbett et al. (2007).

1.2. Intestinal mycobiota in mammals

The GI tract of animals has variable proportions of different members of mycobiota. Initial culture-dependent surveys suggested that it is comprised of a restricted number of species. Recent advances in molecular approaches have allowed for the detailed investigation of the diversity of mycobiota and their association with health and disease status of several mammalian hosts, including humans, mice, pigs, dogs and ruminant and non-ruminant herbivores (Foster et al. 2013, Huffnagle and Noverr. 2013, Iliev et al. 2012, Qiu et al. 2015, Liggenstoffer et al. 2010, Huseyin et al. 2017a).

1.2.1. Human

The GI tract of a healthy adult human comprises a diverse population of fungal community of approximately 13 % of the total gut microbial volume; with around 247 species belonging to 126 different fungal genera (Gouba and Drancourt. 2015). It is dominated by *Candida, Saccharomyces, Trichosporon, Rhodotorula* and *Cladosporium* spp. (Hoffmann et al. 2013). The genus *Candid*a includes approximately 160 species, most of which are adapted to live in varying conditions of different hosts (Blaschke-Hellmessen 1999). *Candida albicans* consistently ranks as the most successful fungal colonizer in the GI tract (MacCallum 2010).

1.2.2. Mice

A diverse mycobiota was observed in the intestinal contents of mice using oligonucleotide fingerprinting of rRNA genes (Scupham et al. 2006). Early metagenomics studies showed that mouse contains a diverse fungal community and it is home to over 50 genera; *C. tropicalis, Saccharomyces cerevisiae*, *Trichosporon* spp. are the most abundant yeasts (Iliev et al. 2012). These initial studies characterized yeast communities in faecal samples, but we now know that there are remarkable differences in the fungal community composition between mucosa and faeces collected from different gut segments in mice (Qiu et al. 2015).

1.2.3. Pig

Knowledge about yeast colonization in the porcine GI tract is mainly obtained from culture-based studies; *Kazachstania slooffiae*, *Galactomyces geotrichum*, *Candida catenulata* and *Candida glabrata* were the most commonly isolated yeasts (Urubschurov et al. 2008). There are compositional differences between the yeast communities of piglets that were reared in commercial and experimental farms. Piglets maintained at commercial farms have more abundant *G. geotrichum*, *K. slooffiae* and *C. catenulate* in their GI tract, whereas *K. slooffiae* and *C. glabrata* were the predominant yeasts in piglets that were reared in an experimental farm (Urubschurov et al. 2008).

1.2.4. Dogs

A cultivation- and PCR-based study investigated the yeast community in the intestine of healthy dogs. It revealed the predominance of Saccharomycetes in most samples and identified around 51 yeast phylotypes (Suchodolski et al. 2008). Another report described the mycobiota present in faecal samples collected from healthy dogs and dogs with acute, non-hemorrhagic diarrhea. Among the 5 phyla identified, Ascomycota and Basidiomycota were dominant in both groups of diseased and healthy dogs (Foster et al. 2013). An 18S rDNA gene-based pyrosequencing study revealed that Ascomycota was the predominant fungal phylum, accounting for 99.6 % of the total sequences (Handl et al. 2011). *Candida. castelli* is the more abundant fungal species of the class Saccharomycetes. Similar to mice and humans, dogs also have a smaller proportion of mycobiota (Swanson et al. 2011).

1.2.5. Cats

Fungi constitute approximately 0.02 to 0.3% of the total faecal microbiota in cats (Minamoto et al. 2012). In addition, pyrosequencing of the fungal 18S rRNA gene identified 16 fungal genera in faecal samples. Ascomycota represents the dominant phylum and *Aspergillus* and *Saccharomyces* are the most abundant fungal genera found in faeces (Handl et al. 2011).

1.3. Fish-associated microbiota

Fishes represent the most diverse group of all vertebrates and interact intimately with their surrounding aquatic environment; hence, they are in close association with microorganisms that exist there (Clements et al. 2014). Similar to other vertebrates, the GI tract of fish comprises bacteria, archaea, viruses, protozoans and fungi (especially yeasts) (de Bruijn et al. 2018). It harbors around 15 to 17 bacterial phyla (Egerton et al. 2018). Proteobacteria, Bacteroidetes, Tenericutes and Firmicutes are the dominant phyla and constitute 90% of fish intestinal microbiota, indicating their functional significance for the host (Ghanbari et al. 2015). Other common phyla are present in lower proportions, mainly Actinobacteria, Fusobacteria, Deinococcus-Thermus, Verrucomicrobia (Romero et al. 2014). *Aeromonas*, *Pseudomonas, Vibrio, Acinetobacter, Corynebacterium, Alteromonas, Flavobacterium, Micrococcus* and *Bacillus* are frequently reported as dominant colonizers of the GI tract of both fresh and marine fishes (Wang et al. 2017).

Archaea are obligatory anaerobic microorganisms that are phylogenetically distinct from both bacteria and eukaryotic organisms (Figure 2). They are metabolically important and involved in fermentation and production of methane. Members of marine group II archaea were identified in European flounder (*Platichthys flesus*) and grey mullet (*Mugil cephalus*) (van der Maarel et al. 1998). A next-generation sequencing (NGS)-based study reported the occurrence of methanogenic archaea in the gut of grass carp (*Ctenopharyngodon idella*), and their existence was related to the processing of indigestible polysaccharides in diet (Wu et al. 2015).

Most research that explored the interaction between aquatic viruses and their hosts focused mainly on disease-causing viruses (Suttle 2007). Our knowledge of the biodiversity and functional role of viruses in holobiont ecosystem is still scarce. Nevertheless, we are beginning to understand the importance of viral communities associated with marine invertebrates (Laffy et al. 2016). For instance, a recent viral metagenomics study linked differences in viral community composition to cellular, immunological, geographical, and ecological niche of the host (Gudenkauf and Hewson

2016). Bacteriophages were also identified from the digestive tract of fish and shellfish collected from coastal waters of Pacific Ocean near Chile and Mexico (Roberto et al. 2010). Viruses influence the host health by exchanging genes with their host bacteria through infection, which modulates microbial diversity as well as functional potential (Chibani-Chennoufi et al. 2004).

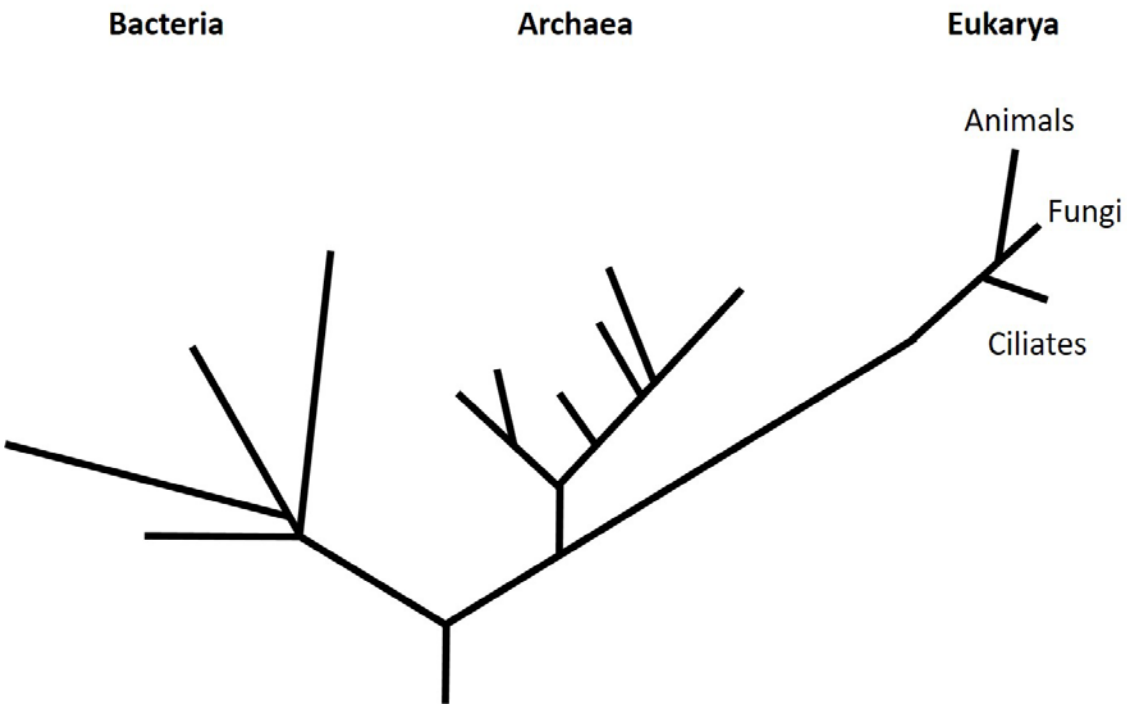

Figure 2. Schematic representation of the phylogenetic tree of life. Organisms are classified into three domains of life based on differences in ribosomal RNA (rRNA) gene sequence. The figure is modified from Woese (2000).

Protozoans are frequently associated with fish gills; members of the orders Clevelandellida and Vestibuliferida (Ciliates) have been identified in the GI tract of surgeonfish species belonging to the family Acanthuridae (Grim et al. 2002). Protozoans also account for a very small proportion of microbiota, and further studies are required to understand their role in health and diseases.

1.4. Mycobiota of fish

Yeasts are recognized as an integral component of the normal microbiota in fish, since they naturally occur in various tissues and organs, including the gut, gills, skin, mouth and faeces (Gatesoupe 2007). The proportion of yeast in fish ranges from non-detectable levels to 10^7 colony-forming units (CFU)/g of intestinal content (Gatesoupe 2007). The most common yeasts found in the GI tract of fish include *Saccharomyces, Debaryomyces, Leucosporidium, Candida, Rhodotorula, Pichia, Cryptococcus* and *Trichosporon* (Gatesoupe 2007, Romero et al. 2014, Raggi et al. 2014). Compared to our knowledge about mammalian fungi, little is known about fish-associated mycobiota and their influence on host physiology.

1.4.1. History of mycobiota studies in fish

To date, only few studies have focused on fish mycobiota. Yeasts were first identified from topsmelt (*Atherinops affinis littoralis*) and Pacific jack mackerel (*Trachurus symmetricus*) during 1960s (Uden and Castelo. 1963) (Figure 3). Yeasts have since been considered as normal members of the fish gut microbiota. In the following decade, there was little progress in this field, apart from the description of *Rhodotorula* sp. from the gut of European flounder (*Platichthys flesus*) (Newman Jr et al. 1972). During the early 1990s several yeast species were isolated from the intestine of rainbow trout (*Oncorhynchus mykiss*) and the properties of *Debaryomyces hansenii* isolated from the GI tract of rainbow trout and turbot (*Scophtalmus maximus*) were documented (Andlid et al. 1995, Andlid et al. 1998, Vazquez-Juarez et al. 1997). From this point onwards, studies on fish yeast became more popular due to their recognised beneficial and immunostimulatory properties; in particular, *S. cerevisiae* and *D. hansenii* were found to enhance growth, feed efficiency and immune response in different fish species (Gatesoupe 2007, Reyes-Becerril et al. 2008a, Ortuño et al. 2002). Subsequently, several reports described the probiotic and extracellular enzyme-producing potential of different yeast species that were isolated from the gut of estuarine water mullet (*Mugil* spp.) and other freshwater fish species, such as Indian carps (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*), Chinese carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon*

idella), common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) (Banerjee and Ghosh. 2014, Laconi and Pompei 2007). Identification and characterization of yeast from the fish GI tract were performed mainly using culture-dependent methods but later on molecular approaches (e.g., PCR) were also adopted (Andlid et al. 1998, Gatesoupe 2007, Laconi and Pompei 2007, Raggi et al. 2014). The first and only comprehensive profiling of fish-associated mycobiota using next generation sequencing was published last year. This report revealed the spatial difference in the mycobiota composition associated with the GI tract of royal panaque (*Panaque nigrolineatus*), and was affected by the wood content in their diet (Marden et al. 2017).

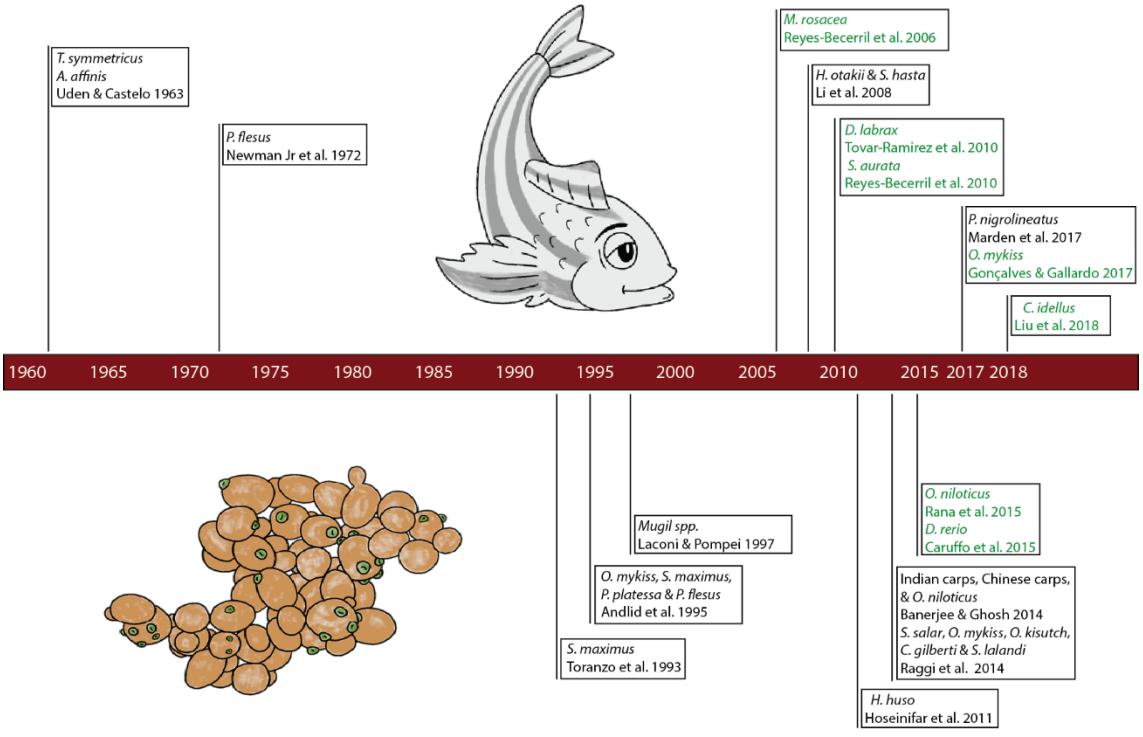

Figure 3. Historical timeline of fish mycobiota studies. Black font indicates yeast characterization studies and yeast colonization through feeding and/or exposure studies are in green font. Multiple publications on the same fish species are mentioned only once.

1.5. Fungal interactions

Polymicrobial communities that inhabit the GI tract engage in dynamic interactions, which are recognized as important determinants of community function (Arvanitis and Mylonakis 2015). Cross-kingdom mutual or antagonistic interactions, especially between mycobiota and bacteria, significantly influence the host health and disease (Fourie et al. 2016). Residents of gastrointestinal microbiota are constantly undergoing physical and chemical interactions among themselves or with the host cells for nutrients and space (Arvanitis and Mylonakis 2015). These complex interactions influence survival, colonization, and virulence of other microorganisms. Interestingly, the discovery of penicillin by Alexander Fleming, is a consequence of the interaction between bacteria (*Staphylococcus aureus*) and fungi (*Penicillium notatum*).

1.5.1. Fungal – bacterial interactions

Quorum sensing and morphology are the main basis for the interaction between fungi and bacteria. For example, the bacteria *Staphylococcus aureus* can successfully invade the host tissues by selectively attaching to the hyphal filaments of *C. albicans* than to the yeast form (Schlecht et al. 2015). Similarly, *Pseudomonas aeruginosa* forms a biofilm on the surface of *C. albicans* hyphae to kill the fungi. In contrast, it neither binds to nor kills the yeast form of *C. albicans* (Hogan and Kolter 2002). Yeasts can also produce various inhibitory molecules such as toxins, ethanol and proteases, which affect the morphology and prevents the growth and colonization of bacteria on the host mucosal surfaces (Hatoum et al. 2012). Exogenous addition of *C. albicans* during antibiotic treatment in mice results in overgrowth of yeast and a substantially altered reassembly of the bacterial community (Downward et al. 2013). It also modulates the expression of virulence genes by producing quorum sensing inhibitory compounds (Rasmussen et al. 2005). Yeast and bacteria naturally occur in close association and are widely used in the production of fermented foods, such as cheese, kefir, kimchi and many others (Hatoum et al. 2012). Some yeasts species are known to produce vitamins, amino acids or purines that favour the growth of lactic acid bacteria (Ponomarova et al. 2017). Subsequently, lactic acid bacteria reduce the pH by producing organic acids, which creates favourable conditions for the growth and proliferation of yeast (Viljoen 2006). Co-existence of

Candida albicans and *Helicobacter pylori* in the GI tract can enhance the pathogenesis of peptic ulcers (Karczewska et al. 2009).

1.5.2. Fungal – fungal interactions

In addition to interacting with bacteria, fungi also interact with other fungi in their niches. For examples, the quorum-sensing molecule farnesol produced by *C. albicans* is able to control the morphology of the pathogenic fungi *Aspergillus niger* and *Fusarium graminearum* by inhibiting their hyphal growth, conidiation and germination (Lorek et al. 2008, Semighini et al. 2008). Farnesol also exhibits antifungal properties and induces cell death of different fungal species (e.g., *Penicillium expansum*) by generating reactive oxygen species (Liu et al. 2010). In addition, yeasts also produce killer toxins or mycocins that are lethal and disrupt the cell membrane function of a wide variety of susceptible yeast varieties (Hatoum et al. 2012). Nevertheless, interactions between yeasts are not as well understood as fungal-bacterial interactions.

1.5.3. Fungal – host interactions

Interaction between host-associated commensal fungi and its mucosal immune system is essential for regulating homeostasis and promoting a mutually beneficial relationship (Iliev and Underhill 2013). The host immune system has different defence mechanisms against fungal invaders, but intestinal mucosal epithelial layers are the first line of defence against fungi that can potentially colonize the GI tract (Pitman and Blumberg 2000). Several components of the host immune system are involved in recognition of fungi and mediation of the anti-fungal responses. These consist of pattern recognition receptors (PRRs), including Toll-like receptors, the C-type lectin receptors, galectin receptors and NOD-like receptors. PRRs are involved in recognizing fungal microbe-associated molecular patterns (MAMPs), such as cell wall carbohydrates (β-1,3-glucans, chitin, zymosan) surface proteins (mannoproteins) and fungal nucleic acids (Romani. 2011). The host immune system can respond differently to the yeast and filamentous form of fungi and this discriminating ability of the host is critical for differentiating other resident microorganisms from potential pathogens (Naglik et al. 2011). Recognition of fungal MAMPs by host PRRs initiate a cascade of intracellular signalling pathways,

eventually leading to the production of a broad range of molecules (e.g., cytokines, chemokines and cell adhesion molecules) to modulate the innate and adaptive immune responses against fungal pathogens (Tang et al. 2018, Brown 2011). Deficiency in the host signalling molecules involved in the antifungal response or fungal recognition increase the likelihood of fungal infection. For example, caspase recruitment domain family member 9 is an essential element in tailoring the antifungal response by receiving signals from Dectin-1 and Dectin-2 receptors. Its deficiency in mice leads to an increased susceptibility to systemic candidiasis (Jia et al. 2014, Tang et al. 2018). Alternatively, fungal species exhibit different strategies to avoid recognition from immune components and thus interfering with the host protective mechanisms. These include shielding of stimulatory PAMPs, modulation of inflammatory signals, shedding of decoy components, complement evasion and escape from phagocytic response (Marcos et al. 2016, Chai et al. 2009). For instance, polymorphic (*C. albicans*) and dimorphic (*Histoplasma capsulatum*, *Paracoccidioides* spp., and *Blastomyces dermatitidis*) fungi have the ability to alter the cell wall architecture during phenotypic switching, which results in differential recognition by PRRs and subsequently different host response (Chai et al. 2009, Marcos et al. 2016). Colonization by mycobiota could training the host immune system, thus promoting a stronger protective response following exposure to an infectious agent. It has been demonstrated that pre-exposure of macrophages to beta-glucan, a fungal cell wall component, leads to a stronger immune response against infection with *C. albicans* (Quintin et al. 2012). Occurrence of *C. albicans* and *S. cerevisiae* among the gut microbiota can educate the host immune system to better cope with secondary infection (Ifrim et al. 2013, Rizzetto et al. 2014).

The zebrafish model has been used extensively to understand the interaction dynamics between host and pathogenic fungi including *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* by real-time visuals (Brothers et al. 2011, Chao et al. 2010, Chen et al. 2015, Knox et al. 2014, Tenor et al. 2015). A complete understanding of the interaction between commensal fungi and the host immune system is necessary to clarify the associated physiological outcomes. The recent development of NGS techniques (host transcriptomics and metatranscriptomics (microbiota)) are expanding

our understanding of the complex relationship that hosts have with their microbial communities.

1.6. Influence of GI mycobiota on their host

GI microbiota can have a major impact on the host physiology, including intestinal homeostasis as well as general metabolic and immunological functions. For example, *S. cerevisiae* in the intestinal tract of mice increases the intestinal permeability and exacerbates colitis in experimental animal models (Chiaro et al. 2017). Disruption of commensal fungi using antifungal drugs increased disease severity in chemically induced and T cell transfer-mediated models of experimental colitis (Wheeler et al. 2016). Administration of *S. cerevisiae* in obese and type 2 diabetic mice modifies host metabolism to reduce fat mass, hepatic steatosis, and inflammatory response by altering their gut microbial composition (Everard et al. 2014). A recent study in mice has displayed the important role of commensal fungi during early life in the maturation and development of gut-associated lymphoid tissues and peripheral lymph nodes (Zhang et al. 2016). Jiang et al (2017) demonstrated that commensal fungi can functionally replace the intestinal bacteria to protect the host against injury of mucosal tissues and inflammatory disorders. In addition, they positively calibrate the activation of circulating immune cells. These observations suggested that fungi are vital components of GI microbiota despite their smaller proportion in the community.

Several studies have shown the beneficial effect of dietary administration of commercial or commensal yeast in fish. In particular, *D. hansenii* is the most frequently isolated commensal yeast from the GI tract of fish. Fish offered a diet supplemented with *D. hansenii* have an enhanced immune response that improves resistance against infection from the dinoflagellate *Amyloodinium ocellatum* and pathogenic *Aeromonas hydrophila* (Reyes-Becerril et al. 2008b, Reyes-Becerril et al. 2011). Feeding tilapia larvae with probiotic *S. cerevisiae* enhances their feed efficiency and improves growth compared to fish fed with probiotic bacteria (Lara-Flores et al. 2003). Also, *Debaryomyces hansenii* dietary supplementation enhances gut maturation and digestive enzyme activities in *Dicentrarchus labrax* larvae (Tovar et al. 2002). The combined use of *S. cerevisiae* and

Bacillus amyloliquefaciens at low doses improves gut mucosal morphology and protects juvenile *C. carpio* (Huang et al. 2015). A recent study has also showed that dietary inclusion of yeasts could modulate the intestinal microbiota of rainbow trout (Huyben et al. 2017). Administration of yeast cell wall components can also enhance growth and immune response in different fish species, including sea bream (*Sparus aurata*), channel catfish (*Ictalurus punctatus)*, *O. niloticus,* yellow croaker (*Pseudosciaena crocea*) and Atlantic salmon (*Salmo salar*) (Gatesoupe 2007, Navarrete and Tovar-Ramírez 2014).

1.7. Factors influencing the mycobiota

Several factors are known to influence the structure and diversity of microorganisms that live in the GI tract of humans and animals. In particular, diet, host genotype, host physiology and environmental factors contribute to determining the composition of GI mycobiota (Cui et al. 2013). Diet is considered as a main driving factor that shapes the GI fungal communities. In human studies, consumption of high amount of carbohydrates can positively influence the colonization by *Candida* sp. whereas diets with high protein, fatty acids and amino acids had the opposite effect (Hoffmann et al. 2013). Consumption of more short chain fatty acids also reduces the abundance of *Aspergillus* (Hoffmann et al. 2013). Eating a diet consisting of meat facilitates the enrichment of *Penicillium* spp, and consumption of nuts (almond and pistachio) lowers the proportion of *Candida* and *Penicillium* species (Ukhanova et al. 2014, David et al. 2014).

Mice offered a high fat diet harbour significantly different gut fungal communities than the control mice that were fed with standard chow (Heisel et al. 2017). Fungal populations in the GI tract are more variable compared to commensal bacteria. For instance, the fungal community in the GI tract of mouse has shown episodic variation over several months but their bacterial community was constant (Dollive et al. 2013). Similarly, Hallen-Adams et al (2015) also observed lack of fungal community persistence that is typically observed for the GI tract bacterial communities.

The gastrointestinal tract of infants was predominantly colonized by *Candida* species presumably acquired by both horizontal and vertical transmission from the mother either during time of birth or breast feeding (Bliss et al. 2008, Waggoner-Fountain et al.

1996, Ward et al. 2017). It is reported that, *Candida* spp. are detectable in about 96% of neonates by the end of the first month of life (Kumamoto and Vinces 2005). The relatively low fungal diversity in infant gut compared to adults is likely due to lack of exposure to a variety of fungal species (Heisel et al. 2015). As the child grows, the composition and diversity of fungal species resemble the maternal mycobiota (Schei et al. 2017). It has been recently reported that in human, gender can also affect the abundance of some fungal taxa. Females had a higher number of fungal isolates compared to males (Strati et al. 2016).

1.7.1. Mycobiota of wild individuals

Our understanding of GI mycobiota is mostly derived from findings in humans, laboratory and farmed animals, but few studies have characterized the fungal composition in wild animals. For example, studies that used insects from their natural habitat revealed a symbiotic interaction between fungi and their host (Blackwell. 2011). The digestive tract of insects has a considerable yeast diversity and it alters depending on the diet changes during their ontogeny (Boekhout 2005, Suh et al. 2005, Suh and Blackwell 2004). In addition, previous studies characterized the fungal communities in the cloaca of marine turtles (*Natator depressus, Chelonia mydas, Caretta caretta* and *Eretmochelys imbricate*) (Phillott et al. 2002), and wild crocodile (*Caiman latirostris*) (Betiana Núñez-Otaño et al. 2013). Cloaca of wild crocodile contains a total of 14 fungal species, among which *Aspergillus brasiliensis*, *Alternaria alternate*, *Fusarium redolens* were the most abundant. Ascomycota (68%) was the dominant phylum followed by Basidiomycota (13%) and Zygomycota (6%) in the gut of tropical butterflies from Costa Rica (Ravenscraft et al. 2017).

Laboratory and farmed animals are offered diets and maintained in controlled environmental conditions that often differ from their wild counterparts. The controlled conditions limit their ability to exhibit natural or social behaviours, which may have profound impact on gut microbiota. However, a comparative study of the gut fungal communities between domesticated silkworm (*Bombyx mori*) and wild *Acronicta major* and *Diaphania pyloalis* did not reveal any major changes in the gut fungal composition

at phylum level (Chen et al. 2018). A molecular and culture-based study has also not shown differences in fungal composition between wild and farmed carnivorous fishes, namely Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), corvina drum (*Cilus gilberti*) and Cape yellowtail (*Seriola lalandi*) (Raggi et al. 2014). These observations contrast with the differences in the gut bacterial composition noted between wild and captive animals, including fish (Uren Webster et al. 2018, Ramírez and Romero 2017).

1.8. Methods for mycobiota analysis

The methods used to identify and analyse fungal communities have greatly evolved over the last decade. Over the years, advances in methods have improved our understanding of the fungal ecosystem and a wide range of techniques are now available to identify fungi, all differing in specificity, reproducibility, time and cost. Traditionally researchers have relied on microbiological techniques such as growth on selective media, biochemical assay and microscopy. These methods are less expensive and simple to perform (Halwachs et al. 2017, Kong and Morris 2017, Huseyin et al. 2017b) but they have several disadvantages, such as being laborious and time consuming. Also, culture-based methods tend to favour fast growing and abundant fungi and mask the detection of low abundant organisms. The majority of symbiotic fungi are non-culturable using traditional methods (Gouba et al. 2013). Despite this limitation, the importance of culture methods cannot be ignored. Researchers have started combining them with other methods such as mass spectrometry and DNA-based analysis for accurate identification and improved resolution. Introduction of culture-independent methods has revolutionized our view of microbial ecology. Polymerase chain reaction (PCR) and sequencing-based methods have revealed some previously unidentified fungi in the environment. In particular, NGS methods have enabled in-depth analysis by providing massive amounts of data on microbial community composition (Barriuso et al. 2011). A range of NGS platforms are available; each has its own sequencing approach but they share a similar workflow involving either DNA fragmentation or amplicon sequencing (Reuter et al. 2015). The internal transcribed spacer (ITS) regions of the ribosomal DNA are considered as a suitable molecular target for compositional analysis of the

mycobiota by high-throughput amplicon sequencing (Schoch et al. 2012). The ITS region lies between the 18S (small sub unit) and 28S (large subunit) genes in the nuclear rDNA cistron and it includes the ITS1 and ITS2 regions separated by the 5.8 S gene (Figure 4). The entire ITS region has commonly been targeted with traditional Sanger sequencing approaches and typically ranges between 450 and 700 bp, whereas the length of each region varies from 300 to 400 bp (Bellemain et al. 2010, Halwachs et al. 2017). The precise target for amplicon sequencing to characterize fungal communities can be the entire ITS region including 5.8S, or either ITS1 or ITS2 (Halwachs et al. 2017, Abdelfattah et al. 2015, Ghannoum et al. 2010, Hamad et al. 2012, Luan et al. 2015, Meason-Smith et al. 2017, Schmidt et al. 2013). The first primers to target ITS region were developed during 1990s (White et al. 1990, Gardes and Bruns. 1993).They are not ideal, as they show amplification bias towards several fungal groups (Diakarya vs non-Diakarya, Ascomycota vs Basidiomycota) (Bellemain et al. 2010) and many researchers have developed several other ITS primers to address this issue (Ihrmark et al. 2012, Toju et al. 2012, Leho and Björn. 2016). The choice of primers and target regions have a significant impact on the fungal community analysis.

Figure 4. Schematic representation of the conserved (18S, 5.8S and 28S) and variable (internal transcribed spacer; ITS1 and ITS2) regions of fungal ribosomal RNA gene. The figure is modified from Halwachs et al (2017).

2. Objectives

The overall aim of this dissertation was to characterize the intestinal mycobiota in order to understand their relevance for zebrafish.

In spite of their known importance for host physiology in mammals, our knowledge about the fish mycobiota is very limited. In fact, at the start of this dissertation no gut-associated fungal communities had been characterized in fish using state-of-the-art methods. The hypothesis underlying this dissertation is that zebrafish intestine may harbour a fungal community that affects the physiology of the fish, similar to the intestinal bacteria.

Specific objectives are as follows:

1. To characterize the intestinal fungal community in zebrafish — mycobiota profiles of laboratory-reared, wild and wild-caught-laboratory-reared individuals (**Paper I**).

2. To determine the influence of yeast exposure on the intestinal bacterial community of zebrafish larvae (**Paper II**).

3. To understand the transcriptome responses in zebrafish larvae exposed to selected host-derived yeasts (**Paper III**).

3. General discussion

The GI fungal communities of vertebrates and the physiological responses elicited by them can influence the health status of the hosts. Furthermore, the fungal communities affect other microbial assemblies, including those of bacteria. Therefore, we profiled the GI mycobiota of zebrafish (**Paper I**) and examined the role of fish-associated yeasts in modulating the intestinal bacterial community (**Paper II**) and the host transcriptome responses (**Paper III**) by employing the germ-free (GF) and conventionally-raised (CR) zebrafish models and high-throughput 16S amplicon (**Paper II**) and RNA-sequencing (**Paper III**).

3.1. Intestinal mycobiota of zebrafish

Our comprehensive characterization of GI mycobiota of wild-caught, laboratory-reared and wild-caught-laboratory-kept zebrafish is perhaps one of the first descriptions in this field (**Paper I**). Regardless of the fish origin, Ascomycota was the predominant fungal phylum (87.5%) followed by Basidiomycota (6.8%), unidentified fungi (5.7%), and an even smaller proportion of Zygomycota. Higher abundance of Ascomycota and Basidiomycota corroborates with results of previous studies related to yeast communities in vertebrates (Foster et al. 2013, Huffnagle and Noverr 2013, Qiu et al. 2015) and fishes (Gatesoupe 2007, Navarrete and Tovar-Ramírez 2014, Romero et al. 2014). In the current study, we have observed more than 15 fungal classes; among these Dothideomycetes and Saccharomycetes were the most abundant ones. The GI tract harbours diverse fungal species, but exact number of species has not yet been clarified. Around 247 species are present in the human GI tract and we have observed more than 200 species in zebrafish. However, dominant members accounted for higher proportion of reads from all the samples. We have found many rare genera by assessing the proxies for fungal abundance. Dominant members have a significant importance to the host, but rare microbial members are vital for maintaining the stability of the microbial community during environmental fluctuations (Jousset et al. 2017).

A previous fish study has identified 43 yeast species from 5 different fishes using both culture-dependent and/or -independent methods (Raggi et al. 2014). Interestingly, 35

of these were identified by culture methods and 17 had to be discovered through culture-independent techniques. This indicates the needs to employ different methods to characterize mycobiota. Nevertheless, the diversity of microbiota (including fungi) inhabiting in the GI tract varies considerably from individual to individual and from species to species; moreover, it is thought to be dependent on various nutritional and environmental factors.

3.2. Compositional difference in the intestinal mycobiota

Several surveys, including those in fish, have shown that the bacterial composition varies with geography and/or habitat (Ramírez and Romero 2017, Salas-Leiva et al. 2017, Eichmiller et al. 2016, Kormas et al. 2014). However, a previous study has reported that there was no difference in the yeast community profiles of wild and farmed fish (Raggi et al. 2014). Although debatable, the authors concluded that yeast community is shaped by the carnivorous diet rather than the fish habitat. This study is limited by its PCR-TTGE molecular approach, which detected only a small fraction of fungi. In the present study, we found dissimilarities in the yeast communities of wild-caught and laboratory-reared zebrafish. Dothideomycetes were the abundant fungal class in the wild fish compared to Saccharomycetes in the laboratory specimens. Moreover, fungal diversity in wild samples was significantly higher than that in the laboratory-reared fish (**Paper I**). Diverse mycobiota of the wild samples could be pointing to the inherent microbial pool of the natural habitat and diverse diet, which might have a different influence on GI colonization and growth of certain species. For instance, Uenishi et al (2007) have described the influence of microbe-rich food sources on the composition of intestinal microbiota of captive and wild-chimpanzees.

Zebrafish is an omnivorous fish, and their diet consists mainly of zooplankton, insects phytoplankton and filamentous algae (Spence et al. 2008). In a recent study, it was demonstrated that the xylivorous fish *P. nigrolineatus* had a higher proportion of Dothideomycetes in their GI tract when fed a wood-based diet compared to a mixed diet (Marden et al. 2017). Therefore, we could speculate that a larger proportion of plant-derived food components may have led to the increase of Dothideomycetes in zebrafish.

However, we do not have any information about the mycobiota associated with the diet or surrounding habitat (water) of the wild-caught zebrafish (**Paper I**).

Laboratory-reared fish had a higher abundance of Saccharomycetes, which mainly comprised the genus *Debaryomyces*. This genus has been frequently found in the GI tract of fishes (Raggi et al. 2014) and is considered as a natural inhabitant of their GI (Navarrete and Tovar-Ramírez 2014). Its potential as a probiotic candidate in different fish species has been widely investigated due to their positive effect on host growth performance and feed digestion (by synthesising digestive enzymes such as amylase and trypsin) (Tovar et al. 2002). In addition, these yeasts can improve the disease resistance of leopard grouper (*Mycteroperca rosacea*) and *S. aurata* (Reyes-Becerril et al. 2011, Reyes-Becerril et al. 2008a, Reyes-Becerril et al. 2008b). We have reported a higher abundance of Saccharomycetes (**Paper I**) in laboratory-reared and wild-caught-laboratory-kept zebrafish. *Debaryomyces* helped to increase the proportion of beneficial bacteria (*Pediococcus* and *Lactococcus*) in the intestine of zebrafish larvae (**Paper II**), which could have an effect on their well-being.

Wild-caught fish reared in captivity (aquarium) and laboratory-reared fish had similar fungal composition, which was distinct from the wild-caught individuals. This indicates the impact of captivity on the alteration of fungal composition **(Paper I)**. Previous studies have also illustrated the changes in microbial composition after rearing animals in captivity (Xie et al. 2016, Dhanasiri et al. 2011, Kohl et al. 2014). The co-evolutionary relationship maintained between vertebrates and their microbes in the natural habitat can be disrupted by domestication and captivity; this is likely to have major consequences to host health. In a recent study, the intestinal bacteria of laboratory mice were reconstituted with those of wild mice; the implantation led to protection against infection from influenza virus and resistance to mutagen/inflammation-induced colorectal tumorigenesis (Rosshart et al. 2017). Laboratory-reared animals lack a complete pool of symbiotic microbes, which leads to a dearth of immune activity and reduction in inflammation-reducing abilities (Feng and Elson 2011). Moreover, laboratory animals are poor models to understand the aspects of biology and intestinal microbes of wild animals. Ideally, we should gather information of mycobiota of wild

populations before attempting to explain the changes in animals under captive conditions. The distinct GI mycobiota composition of the wild fish is likely to have a functional significance for the host. The occurrence of certain bacteria (e.g., Firmicutes) in the GI tract is associated with nutrient absorption and energy regulation (Krajmalnik-Brown et al. 2012). Similarly, the co-occurrence of saprotrophic and pathotrophic yeast indicates their ecological relevance for the fish (**Paper I**).

Even though, amplicon sequencing is a powerful approach to characterize yeast communities, it has inherent limitations, including PCR bias, unequal copy number of the ribosomal repeats (target region) and restricted ability to distinguish taxa to the genus level (Bokulich and Mills 2013, Pinto and Raskin 2012). Shotgun yeast metagenomics is still in its infancy but it represents an exciting alternative to amplicon sequencing in the future, since it allows simultaneous description of high-resolution identification and functional classification (Quince et al. 2017). Hence, it would provide an overview of the potential role of GI microbial communities and their complex interaction on host physiology.

3.3. Yeast exposure alters the intestinal bacterial communities

Microbial colonization of GI tract during early life can profoundly influence the development and maturation of host metabolism and immune system (Gensollen et al. 2016, Lu et al. 2018, Rawls et al. 2006, Rawls et al. 2004). There are various factors that determine the structure of early life microbiota. In fish, microbiota colonization is initiated upon hatching, when the larvae emerges from their protective chorions. Microbes associated with eggs and surrounding water could dictate the initial colonization patterns. Subsequently, when the larvae commence drinking and feeding their microbial pool diversifies. In addition, host deterministic factors also affect the structure of early microbial community (Llewellyn et al. 2014, Vadstein et al. 2013). Therefore, early-life is a critical period for host metabolic and immunologic development, and microbial community alteration during this early life window could have persistent and irreversible influence on the development of certain elements of immune system (Gensollen and Blumberg 2017, Gensollen et al. 2016). Most examples

in the literature focused on bacterial colonization events during early life, but recent investigations have revealed the importance of early exposure to fungi and its consequences on host health. For example, exposure to environmental fungi during infancy reduces the incidence of developing wheezing and asthma at later ages (Behbod et al. 2015).

Beneficial effects of yeast and its derivatives in different animals, including fish, are well documented (Navarrete and Tovar-Ramírez 2014, Vohra et al. 2016). Yeasts are capable of modulating host bacterial composition, which concurrently elicit host immune responses that ensure protection against infection. However, some studies have not found any significant effect on the growth and performance of weanling pigs after feeding with yeast-supplemented diet (van Heugten et al. 2003). We investigated the effect of fish-derived yeasts on intestinal microbial community structure and host physiology of zebrafish larvae. We employed *Debaryomyces* sp. that is frequently found in GI tract fish including zebrafish (**Paper I**) and *Pseudozyma* sp. isolated from zebrafish to understand their effects on host responses and their microbiota. They can be considered as natural inhabitants of the GI tract of zebrafish. GF animals are excellent models for understanding the interaction between host and its microbiota. In our study, we used a GF zebrafish model to determine the role of fish- derived yeasts by exposing the fish with no intestinal microbes (germ-free fish) to one of the yeast strains. In **Paper II** we revealed the effect of the yeast species *Debaryomyces* sp. or *Pseudozyma* sp. on intestinal microbial community structure of GF and conventionally-raised (CR) zebrafish larvae. Furthermore, we investigated the effect of *Pseudozyma* sp. exposure on the host transcriptome by RNA-sequencing analysis **(Paper III).** Two separate experiments (**Paper II** and **Paper III**) were performed, and intestinal samples and whole larvae RNA were collected for microbial (**Paper II**) and transcriptomic analyses (**Paper III**), respectively.

Consistent with previous studies (Dahan et al. 2018, Stephens et al. 2016), Proteobacteria was the dominant bacterial phylum in the GI tract of zebrafish larvae (**Paper II**). Bacteroidetes, Actinobacteria, Firmicutes, Fusobacteria and other rare phyla were also present. At the class level, Gammaproteobacteria was the dominant class and *Aeromonas*, *Acidovorax, Pseudomonas*, *Rheinheimera*, *Shewanella*, *Sphaerotilus*,

Gemmobacter and *Zoogloea* were the abundant and core microbiota in the intestinal tract of zebrafish. In particular, *Aeromonas* and *Pseudomonas* are commonly associated with freshwater habitat and many fish species (Wang et al. 2017). The consistent detection of these genera suggests that they are part of the indigenous population of the GI tract of fish (Egerton et al. 2018). However, they often cause infections in fishes (Austin and Austin 2007).

Intestinal bacterial communities were distinct from those in rearing water, which mainly comprised the phyla Proteobacteria, Bacteroidetes and Actinobacteria. The phylum Proteobacteria was dominant in all the samples including water and intestinal tract of zebrafish larvae; we observed predominance of Gammaproteobacteria and Betaproteobacteria in the intestine and water, respectively. These results are consistent with the work of Stephens et al (2016), who have shown that zebrafish harbours a distinct bacterial community compared to the rearing water, and the community differences become more distinct at the later stages of development.

Our data demonstrated that early exposure to yeasts alters the intestinal bacterial community (**Paper II**). The bacterial composition of the yeast-exposed and control larvae was significantly different, as we observed a shift in the abundance of certain genera. *Pseudomonas* abundance positively correlated with yeast exposure, whereas *Aeromonas* were less abundant in yeast-exposed larvae. In a previous study, the proportions of certain genera including *Pseudomonas* were higher in yeast treated fish compared to control (Liu et al. 2018). In addition, distinct changes in the microbial community at the genus level were found (Liu et al. 2018); reductions in the proportion of *Cetobacterium* and increase in the proportion of *Stenotrophomonas*, *Pseudomonas*, *Phyllobacterium* and *Rhodococcus* were observed in grass carp fed 12% of yeast culture compared to control diet. This result is consistent with our findings that yeast exposure-drove relative changes of certain genera and an enrichment of *Pseudomonas*. It is difficult to fathom the effect of the increase and decrease of *Pseudomonas* and *Aeromonas* (i.e., whether they are favourable to the host), since both bacteria are opportunistic pathogens and are widely present in the fish (Austin and Austin 2007).

Nevertheless, the dominance of *Pseudomonas* might point to their important role in the intestinal ecosystem.

We observed the impact of yeast on the diversity of bacterial communities in CR larvae (**Paper II**). In contrast, effect of yeast exposure was not much evident in GF zebrafish larvae. Similarly, introducing *C. albicans* into antibiotic-treated mice (disturbed microbial community or reduction of bacteria) further reduced the bacterial diversity of animals that received the antibiotics (Erb Downward et al. 2013). The intestinal ecosystem of CR animals is quite different to GF individuals, and the existing bacterial consortia in the intestine of CR animals could be the reason for the difference in diversity results. In a previous study, yeast supplementation did not significantly influence the species richness and diversity of bacterial communities of grass carp (Liu et al. 2018). In another study, supplementation of yeast significantly altered the diversity of bacterial communities in the GI tract of Nile tilapia (Ran et al. 2015). Differences in the effect of yeast exposure on alpha diversity suggest that both abundant and rare microbial communities have specific requirements and are unable to persist in the host GI tract. In other words, yeast exposure only favours the presence or growth of certain bacteria.

3.4. *Debaryomyces* but not *Pseudozyma* enriches the intestinal beneficial bacteria

We observed significantly more diverse bacteria in the *Debaryomyces*-exposed than in control and *Pseudozyma*-exposed larvae. A stable and diverse microbiota is important for the colonization resistance against invading pathogens and to improve the host health (Lawley and Walker. 2013). Diversity alone cannot determine the microbial stability and the well-being of host. Furthermore, ecological stability is based on functional dependence and may sometimes lead to negative feedbacks and ecosystem imbalance (Coyte et al. 2015). Therefore, microbial stability and the presence of certain species, particularly beneficial ones are important for maintaining microbial homeostasis. We have observed significant enrichment of lactic acid bacteria, such as *Pediococcus* and *Lactococcus* in *Debaryomyces*-exposed larvae. Similarly, higher relative proportion of beneficial bacteria was observed in grass carp after feeding diet supplemented with yeast (Liu et al. 2018), suggesting the ability of yeast to favour the

growth of beneficial bacteria. Previous studies have also shown that lactic acid bacteria are crucial for the host because of their inherent properties to inhibit the growth of putative pathogens and to stimulate the host immune system (Merrifield et al. 2014). Exposure to the fish-derived *D. hansenii* significantly increased the survival of zebrafish larvae challenged with *V. anguillarum* (Caruffo et al. 2016). In addition, growth and antioxidant enzyme activities were enhanced in *D. labrax* larvae fed diets supplemented with *D. hansenii* (Tovar-Ramírez et al. 2010). Furthermore, it could also be considered as an alternative diet (replacing microalgae) for rotifers that are used as live food for rearing zebrafish larvae (Rafael et al. 2017).

Our results reinforce the emerging concept of manipulating intestinal microbiota in fish by modifying specific bacterial groups via live yeast supplementation during early stages of life. To date, a number of bacterial and yeast species have been tested as probiotic candidates in aquaculture; to manipulate intestinal microbiota and improve both growth and the function of the immune system of fish (Hai 2015, Navarrete and Tovar-Ramírez 2014). Exploring yeast and its derivatives will be considered as an ideal approach because of their low risk and safety assurance in usage, since most yeasts show limited horizontal gene transfer and resistance to antimicrobial agents (Czerucka et al. 2007). Thus, they can be safely and effectively used in parallel with antibacterial agents to combat fish bacterial infections.

3.5. Modulation of bacterial community assembly and host response in GF larvae exposed to *Pseudozyma*

We have not observed a significant enrichment of the beneficial bacterial community in zebrafish after exposure to *Pseudozyma*, in contrast to *Debaryomyces*-exposed larvae. However, it modulated the bacterial communities in both GF and CR zebrafish larvae (**Paper II**). Although *Pseudozyma* sp. exposure did not significantly influence the host transcriptome response of CR larvae, it did affect the host response in GF zebrafish larvae (**Paper III**).

A total of 59 genes were differentially expressed in yeast-exposed GF larvae compared to the control group. Of these 59 genes, 57 were up-regulated and 2 were down-regulated. Peroxisome proliferator-activated receptors (PPARs), steroid hormone biosynthesis, phototransduction pathways and metabolism-related pathways including drug (xenobiotic) metabolism and primary bile acid synthesis pathways were the enriched KEGG pathways. None of the immune-related pathways were enriched. However, we found that some immune-related genes, such as *complement component* (*c3a*), *galectin* (*lgals2b*), *ubiquitin specific peptidase 21* (*usp21)* and *aquaporins* (*aqp8a* and *aqp9b*) were differentially expressed (**Paper III**).

Cytochromes P450 large family of enzymes are expressed in many tissues mainly intestinal and hepatic tissue of vertebrates (Uno et al. 2012, Stavropoulou et al. 2018). These enzymes are involved in many functions and are modulated by several factors. In the current study, we observed the up-regulation of genes such as *cyp7a1*, *cyp8b1*, *cyp2r1* and *cyp2p8* that belongs to cytochromes P450 family. Therefore, we observed a significant enrichment of pathways involved in drug (xenobiotic) metabolism and primary bile acid synthesis. A previous report on zebrafish by Rawls et al (2004) has demonstrated 'conventionalisation-driven' enrichment of nutrient metabolism, xenobiotic metabolism and immune response.

Recently, another study demonstrated the contribution of commensal yeast in the regulation of purine metabolism in GF mice after monoassociation with either *S. cerevisiae* or *R. aurantiaca* (Chiaro et al. 2017). Both yeasts induced similar transcriptomic profiles and yeast exposure significantly affected metabolic pathways and regulated the genes involved in intestinal barrier maintenance. They also observed a significant difference in immune-mediated pathways. The GF mice colonized with *R. aurantiaca* exhibited higher expression levels of antimicrobial peptides and genes involved in cellular tight junction formation compared to *S. cerevisiae*-exposed GF mice. These studies suggest that the immune system of a GF animal can be partially restored by colonization of commensal microorganisms (Wagner 2008). We have observed the upregulation of few immune-related genes, suggesting a moderate immune response through yeast priming. The up-regulated immune genes such as complement

component (*c3a*) and *galectin* (*lgals2b*) indicate their involvement in recognition and mediation of immune response.

The effect of yeast on the transcriptome of CR zebrafish was not obvious although it altered the GI bacterial composition of the larvae. Probably, *Pseudozyma* sp. lacks the ability to modulate host signaling pathways (**Paper II**). Alternatively, the host might have developed the tolerance to *Pseudozyma* sp., thus diminishing the host response during exposure. *Pseudozyma* sp. and many other yeast species are commonly associated with and are considered as normal members of the zebrafish GI tract. The host immune system has developed discriminating mechanisms to prevent infection by pathogenic yeast and to remain tolerant to natural (commensal) yeast that inhabit in the host. For instance, *Candida* sp. has a dual lifestyle as a commensal or pathogenic yeast; only commensal forms are tolerated by the host (Romani 2011, Cauchie et al. 2017).

Many previous studies have shown the role of beneficial microbes in the development of host physiological processes. It is important to know the contribution of other microbes including yeast and their metabolites, to host physiology. **Paper III** is the first study to demonstrate the ability of host-derived yeast to modulate the transcriptomic response in GF zebrafish larvae by RNA-sequencing. This response can be specific to a particular tissue or ubiquitous to regulate the homeostasis required for maintaining the mutualistic relationship. It seems also important to expand future research to non-immune interactions between host and mycobiota in order to understand the overall role of fungi in this complex relationship with the host.

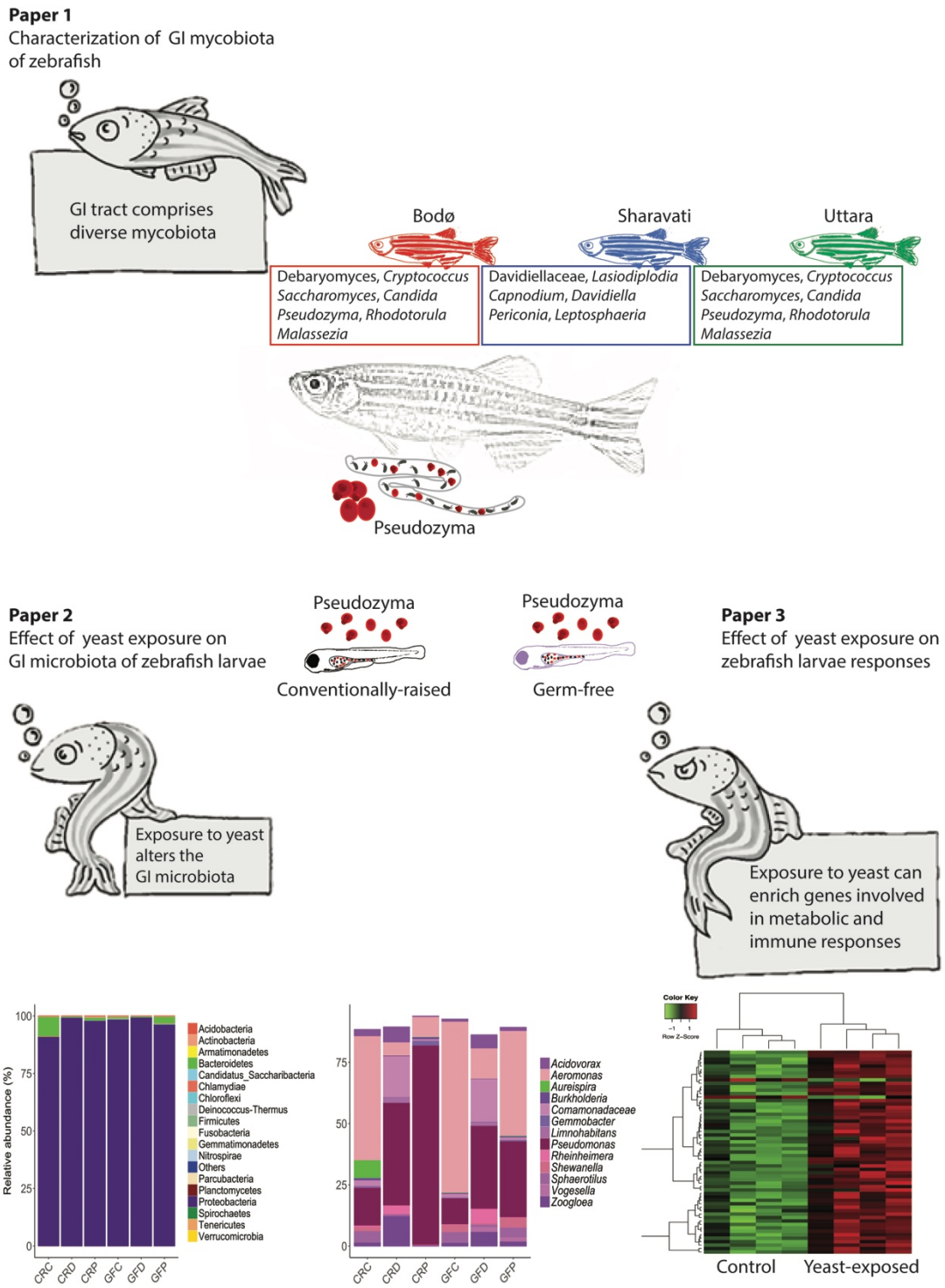

Figure 5. Graphical representation of the yeast studies presented in this thesis

4. Conclusions

This study provides the first insight into the GI tract mycobiota of zebrafish, characterized using a state-of-the-art next-generation sequencing approach. We have succeeded in demonstrating that early exposure to fish-associated yeast can influence the bacterial communities and potentially host physiology, based on transcriptomic responses of zebrafish larvae.

1. The GI tract mycobiota of wild-caught and laboratory-reared zebrafish were significantly different.
2. Rearing wild-caught zebrafish in captivity induced a shift in the mycobiota compared to wild individuals. Fungal diversity and compositional differences could possibly be due to nutritional and environmental factors.
3. Zebrafish larvae possess a typical fish core gut microbial community. Transient exposure to yeast during early ontogeny dramatically altered different bacterial genera and increased the abundance of beneficial bacteria.
4. Shift in the relative abundance of core bacterial genera by yeast exposure could be exploited as a novel strategy to selectively manipulate certain genera of fish intestinal bacteria.
5. Metabolic and immune responses of zebrafish larvae (GF) were modulated upon exposure to host-derived yeast, highlighting the importance of the mycobiota in early-life development and modulation of biological processes in the host

5. Future perspectives

The present thesis lays the foundation to understand the functional significance of the intestinal mycobiota of fish. GI mycobiota has been attracting attention as they can contribute to the wellbeing of hosts. Greater efforts are needed to gather more information about taxonomic and functional aspects of mycobiota that inhabit the GI tract. Complex interactions between yeasts and bacterial communities is likely a crucial factor for maintaining intestinal homeostasis. Therefore, future studies should be directed to understanding the fundamental factors underlying host–microbial (bacteria as well as fungi) interactions in the GI tract. Bacterial members are known for their ability to prime the immune system of hosts. More efforts are required to understand the equivalent role of mycobiota due to the inherent immune stimulating properties of their cell wall components. The present findings are mainly based on early developmental stages before the host adaptive immune system becomes fully functional. New investigations on similar lines should address the immunological response to yeast in adult fish. Further research is essential to develop a standard methodological strategy for circumventing pitfalls in the current techniques (e.g., low DNA recovery, sequencing depth) and minimizing confounding effects related to methodological differences (e.g., bioinformatic tools and database quality). Overall, adopting a hologenomics approach that integrate molecular data generated from the analysis of fish and their mycobiota will broaden our knowledge on the relevance of mycobiota to host.

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