THE EFFECTS OF PREBIOTICS ON THE GROWTH PERFORMANCE AND GASTROINTESTINAL MICROFLORA MODULATION IN *OREOCHROMIS MOSSAMBICUS* IN HARDAP REGION, NAMIBIA

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ABSTRACT

Aquaculture across the world is growing exponentially, and has certainly gained popularity among small scale farmers in Namibia. Aquaculture aims to take on the increasing demand of fish as a food source in light of depleting fish stocks in natural water systems. Therefore, studies aiming to improve aquaculture production in eco-friendly and sustainable ways, such as, adopting measures that improve fish performance and health are necessary, for example, the use of feed additives such as prebiotics, probiotics and synbiotics. This study aimed to investigate the prebiotic effect of dietary inulin and *Termitomyces schimperi* mushroom powder incorporated in fish feed on the growth performance and modulation of gastrointestinal microflora in *Oreochromis mossambicus*. Three experimental diets; inulin, inulin+ mushroom and control were made at 1% dry weight of feed inclusion level. Furthermore, initial and final body mass and daily feed consumption records were used to determine growth performance and feed utilization parameters; while fish tissue samples were used to determine proximate composition analyses. 16S metagenomics sequencing of the bacterial communities available in the intestinal samples were carried out on Illumina Miseq with primers targeting the V3-V4 region of ribosomal ribonucleic acid (rRNA). The results of this study showed that the weight gain rate (WGR) and specific growth rate (SGR) of fish fed the control diet (152.59 ± 9.61 & 2.15 ± 0.09 respectively) were significantly higher (P value ≤ 0.05) than those fed the inulin diet (117.25 ± 6.20 & 1.80 ± 0.07 respectively). However, the results indicated that there were no significant differences (P values ≥ 0.05) among the experimental diets in terms of feed conversion ratio (FCR), survival rate (SR), fat, moisture, ash and protein contents. The colony-forming units (CFU) in the inulin group

(3.54 ± 0.05 log 10 CFU/g) were significantly lower (P value ≤ 0.05) than the control group (3.54 ± 0.05 log 10 CFU/g). On the other hand, it was discovered that I group showed higher alpha and beta diversity indexes in comparison to the control and inulin + mushroom groups. Typical examples of phyla identified include *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*. At the genus level, relative abundance showed higher variation in the most abundant genera between samples. This study established that the inclusion of inulin and *T. schimperi* powder had no significant effect on the growth performance and proximate composition of *O. mossambicus*. On the other hand, the inclusion of inulin powder resulted in the reduction of viable gut microbiota counts in culture dependent methods, as well as higher alpha & beta diversity indices in culture independent methods in comparison to mushroom powder and the control. To the best of our knowledge, this is the first study to document the inclusion of *T. schimperi* powder as a prebiotic in fish feed. This study recommends the incorporation of prebiotics in fish feed before extrusion to ensure homogeneity of prebiotics and feed mixture. In addition, future studies should consider doing stress challenges to the fish to test the health benefit (resistance to stress) of fish fed with prebiotics. Moreover, further sequence analyses are recommended to identify the amplicon sequence variants (ASVs) without taxonomic classification in this study.

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List of Abbreviations and/or Acronyms

WGR	Weight gain rate
SGR	Specific growth rate
FCR	Feed conversion ratio and
SR	Survival rate
DNA	Deoxyribonucleic acid
CFU	Colony forming units
FAO	Food and Agriculture Organisation
MFMR	Ministry of Fisheries and Marine Resources
EU	European Union
HIAC	Hardap Inland Aquaculture Centre
GRAS	Generally Regarded as Safe
IBS	Irritable bowel syndrome
HMO	Human Milk Oligosaccharides
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
XOS	Xylooligosacharides
IMO	Isomaltooligosaccharides

SCFA	Short-chain fatty acids
scFOS	Short-chain fructooligosaccharides
MOS	Mannanoligosaccharides
TOS	Trans-galactooligosaccharides
AXOS	Arabinoxylooligosaccharides
GI	Gastrointestinal
MAMP	Microbe-associated molecular patterns
GALT	Gut-associated lymphoid tissues
GPR41	G-protein-coupled receptors 41
GPR43	G-protein-coupled receptors 43
GPR109A	G-protein-coupled receptors 109A
OLFR78	Olfactory receptor 78
NBT	Nitro blue tetrazolium
MPO	Myeloperoxidase
IgA	Immunoglobulin A
PRR	Pattern recognition receptors
LAB	Lactic acid bacteria
50AMPK	50 adenosine monophosphate-activated protein kinase

GLP-1	Glucagon-like peptide 1
H2O2	Hydrogen peroxide
IL-10	Interleukin 10
IL-2	Interleukin 2
ROS	Reactive oxygen species.
EFSA	European Food Safety Authority
PAMP	Pathogen- associated molecular pattern
ZERI	Zero Emissions Research Initiative
BW	Body weight
PCA	Plate Count Agar
SE	Standard error
ANOVA	One-way analysis of variance
WBMP	White bottom mushroom powder
C1	Control day 0
I29	Inulin Day 29
IM29	Inulin + Mushroom Day 29
I57	Inulin Day 57
IM57	Inulin + Mushroom Day 57

C57	Control day 57
QIIME2	Quantitative Insights into Microbial Ecology 2
DADA2	Divisive Amplicon Denoising Algorithm 2
ASVs	amplicon sequence variants
Faith's PD	Faith's phylogenetic diversity
OTUs	operational taxonomic units
PCoA	Principal Coordinate Analysis
PERMANOVA	Permutational Analysis of Variance
RDP	Ribosomal Database Project
rRNA	ribosomal ribonucleic acid
HSD	honestly significant difference
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
DNA	deoxyribonucleic acid
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
FISH	fluorescent in-situ hybridization

DGGE	denaturing gradient gel electrophoresis
TTGE	temporal temperature gradient electrophoresis
NGS	next generation sequencing
LDO	luminescent dissolved oxygen
DO	dissolved oxygen
PES	paraacetic acid

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Dedication

I would like to dedicate this research to my family, friends and anyone who assisted me in this research. Thank you all so much.

Declarations

I, Kaulo Salushando, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

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Name of Student	**Signature**	**Date**

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Fisheries and aquaculture sectors have been recognised to play a significant role in the eradication of poverty and towards the achievement of food security by providing food and income in many developing countries (Allison, 2011). Capture fisheries is reportedly declining due to over-exploitation and over-capitalization of natural fish stocks (Allison, 2011). On the contrary, aquaculture is likely the fastest growing food production sector in the world (Food and Agriculture Organisation (FAO), 2018). Therefore, the focus has shifted from increasing capture fisheries production towards the need for sustainable and improved environmental performance in a rapidly growing aquaculture sector (Allison, 2011). Besides, it has been observed that freshwater aquaculture in Namibia is still in its infancy and is gaining popularity amongst small scale farmers. This is mainly due to efforts from the Ministry of Fisheries and Marine Resources (MFMR) through aquaculture promotion campaigns and extension services.

In developing countries, challenges in aquaculture include lack of quality fish seeds and feeds; high cost of fish feeds; losses due to poor fish health management interventions sometimes leading to disease outbreaks; food safety and quality of products; and slow growth of some farmed species (Hongskul, 1999; Nsonga & Simbotwe, 2014). Nevertheless, production can be increased through expansion, intensification, diversification and improved integration of fish production into current land and water use (Nsonga & Simbotwe, 2014; Benedict, 2017). Moreover, there is a need to establish sustainable technologies that could boost rapid increase in growth rate, while minimizing disease outbreaks in aquaculture such as the use of functional ingredients as feed additives

in fish feed production (Bharathi et al., 2019). Typical examples include prebiotics, immunostimulants, and probiotics which have been found to improve immunity, feed efficiency and growth performance of fish through the modulation of the gut microbiota (Ganguly et al., 2013).

Gibson et al. (2017) defined a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit". Furthermore, there are many types of prebiotics, and sources include honey, banana, onion, wheat, human's and cow's milk, barley, tomato, asparagus, garlic, peas and beans (Davani-Davari et al., 2019). In particular, inulin is a fructan-type prebiotic commonly found in chicory roots and Jerusalem artichokes. In fact, several studies have reported beneficial effects of inulin on growth performance and health of fish in aquaculture (Eshaghzadeh et al., 2015; Tiengtam et al., 2015; Yones et al., 2020).

Similarly, in aquaculture fungal polysaccharides are generally recognized as nutritional ingredients to modulate growth and health condition of fish (Mohan et al., 2019). Several studies have reported significant improvements in various parameters from the use of fungi and their polysaccharides incorporated in aquaculture feeds (Liu et al., 2015; Chithra et al., 2016; Ahmed et al., 2017). In addition, Amadhila (2019) reported prebiotic effects of polysaccharides extracted from *Termitomyces schimperi* mushrooms. This shows great potential inulin and *T. schimperi* mushroom powder for use as prebiotics in fish feed.

O. mossambicus commonly known as Mozambique Tilapia is a worldwide distributed fish belonging to the Cichlidae family. In Namibia, *O. mossambicus* is indigenous to the Zambezi streams, it was translocated to southern Namibia in state dams, farmed dams and

natural waters for aquaculture and sport fishing (Moor & Bruton, 1988; Pullin et al., 1997). The MFMR is leading freshwater aquaculture production in Namibia, it produces and supplies fish feed with locally sourced ingredients to local small-scale fish farmers. Despite the proven benefits of adding prebiotics to fish feed, there are no prebiotics are added to the fish feeds produced by the MFMR. Therefore, this study intends to investigate the prebiotic effects of inulin and *T. schimperi* mushroom powder on the growth performance and gastrointestinal microbiota modulation of *O. mossambicus* fingerlings.

1.2. Statement of the problem

The main challenge aquaculture in Namibia is facing is the slow growth rate of the *O. mossambicus* fish species, this often results in longer production seasons culminating into increased production cost due to the high cost of fish feed. In the light of widespread antibiotic resistance in fish pathogens, vaccines and antibiotics have been banned from use in aquaculture to improve growth and control disease outbreaks (Pérez-Sánchez et al., 2018). The use of prebiotics in aquaculture have resulted in enhanced growth performance, survival, intestinal absorption, health of fish and immunomodulation (González-félix et al., 2018). Therefore, the use of prebiotics in aquaculture can lead to eco-friendly and sustainable production of quality, nutritious and healthy fish while limiting disease outbreaks.

Despite well-known benefits of prebiotics on fish's overall health and growth performance, very little research was found that investigated the effects of prebiotics on freshwater aquaculture fish species in Namibia and none on *O. mossambicus.* Particularly, the effects of garlic extract and aloe vera polysaccharides on catfish has been investigated (Gabriel et al., 2019, 2021). On the other hand, there have been no reports of the use of *T.*

schimperi powder as a feed additive in fish feed formulation in Namibia. This research proposes to fill this gap of knowledge and improve our understanding of the effects of prebiotic inulin and *T. schimperi* mushroom incorporated in fish feed on the growth performance and gastrointestinal microflora modulation in *O. mossambicus* in Namibia

1.3. Objectives of the study

The aim of this study is to investigate and compare the effects of prebiotic inulin and *T. schimperi* mushrooms incorporated in fish feed on the growth performance and modulation of gastrointestinal microflora in freshwater *O. mossambicus*. The specific objectives of this research are as follows:

a) To determine the effects of prebiotic inulin and *T. schimperi* mushroom incorporated in fish feed on the growth performance and feed utilization of *O. mossambicus*.
b) To determine the proximate composition of *O. mossambicus* fed with prebiotic inulin and *T. schimperi* mushroom incorporated fish feed.
c) To determine the effects of prebiotic inulin and *T. schimperi* mushroom incorporated in fish feed on the gastrointestinal microbiota modulation of *O. mossambicus*.

1.4. Hypotheses of the study

- Prebiotics promote the growth performance and feed utilization of *O. mossambicus*.
- Prebiotics modulate of gastro intestinal microbiota of *O. mossambicus.*

1.5. Significance of the study

The aim of aquaculture is to maximise the efficacy and optimise the profitability of fish production (FAO, 2014). To aid this goal, this study proposes to improve aquaculture production in Namibia while limiting disease outbreaks by incorporating prebiotics inulin and *T. schimperi* in fish feed. The results of this study will offer insight on the effects of prebiotics inulin and *T. schimperi* powder on the growth performance, feed utilization and gut microbiota modulation in *O. mossambicus* fingerlings in Namibia. A gap much needed to be filled if aquaculture in Namibia aims to grow alongside global aquaculture. Most importantly, this study will be the first to ever document the use of inulin and *T. schimperi* mushrooms as feed additives in Namibia. Overall, it will elucidate the potential of prebiotics application to aquaculture production in Namibia.

1.6. Limitation of the study

The study will concentrate on Hardap Inland Aquaculture Centre (HIAC)'s *O. mossambicus* therefore, the obtained results could only be applicable to that site and not to all Namibian fresh water fish which need further confirmation.

1.7. Delimitation of the study

For the purpose of this research, feeding trials will last 8 weeks and only two prebiotics (inulin and *T. schimperi* mushroom) will be studied for their effects on the fish growth and gastrointestinal modulation.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The microbiome forms an intricate, organ-specific and adaptive ecosystem which greatly influences the host physiology (Wang et al., 2018). While host genetics play a vital role, the composition of the gut microbiome is also greatly influenced by environmental and diet factors (Romero et al., 2014) Thus, improving human and animal health via modulation of the microbiome is an evolving strategy that is part of a comprehensive, holistic approach to lifestyle awareness (Gibson et al., 2017). This can be achieved through the introduction of consortia or single beneficial microorganisms (probiotics), supply of substrates to support growth of commensal microorganisms (prebiotics) and fecal transplants (Enam & Mansell, 2019).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). The common mechanisms of action of probiotics are namely: competitive exclusion of pathogenic bacteria; enhancement of host nutrition; enzymatically contribute to nutrient digestion and stimulate host immune response (Guerreiro et al., 2018). The common probiotics produced are Lactic Acid Bacteria and Bifidobacterium species, they have been reported to ferment prebiotics in the large intestine and therefore contribute to the improvement of overall health of hosts (Krumbeck et al., 2016). It is important to understand probiotics and their relationship with prebiotics to fully comprehend the concept of prebiotics, basically, prebiotics are food for probiotics.

Prebiotics are defined as substrates that are selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). Prebiotics are a sub-category of functional food ingredients that can be included in foods, drinks animal feeds, as well as supplements (Gibson et al., 2010). They cause shifts in the gut microbiota by supporting the growth of beneficial communities, as well as serve as substrates for the production of biologically active metabolites resulting in positive systemic and specific health outcomes (Krumbeck et al., 2016). Benefits of prebiotics include, reducing the prevalence and duration of infectious and antibiotic-associated diarrhoea; reducing the inflammation and symptoms associated with inflammatory bowel disease; and exerting protective effects to prevent colon cancer (Slavin, 2013). Moreover, they enhance the bioavailability and uptake of minerals, including calcium, magnesium, and possibly iron; lower some risk factors for cardiovascular disease; and promote satiety, weight loss and prevent obesity (Slavin, 2013).

Another concept worth noting is synbiotics, they are a mixture of live microorganisms and substrates selectively utilized by host microorganisms that confers a health benefit on the host (Swanson et al., 2020). This translates to a combination of prebiotics and probiotics, resulting in a single product that ensure a superior effect compared to the activity of either one on its own (Markowiak & Ślizewska, 2018). Synbiotics imply synergy, they provide an effective approach to enhancing the persistence and metabolic activity of specific beneficial probiotic strains (Krumbeck et al., 2016).

In aquaculture, prebiotics have been reported to improve growth performance, feed utilization, survival, carcass composition, health status, disease resistance, immune functions, microbiota modulation, and reduced oxidative stress (Ganguly et al., 2013;

Ringø, Dimitroglou, et al., 2014; Akhter et al., 2015; Guerreiro et al., 2018). The main focus of this study is to investigate how prebiotics inulin and *T. schimperi* powder influence the growth performance, feed utilization, and gut microbiota modulation (diversity) of *O. mossambicus* fingerlings. Therefore, this chapter aims to review the relevant literature on the topic of prebiotics and their application in aquaculture, subsequently, narrowing it down to the specific prebiotics applied in this study. Additionally, a brief review of the methods used in assessing fish gastrointestinal microbiota composition, all the while highlighting what has been done, what remains to be done, the gaps in knowledge and literature, disagreements in literature.

2.2 Prebiotics in Aquaculture

The current aquaculture intensification may lead to water quality deterioration and subsequently to disease outbreaks (Dawood & Koshio, 2016). In the past, diseases outbreaks were controlled by the use of antibiotics and vaccines, but due to the widespread emergence of antibiotic resistance in fish pathogens, they were banned by the European Union (EU) (Pérez-Sánchez et al., 2018). Prebiotics and probiotics offer a distinct strategy to avoiding disease outbreaks in aquaculture and positive impacts have been reported such as enhancing growth, survival, intestinal absorption, well-being of fish and immunomodulation (González-félix et al., 2018) (figure 1).

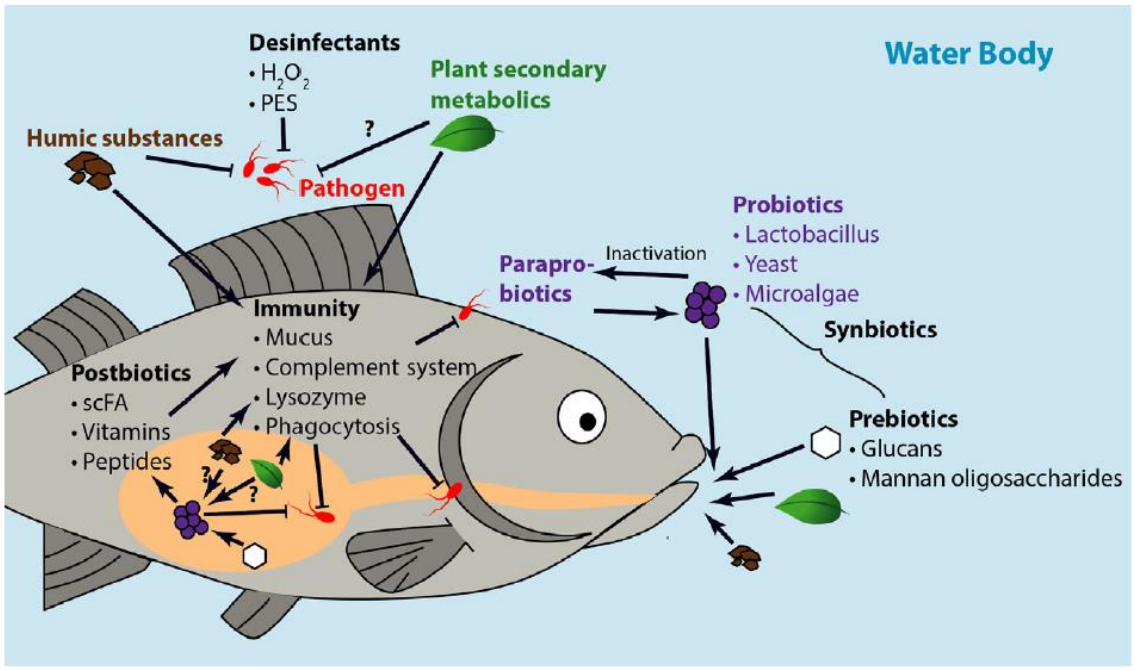

Figure 1: Environmental treatments used to prevent or treat diseases (→stimulation; ⊣ inhibition; proposed pathway; PES; paraacetic acid) (source: (Lieke et al., 2020).

The application of probiotics as feed additives in aquaculture has been limited due to low viability of the bacteria after pelleting and storage along with problems with feed handling and preparation (Ringø, Olsen, et al., 2014). Also, there is the possibility of probiotics entering and affecting the environment (Ringø, Olsen, et al., 2014). Prebiotics on the other hand, present an alternative way of manipulating endogenous microbes to improve host health and as well overcome issues of bio security associated with probiotics (Ringø et al., 2010; Dimitroglou et al., 2011). Furthermore, they stimulate selected favorable indigenous microbial populations by altering conditions in favor of beneficial species which potentially enhance fish growth efficiency and reduce disease susceptibility (Ringø et al., 2010; Dimitroglou et al., 2011). Ringø et al. (2010) further hypothesized the potential of prebiotics in increasing efficiency and sustainability of aquaculture production. In fact, several studies have documented the benefits of prebiotics in

aquaculture (Ortiz et al., 2013; Eshaghzadeh et al., 2015; Tiengtam et al., 2015). However, further studies are required to elucidate prebiotic's role in aquaculture and Namibia is not an exception.

Over the past decades, the definition of what constitutes a prebiotic has evolved, particularly, the prebiotic concept has expanded applications to include not only humans but other animals and other target sites apart from the gastrointestinal tract (Gibson et al., 2017). Previously, prebiotics were defined and characterized based on human applications, this has been the case with the criteria to classify an ingredient as a prebiotic. Defining, using and setting up criteria of prebiotics in aquaculture is complex, (Lauzon et al., 2014) suggested criteria for classifying prebiotics in aquaculture (aquatic organisms) as follows:

- Resistance to hydrolysis by the host's enzymes and gastrointestinal absorption;
- Improvement in intestinal microbial balance, and
- Conferring host benefits (such as improved disease resistance, non-specific immunity, gut morphology, growth, survival and/or nutrient digestibility).

The fact that mammals and aquatic organisms significantly vary physiologically justifies the need to define the criteria specific to particular groups of organisms and consensus on classifying prebiotics is needed to enable correct applications of prebiotics in different animals. Specifically, aquatic organisms lack a well-defined colon; and some fish species have a distinct stomach while some do not (Lauzon et al., 2014). Moreover, pyloric caeca numbers can vary from nil to 1000 for some species, intestinal length ranges from short or up to 20 times the body length, and some species have a hindgut (fermentation) chamber

(Lauzon et al., 2014). The criteria by Lauzon et al. (2014) is very similar to the established criteria except its more applicable to aquatic animals.

2.2.1 Benefits and mode of action of prebiotics in aquaculture

Most prebiotics (oligosaccharides) are made up of beta-glycosidic bonds that resist hydrolysis by endogenic enzymes of animals, these enzymes only recognize the alpha-glycosidic linkages like the ones in starch (Lauzon et al., 2014). The most commonly used prebiotics in fish include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMO), short-chain fructooligosaccharides (scFOS), oligofructose, mannanoligosaccharides (MOS), trans-galactooligosaccharides (TOS), arabinoxylooligosaccharides (AXOS), and different commercial prebiotic mixtures (Ringø, Dimitroglou, et al., 2014; Zoumpopoulou et al., 2018). Reported benefits of prebiotics in aquaculture are improved growth performance, survival, feed utilization, carcass composition, health status, disease resistance, gut morphology, digestibility, gastrointestinal enzyme activities, immune functions, glucose and lipid metabolism, microbiota modulation, and reduced oxidative stress (Ganguly et al., 2013; Ringø, Dimitroglou, et al., 2014; Akhter et al., 2015; Guerreiro et al., 2018).

Due to the several modes of actions and synergies that often occur, the elucidation of the mechanisms by which the benefits of prebiotics are conferred is often difficult to explain (Guerreiro et al., 2018). Nevertheless, Merrifield et al. (2010) suggested that the mechanism by which prebiotics confer benefits in fish is through changes in bacterial communities resulting in an increase in beneficial bacteria that promote the production of inhibitory compounds. Further mechanisms of action include, competition for chemicals, available energy and adhesion sites; and inhibition of virulent gene expression or

disruption of quorum sensing (Merrifield et al., 2010). What is more, prebiotics acts as energy sources for selective (beneficial) bacterial species in the gut that ferment these prebiotics and the end products of this fermentation (short-chain fatty acids (SCFAs), vitamins and peptides) are responsible for the majority of the beneficial effects of prebiotics in fish (Lauzon et al., 2014; Caipang and Lazado, 2015; Guerreiro et al., 2018; Lieke et al., 2020). In this review, it is of outmost importance to further elucidate the role of gut microbiota in fish health and the mechanism of action of SCFAs production, immunomodulation, growth performance and intestinal microflora modulation.

2.2.2 Role of fish gut microbiota

Intestinal microbiota performs a major function in nutrition, development of immunity, disease resistance, health and well-being of fish similarly to terrestrial animals (Lauzon et al., 2014; Romero et al., 2014; Montalban-Arques et al., 2015). The microbial community of the fish gastrointestinal tract is made up of viruses, Archae, protozoa, yeast and bacteria (Guerreiro et al., 2018). However, bacteria are the most abundant in the gut (Guerreiro et al., 2018). As adapted from Llewellyn et al. (2014) (figure 2) is a schematic generalized lifecycle of a teleost and accessory indigenous microbiota with different taxa represented by colored ellipses and is summarized as follows:

(1) immediately when the eggs are laid, the interactions and colonization of fish progeny occurs and the diversity of the microbiota that develops reflects the microbial composition of the water;

(2) once the egg hatches, environmental microbiota colonizes the larvae;

(3) once the larvae begins to feed, early digestive tract colonization occurs with microbial taxa resembling those associated with feed;

(4) development of the microbiome occurs, accumulating diversity and reaching maturity and finally

(5) the adult microbiome is a diverse assemblage of microbial taxa.

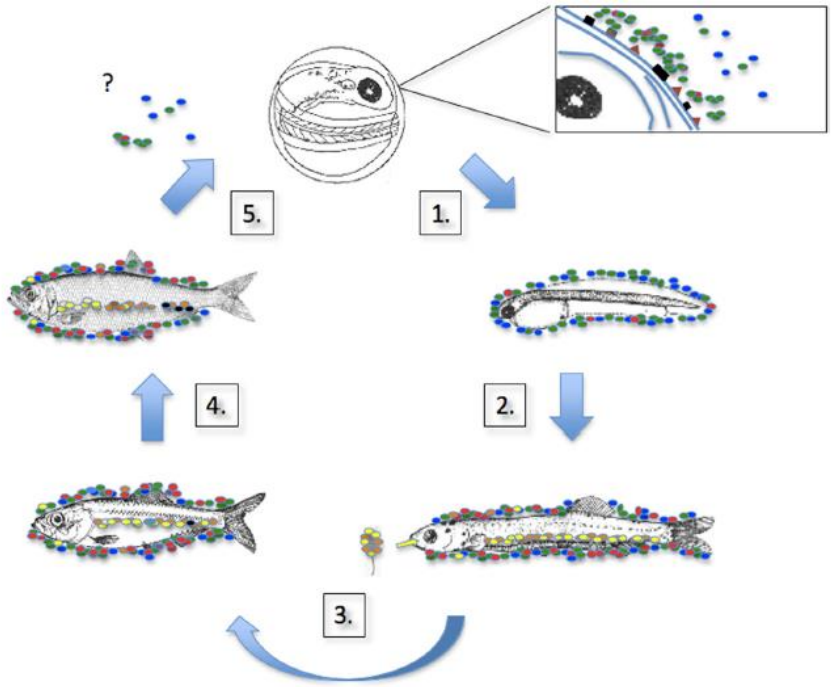

Figure 2: General lifecycle of a teleost and accessory indigenous microbiota. Source: (Llewellyn et al., 2014).

Furthermore, gut microbiota of fish is to a greater extent fluidic compared to terrestrial vertebrates on account of the fluctuating environmental conditions, variational factors, high influx of microbe-laden aqueous media and dietary changes (Ringø et al., 2016; Li, Ringø, et al., 2019). The factors affecting the composition of the gut microbiota can be divided into three categories namely host, environmental and diet factors (Romero et al., 2014). Host factors include species, genetics, gender, weight, age, immunity, different regions of the gastrointestinal tract (Li et al., 2012; Romero et al., 2014; Ringø et al., 2016). While environmental factors include seasonal changes, water quality, temperature,

salinity, stress and cultured versus wild (Romero et al., 2014; Ringø et al., 2016; Wang et al., 2018). And diet factors include dietary form, dietary lipid, protein sources, functional glycomic ingredients, nutraceuticals (prebiotics, probiotics, synbiotics and immunostimulants), antibiotics, dietary iron and chromic (Li et al., 2012; Romero et al., 2014; Ringø et al., 2016; Wang et al., 2018; Tapia-Paniagua et al., 2019). Diet is the chief source of energy for microbial growth, making it a significant factor in modulating the gastrointestinal microbiota composition of fish (Davani-Davari et al., 2019; Enam & Mansell, 2019). Nutraceuticals such as probiotics and prebiotics have been proposed and used to affect the microbiota composition positively resulting in benefits to host health and well-being (Lauzon et al., 2014; Tapia-Paniagua et al., 2019).

Among fish gut microbiota *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes* and *Fusobacterium* are the dominant phyla and *Aeromonas, Pseudomonas* and *Bacteroides* type A dominate fresh water fish gut microbiota (Wang et al., 2018). Fish gut microbiota potentially has a positive impact in digestive processes of fish. In fact, a broad variety of enzyme-producing microbiota have been isolated and identified in the gastrointestinal (GI) tract of fish (Romero et al., 2014). The enzymes produced by gut microbiota have a significant role in digestion through hydrolysis of feed components and they include amylase, cellulase, lipase, proteases, chitinase and phytase (Wang et al., 2018; Tapia-Paniagua et al., 2019). In addition, intestinal microbiota participate in epithelial renewal, enhances the stability of β-catenin in intestinal epithelial cells and promotes cell proliferation in the developing vertebrate intestine (Wang et al., 2018; Tapia-Paniagua et al., 2019). Moreover, the gut microbiota stimulates nutrient uptake of cholesterol and

regulates fat storage by stimulating fatty acid uptake in intestinal epithelium leading to the buildup of dietary fatty acids in extra-intestinal tissues (Wang et al., 2018).

Furthermore, gut microbes have a vital role in the development, maturation and modulation of the immune response, which in turn mediate a variety of host immune functions (Wang et al., 2018; Tapia-Paniagua et al., 2019). Gut microbiota and derived products stimulate systemic and mucosal immune response through microbe-associated molecular patterns (MAMP) interaction with pattern recognition receptors (Tapia-Paniagua et al., 2019). In addition, fish gut-associated lymphoid tissues (GALT) have significant numbers of antigen- presenting cells that participate in the initial stages of the immune response (Tapia-Paniagua et al., 2019). They further modulate the levels of cytokine and immunoglobulin as well as activating cellular response including B and T cells (Tapia-Paniagua et al., 2019). Equally important, the gut microbiota prevents pathogenic microbes from colonizing the gastrointestinal tract by competitive exclusion or production of toxic secondary metabolites (Llewellyn et al., 2014).

Finally, some species in the phylum *Fusobacteria* are capable of excreting butyrate or synthesizing vitamins which positively affect the health of the fish (Romero et al., 2014). Although there is an increase in research studying the gastrointestinal microbiota communities of fish (Wang et al., 2018), fewer studies have been done on *O. mossambicus* in general and particularly in Namibia. Wang et al. 2018) postulated that the lack of gut microbiota in aquatic organisms could result in damaged physiological functions, for instance, intestinal epithelial cell dysfunction, compromised nutrient absorption, metabolism and weaker immune responses. Therefore, investigating the role gut microbiota play in metabolism is useful in improving the development of commercial

feeds, fish feed efficiency and overall health and well-being of fish in aquaculture (Li et al., 2014).

2.2.3 SCFAs production

SCFAs are carboxylic acids made up of aliphatic tails of less than 6 carbons and are the end products of fermentation of prebiotic carbohydrates that escape digestion in the upper GI tract by anaerobic microbiota (Montalban-Arques et al., 2015; Tran et al., 2020). The most abundant SCFAs in the fish gastrointestinal tract are acetate, propionate and butyrate (Lauzon et al., 2014). Among fish and gut regions within a species, differences in the concentration of SCFAs such as acetate, propionate and butyrate are evident (Tran et al., 2020).

The factors that influence the production of SCFAs according to Tran et al. (2020) are as follows; composition of the gut microbiota, environmental conditions (such as pH, hydrogen partial pressure and available substrates) and species of fish. For instance, freshwater herbivorous fish are reported to have lower SCFAs levels compared to marine herbivorous fish, while, carnivorous fish generally have higher concentrations of SCFAs than herbivorous and omnivorous fish (Hao et al., 2017). Other factors include, living environments, seasons, intestinal morphology, intestinal regions, gut transit time, rate of SCFA transport across the gut epithelium however, in aquatic animals, limited data is available on the biosynthesis of SCFAs (Tran et al., 2020).

Furthermore, SCFAs play a crucial role in maintaining energy homeostasis, metabolism and gut health (Tran et al., 2020). Nearly all SCFAs produced are absorbed in the hindgut as an energy source and aids in various biosynthetic processes within the vicinity of the gut (Tran et al., 2020). Nonetheless, small proportions of propionate and acetate reaches

the liver and is used as substrates for the energy-producing tricarboxylic acid cycle and gluconeogenesis (Tran et al., 2020). Moreover, in fish, acetate is carried into the portal blood and utilized as energy source for skeletal muscle or lipid synthesis and butyrate is attributed for most of the oxygen requirements for gut (Lauzon et al., 2014; Tran et al., 2020).

SCFAs production leads to the modulation of blood lipid metabolism, gastrointestinal/systemic immunomodulation, energy sources resulting in intestinal cell proliferation, and improved intestinal barrier function (Lauzon et al., 2014). In addition, their synergistic stimulation of commensal and symbiotic bacteria grants competitive exclusion of pathogens, incidentally heightening pathogen resistance, decreasing toxic microbial metabolites and repressing intestinal inflammation (Lauzon et al., 2014; Llewellyn et al., 2014). Specifically, SCFA production leads to a lowered pH in the gastrointestinal tract which aids in mineral absorption and general nutritional support by enhancing digestive enzymes and the bioavailability of dietary minerals cells (Lauzon et al., 2014; Hoseinifar et al., 2017) . To add, SCFAs inhibits the growth of pathogenic microbes, for instance Gram-positive bacteria are inhibited by dissociating acids and the anions produced in bacterial cells (Ebrahimi et al., 2017; Hoseinifar et al., 2017).

SCFAs contribute significantly to the energy requirements of fish, they are absorbed and metabolized by the enterocytes; accounting for a large proportion of enterocyte energy needs and stimulating growth of gut beneficial bacteria resulting in proliferation of epithelia and maintainance of gut health homeostasis (Ebrahimi et al., 2017; Hoseinifar et al., 2017; Guerreiro et al., 2018; Nawaz et al., 2018). In mammals SCFAs (mostly acetate, butyrate and propionate) seem to act as signaling molecules in cellular activities via the

binding of receptors, G-protein-coupled receptors 41 (GPR41), GPR43, GPR109A and Olfactory receptor 78 (OLFR78) (Tran et al., 2020).

The expression of these receptors in different tissues and cell types indicate that SCFAs have a role in modulating substrate and energy metabolism together with the immune response of the host by leading to a rise in immune components and improved regulation of immune-related gene expression (Tran et al., 2020). While these receptors have not been substantiated in fish to date, it is likely that the fish mucosal immune response is regulated in the same way (Hoseinifar et al., 2017).

The dietary supplementation of SCFAs and their salts led to the immunomodulation of fish and shrimp ensuing the up-regulation of favorable immune components, at the same time matching a reduction in immune components that are harmful to the host, subsequently aiding fish in the fight against pathogens (Hoseinifar et al., 2017). Figure 3 below shows the effects of dietary supplementation of SCFAs and their salts on the modulation of the immune responses in fish such as cellular, humoral and molecular responses. Therefore, consideration should also be taken into the incorporation of SCFAs as dietary supplementation due to their positive impact on growth performance and feed utilization and the health status of the fish as well as in maintaining intestinal homoeostasis, acting as energy sources and anti-inflammatory agents (Hoseinifar et al., 2017; Tran et al., 2020).

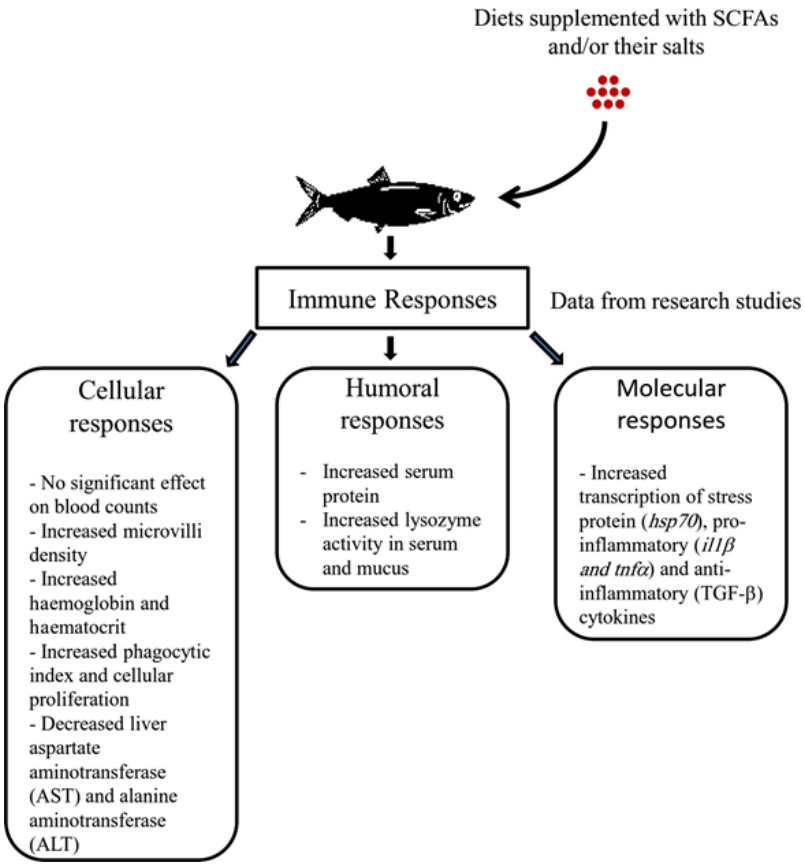

Figure 3: The impact of dietary supplementation of SCFAs on immunomodulation of fish. Source: (Hoseinifar et al., 2017).

2.2.4 Immunomodulation

The fish immune system is a sophisticated network made up of genes, proteins and cells that perform a vital role in protecting the fish against invading pathogens and stress (Caipang & Lazado, 2015). In fish, cellular and humoral immunity consists of phagocytic cells, neutrophils, natural killer cells, lymphocytes, lysozyme, hemolysin, immunoglobulin and complement molecules (Wang et al., 2017; Dawood et al., 2018). There are four mechanisms of fish immunity. Firstly, phagocytosis, whereby neutrophils, monocytes, and macrophages engulf pathogens then secrete degradative enzymes and antimicrobial peptides, which helps destroy pathogens (Song et al., 2014; Akhter et al., 2015; Caipang & Lazado, 2015; Carbone & Faggio, 2016). Secondly, respiratory burst,

the invigorated release of reactive oxygen species (hydrogen peroxide, superoxide anions and hydroxyl radicals) by phagocytic cells responsible for degrading engulfed material and is measured by nitro blue tetrazolium (NBT) or myeloperoxidase (MPO) tests (Song et al., 2014; Akhter et al., 2015; Caipang & Lazado, 2015; Carbone & Faggio, 2016). Thirdly, lysozyme, responsible for the degradation of peptidoglycan in bacterial cell walls by hydrolyzing of β-(1, 4) glycosidic linkages in N-acetylmuramic acid and N-acetylglucosamine; they are synthesized by macrophages and neutrophils and are found in mucus, plasma and lymphoid tissues (Song et al., 2014; Akhter et al., 2015; Caipang & Lazado, 2015; Carbone & Faggio, 2016). Lastly, cytokines, produced by macrophages, lymphocytes and other cells, examples include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors; and they regulate and link innate and adaptive immune responses for maximum immunity as well as stimulate hematopoiesis (Song et al., 2014; Akhter et al., 2015; Caipang & Lazado, 2015; Carbone & Faggio, 2016).

Prebiotics modulate the immune system by directly stimulating the innate immune system or indirectly by enhancing the growth of beneficial microbiota (Song et al., 2014). The mucosal immune response is the line of defense where pathogens may enter the host and is vital in securing the host immunity for better health (Nawaz et al., 2018). Prebiotics directly impact the epithelial barrier by interlinking epithelial cells via tight junctions reducing the penetration of pathogens; they block pathogen adhesion on mucus lowering the number of pathogens that invade the mucosa; they as well bind the active site of pathogens to decrease their activity; and they facilitate the initiation of immunoglobulin A (IgA) and dendritic cells increasing permeability (Nawaz et al., 2018). Furthermore, prebiotics directly activate the innate immunity by interacting with pattern recognition

receptors (PRR) (β-glucan or dentin-1) expressed on macrophages (Song et al., 2014; Hoseinifar et al., 2015). Figure 4 below shows some of the direct impacts of prebiotics on the immune system.

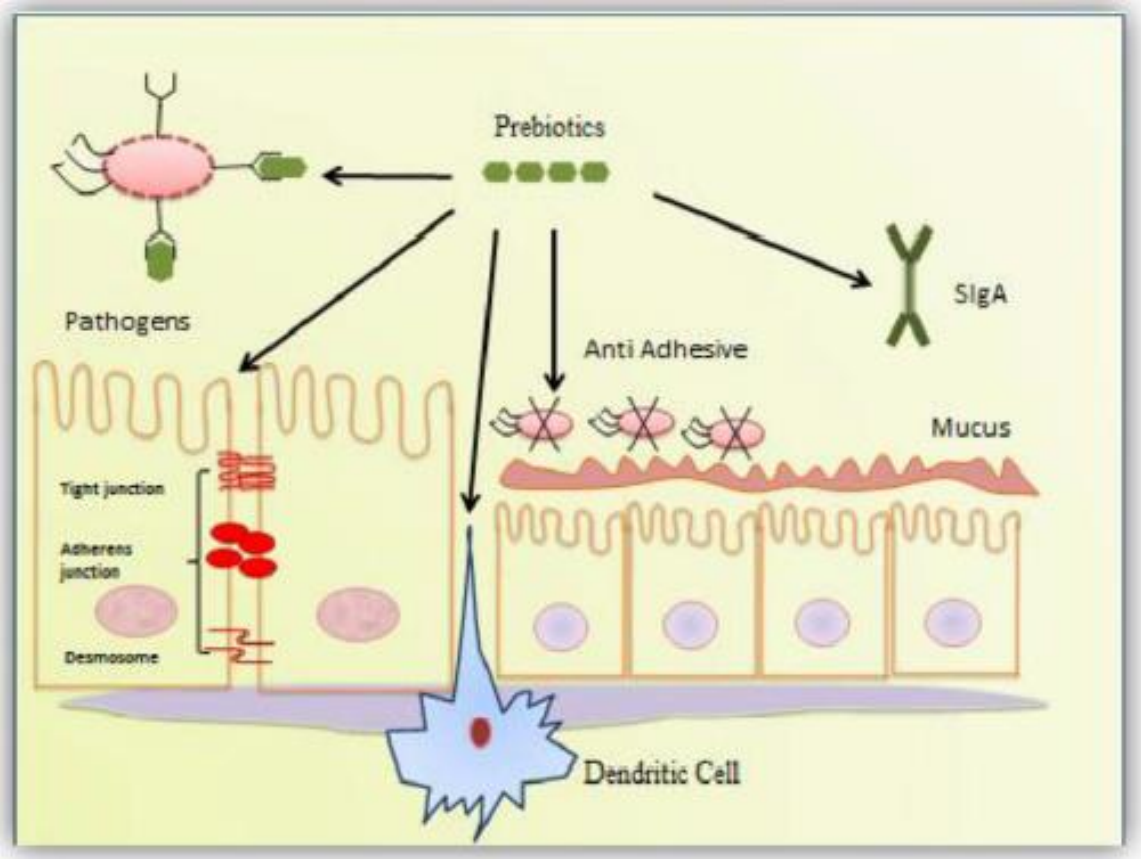

Figure 4: Direct impacts of prebiotics on immune response. Source: (Nawaz et al., 2018).

On the other hand, prebiotics indirectly effect immunity by promoting the growth of beneficial bacteria which hinder the adhesion and invasion of pathogens in the epithelia by, competing for adhesion sites in epithelia, lowering the pH, enhancing mucus production, producing SCFAs and antimicrobial peptides and stimulating cytokine production (Akhter et al., 2015). The SCFAs produced by fermentation of prebiotics seem to be a significant mechanism of this indirect impact on immunity. SCFAs provide energy

to epithelial cells and they positively impact anti-inflammatory properties and cytokine expression (Nawaz et al., 2018). To add, because they lower the pH in the intestine, they prevent pathogen growth. Prebiotics may also associate with MAMPs (teichoic acid, peptidoglycan, glycosylated protein, or the capsular polysaccharide of bacteria) resulting in the activation of innate immune cells (Song et al., 2014; Hoseinifar et al., 2015). Other indirect effects include the stimulation of natural killer cells, stimulation of phagocytosis, stimulation of neutrophils, stimulation of the alternative complement system, an increase in lysozyme activity and antibody response in the host (Akhter et al., 2015). Without a doubt, a boosted immune status will bring about a heightened immune response, manifested in higher survival rates post bacterial or environmental challenges, disease resistance and improved growth performance and feed utilization (Guerreiro et al., 2018). The effects of prebiotics may vary depending on the fish species investigated, hence effects should not be extrapolated between species, rather, the actual effect should be primarily demonstrated before claiming its potential use (Caipang & Lazado, 2015; Hoseinifar et al., 2015). Figure 5 below shows the indirect impact of prebiotics on immunity.

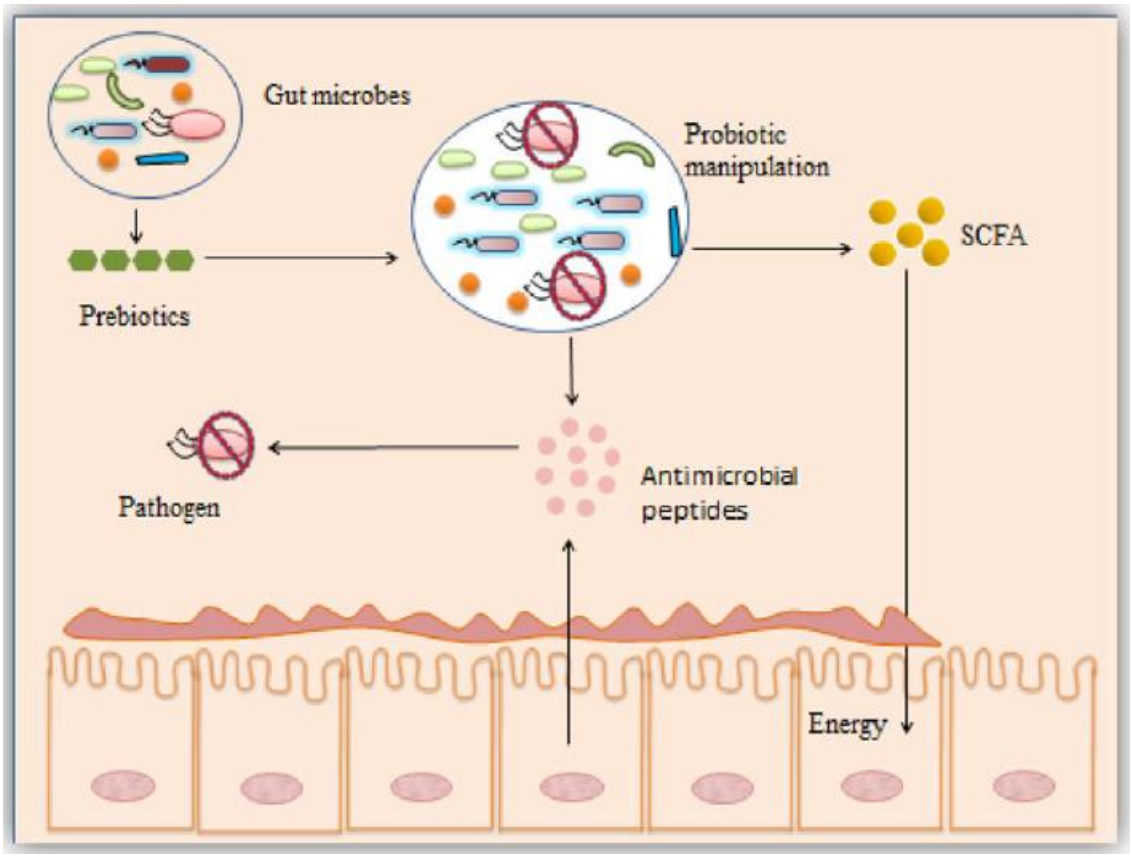

Figure 5: Indirect impact of prebiotics on immune response. Source: (Nawaz et al., 2018)

2.2.5 Selective stimulation of intestinal microbiota

Gut microbiota plays an important role in host health and immunity as discussed above, and most times can be considered an 'extra organ' of the fish due to their significance in development, homeostasis and protection of the gastrointestinal tract (Nawaz et al., 2018). Diet plays a significant role in modulating gut microbiota. Although a substantial number of beneficial microbes can be found in the fish gut, to sustain beneficial microbe homeostasis, the provision of substrates (prebiotics) that stimulate the proliferation of beneficial microbes such as bifidobacterial and lactic acid bacteria is of paramount importance as the basic mechanism of action of prebiotics is via its impact on the gut

microbiota (Guerreiro et al., 2018; Nawaz et al., 2018). The key mode of action of prebiotics on the selective modulation of gut microbiota is as a result of the fermentation of prebiotics by advantageous bacteria such as LAB and *Bifidobacteria* (Huynh et al., 2017; Guerreiro et al., 2018). These bacteria hydrolyze prebiotics using enzymes into respective sugars and use them as a carbon source to increase biomass of bacteria and therefore become favored compared to less beneficial (pathogenic) bacteria in the gut (Huynh et al., 2017; Guerreiro et al., 2018). Moreover, some species (*Lactobacillus*) have adopted various transport mechanisms and catabolic pathways together with intracellular and extracellular hydrolysis of specific oligosaccharide substrates (Goh & Klaenhammer, 2015).

Studies have shown that prebiotics increase lactic acid bacteria, such as *Firmicutes* (*Lactobacillus* and *Enterococcus*) however, they decrease the abundance of pathogenic bacteria for instance, *Proteobacteria* (*Aeromonas* and *Vibrio* spp.) (Boyd et al., 2020). According to Huynh et al. (2017) the mechanism with which prebiotics decrease the abundance of pathogens is through competitive adhesion where prebiotics either bind to receptors on the opportunistic pathogens or they bind to the target sites of opportunistic bacteria. Moreover, pathogens have a complex metabolism making them unlikely to take advantage of prebiotics as substrates because they do not possess the necessary enzymes to hydrolyze them (Caipang & Lazado, 2015). Though, caution needs to be taken because supplying the substrate constantly in the gut could bring about a risk of pathogens acquiring the ability to use either the substrate or its degraded product (Caipang & Lazado, 2015). Equally important, because prebiotics are able promote the growth of beneficial microbes, they can be combined together with beneficial live microorganisms (probiotics)

to facilitate their colonization in the gut to ensure the benefits of the probiotic (Huynh et al., 2017).

2.2.6 Growth performance and feed utilization

Several elements influence the growth of the fish such as nutrient digestion, assimilation rate or feed conversion efficiency, (Torrecillas et al., 2014). The mechanism of action of prebiotics' effect on growth performance and feed utilization is closely linked to greater nutrients availability owing to changes in digestive enzymes activities or in gut morphology (figure 6) (Guerreiro et al., 2018). The improvement in growth performance could be related to prebiotics that stimulate beneficial microbes that produce digestive enzymes and SCFAs that aid in improving nutrient availability and intermediary metabolism (Huynh et al., 2017; Guerreiro et al., 2018). Prebiotics also improve gut morphology by enhancing gut integrity for example microvilli density, intestinal fold height, enterocyte height, and absorption area resulting in heightened functioning of enterocytes and transport of nutrients across the intestine (Gatlin III & Peredo, 2012). Moreover, they improve immune response, confer disease resistance and positively affect oxidative status in fish by either reducing oxidative damage or improving antioxidant potential (Gatlin III and Peredo, 2012; Huynh et al., 2017; Guerreiro et al., 2018; Boyd et al., 2020).

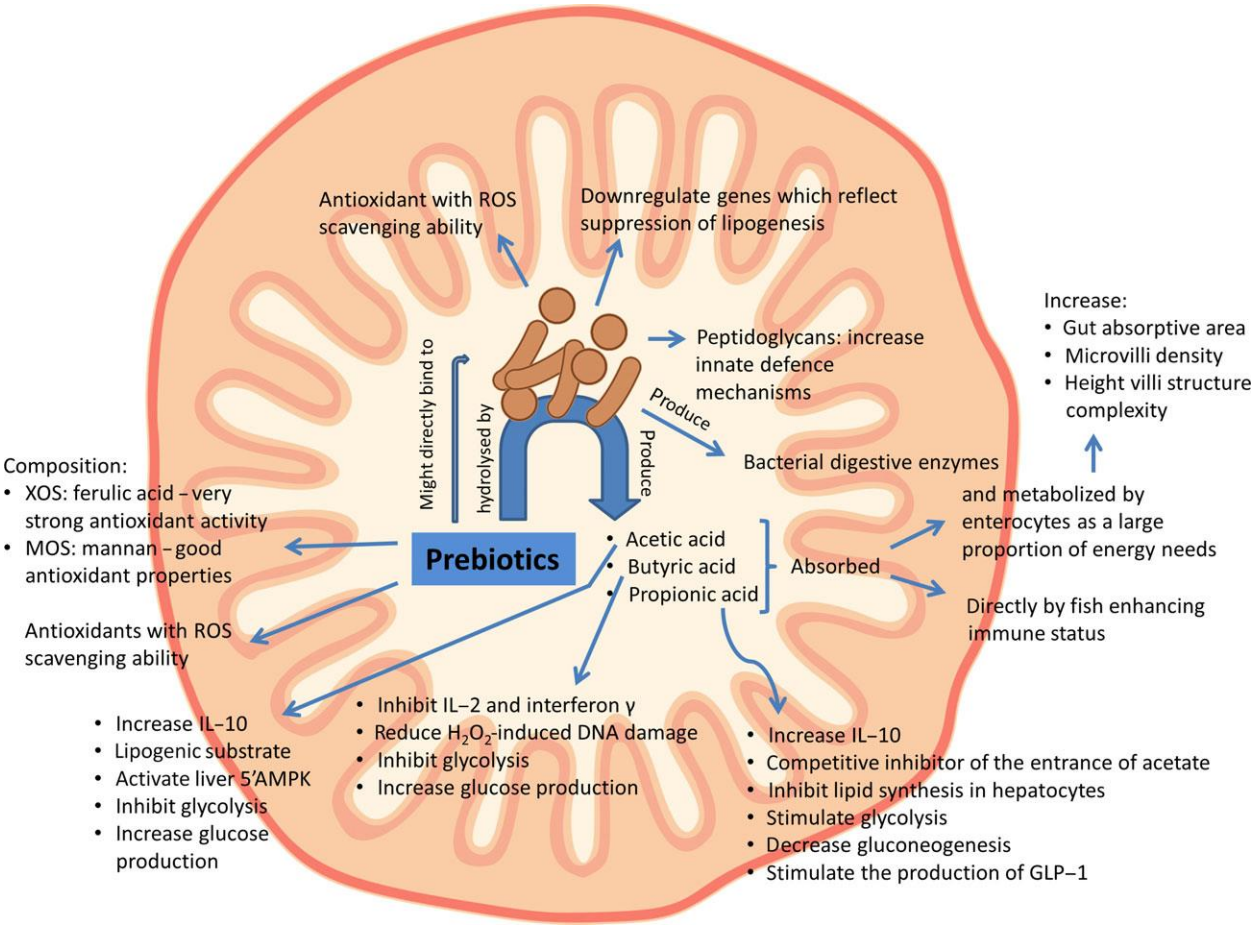

Figure 6: General prebiotic mechanisms of action. Round and rod shapes represent bacteria. 50AMPK (50 adenosine monophosphate-activated protein kinase); GLP-1 (glucagon-like peptide 1); H_2O_2 (hydrogen peroxide); IL-10 (interleukin 10); IL-2 (interleukin 2); ROS (reactive oxygen species). Source: (Guerreiro et al., 2018).

All these effects lead to increased nutrient absorption resulting in improved nutrient utilization and enhanced metabolism culminating in increased weight gain and feed efficiency (Gatlin III & Peredo, 2012). Prebiotic effects on growth and feed utilization varies based on numerous factors for example, prebiotic source, supplementation level, fish species and age, rearing conditions and diet composition; with some having positive effects, no effects and sometimes negative effects on the growth of fish supplemented with prebiotics (Das et al., 2017; Guerreiro et al., 2018). Henceforth, Caipang and Lazado

(2015) proposed the prospect that growth-promoting properties of prebiotics in fish encompass complex underlying mechanisms which might be random in nature and greatly manipulated by the maturity of the physiological status of the host.

2.2.7 Inulin as a prebiotic in aquaculture

Inulin is one of the common prebiotics used in aquaculture, it is a fructan-type polydisperse carbohydrate made primarily of β (2→1) fructosyl-fructose glycosylic bonds with a degree of polymerization between 2-60 (Lauzon et al., 2014; Ringø et al., 2016). Inulin can be derived from natural vegetable or plant sources such as chicory, coneflower, dandelion, wheat, onions, leeks, garlic, asparagus, artichokes and bananas (Davani-Davari et al., 2019). However, it may not occur in fish diet and yet it provides many opportunities for applications in aquaculture to enhance the proliferation of beneficial bacteria, destroy pathogens and stimulate fish immunity (Ringø et al., 2010, 2016; Lauzon et al., 2014). The European Food Safety Authority (EFSA) in 2006 substantiated the health claim that inulin-type fructans and inulin extracted from chicory, with a mean degree of polymerization ≥ 9, "improves bowel function by increasing stool frequency" in humans (EFSA, 2015).

It is the β (2→1) linkages of inulin give that rise to the prebiotic effect of inulin, enabling inulin to withstand enzymatic hydrolysis by gastrointestinal tract enzymes and instead metabolized by β-fructosidase and inulinase producing bacteria resulting in the modulation of the gut microbiota (Caipang & Lazado, 2015; Enam & Mansell, 2019). The byproducts of inulin digestion include, butyric acid, propionic acid, and FOS (an intermediate product) (Song et al., 2014). Therefore, depending on the bacteria that are dominant in the gut of the fish, FOS could also be part of the resulting prebiotic effect of

inulin administration. The beneficial effects in fish include: stimulating innate immune response by increasing hematocrit, NBT and lysozyme activity, improving the alternative complement system, and improving disease resistance and survival for instance against parasitic infections (Song et al., 2014; Tiengtam et al., 2015; Yones et al., 2020). As well as improving growth performance and feed efficiency by increasing SGR, weight gain and lowering FCR (Tiengtam et al., 2015; Yones et al., 2020). And positively modulating gastrointestinal microflora by improving the proliferation of beneficial microbes such as and limiting the growth of pathogenic microbes (Tiengtam et al., 2015; Yones et al., 2020).

2.2.8 Mushrooms

Mushrooms are macroscopic fruiting bodies made by *Ascomycete* and *Basidiomycete* fungi in the course of sexual reproduction, majority of the famous and cultivatable edible mushrooms are from the group *Basidiomycota* and encompass the following genera: *Agaricus*, *Pleurotus*, and *Lentinula* (Dias & de Brito, 2017). Mushrooms are not only a source of food but they have medicinal properties as well (Prasad et al., 2015). Their use can be backdated to the ancient Egyptians and Chinese cultures who used them to promote healthy long lives as well as to treat diseases (Jayachandran et al., 2017). Consumable mushrooms are excellent sources of bioactive substances such as functional polysaccharides, essential amino acids along with minerals, for example, calcium, potassium, magnesium, iron, and zinc; moreover, fibers, terpenes, peptides, glyco-proteins, un-saturated fatty acids, and antioxidants like phenolic substances, as well as vitamin C and E (Jayachandran et al., 2017; Hoseinifar et al., 2019). It is these active substances that contribute to the benefits of mushrooms such as, antitumor, antimicrobial,

antioxidant, antiviral, and immunomodulatory properties; as well as anti-hypertensive activity, hypocholesterolemic activity, liver protection, anti-inflammatory activity and anti-diabetic activity (Jayachandran et al., 2017; Bharathi et al., 2019). Therefore, derivatives of bioactive polysaccharides or polysaccharide-protein complexes from mushrooms are receiving more attention as prebiotics (Singdevsachan et al., 2016).

The different bioactive compounds allow mushrooms to act as natural prebiotics due to the presence of indigestible polysaccharides such as; pleuran, lentinan, schizophyllan, β and α- glucans, mannans, xylans, galactans, chitin, inulin and hemi-celluloses (Singdevsachan et al., 2016; Van Doan et al., 2019). Moreover, the proven multiple health benefits mediated through different mechanisms of action make mushrooms excellent sources and potential prebiotics (Bhakta & Kumar, 2018). Mushrooms and their derivatives have found applications in aquaculture feeds due to their properties and health benefits, as well as the increased awareness among aqua-culturists (Bharathi et al., 2019; Hoseinifar et al., 2019). In addition, they have been used to enhance of growth performance, hematological parameters, innate immunity, and diseases resistance in many cultured species (Bharathi et al., 2019; Hoseinifar et al., 2019). The common mushrooms used in aquaculture include Chaga mushroom (*Inonotus obliquus*), Yun Zhi (*Coriolus versicolor*), Black hoof mushroom (*Phellinus linteus*), Oyster mushrooms (*Pleurotus ostreatus*), Shiitake (*Lentinula edodes*), Eryngii mushroom (*Pleurotus eryngii*), Lingzi mushroom (*Ganoderma lucidum*) and White button mushroom (*Agaricus bisporus*) (Zou et al., 2016).

Polysaccharides from fungi/mushrooms have a significant role in fish health and growth. They stimulate the innate immune system directly or indirectly by association with

pathogen- associated molecular pattern (PAMPs) that interact with PRRs expressed on immune cells (Mohan et al., 2019). Moreover, they enhance the fish's ability to protect itself against infection and sepsis by the activating leukocytes, phagocytic activity, and the production of inflammatory cytokines, chemokines, and reactive oxygen free radicals (Dobšíková et al., 2013). This often results in improved immune responses that are vital for fighting against pathogens as well as proper regulation of physiological function which can regulate survival and growth of an organism (Dobšíková et al., 2013; Mohan et al., 2019). The mechanism by which fungal polysaccharides improve growth performance and feed utilization is through, increasing specific growth rate, weight gain, survival rate and protein efficiency ratio (Van Doan et al., 2019). Furthermore, they reduce feed conversion ratio, enhance the release of digestive enzymes, and improve intestinal integrity; all of these promote digestion, absorption and utilization of nutrients (Van Doan et al., 2019). The mechanism by which they modulate the gastrointestinal microbiota is by competing with and inhibiting the attachment of pathogens (Van Doan et al., 2019). They further promote the growth of beneficial bacteria such as lactic acid bacteria which ferment mushroom polysaccharides to produce SCFAs leading to improved physiological conditions of the gastrointestinal tract and nutrient digestibility (Van Doan et al., 2019; Yin et al., 2020).

Termitomyces is a genus of consumable mushrooms which belongs to the *Lyophyllaceae* family, it is consumed by people in Africa and Asia (Elkhateeb & Daba, 2020; N'guessan et al., 2021). These mushrooms are often collected from the wild (on small hills and mounds) during rainy season. *Termitomyces* mushrooms grow in a symbiotic relationship with termites, they produce several enzymes that facilitate the digestion of lignocellulosic

substrates by termites (Elkhateeb & Daba, 2020; N'guessan et al., 2021; Yang et al., 2021). This symbiosis is vital for the survival of mushrooms and termites and the evolution of this sophisticated association (Elkhateeb & Daba, 2020; N'guessan et al., 2021; Yang et al., 2021). It brings about a challenge in trying to cultivate *Termitomyces* mushrooms besides their mycelium growing on nutritive media and substrates (Elkhateeb & Daba, 2020; N'guessan et al., 2021; Yang et al., 2021). In Namibia, *T. schimperi* commonly known as Omajowa is associated with the termite *Macrotermes michaelseni* (van der Westhuizen & Eicker, 1991). In Namibia it has been recorded to occur during the rainy season in Omaruru, Okahandja, Otjiwarongo, Grootfontein, Tsumkwe, Windhoek,Etosha National Park and the Zambezi, Kavango East and West regions (van der Westhuizen & Eicker, 1991).

Termitomyces are endowed with elevated nutritional value and a variety of medicinal properties. They are rich in proteins, amino acids, ascorbic acid including micronutrients such as vitamins and minerals (Hsieh & Ju, 2018; Elkhateeb & Daba, 2020; Yang et al., 2021). *Termitomyces* species have been used ethnomedicinally to promote good health, as well as to treat illnesses (Hsieh & Ju, 2018). This is mainly due to the presence of bioactive compounds such as phenolic compounds, polysaccharides, cerebrosides, serine protease, ergostanes, saponins, and fatty acid amides (Hsieh & Ju, 2018; Elkhateeb & Daba, 2020). Offering potential to be used as antioxidants, immunomodulators, antitumors, antimicrobials, antimalarials and even as prebiotics (Hsieh & Ju, 2018; Elkhateeb & Daba, 2020). Amadhila (2019) reported the antioxidant and prebiotic effects of polysaccharides extracted from *T. schimperi* and their potential for being functional foods.

The need for functional feed additives in aquaculture is on the rise as evident from literature as they promote good health in farmed species and improve the growth performance and feed utilization (Dawood et al., 2018; Van Doan et al., 2019; Boyd et al., 2020; Monica and Jayaraj, 2021). A literature search found no publications on the use of mushrooms as feed additives in aquaculture in Namibia. The benefits of including mushrooms or their derivatives have been reported in many studies (Liu et al., 2015; Chithra et al., 2016; Ahmed et al., 2017). Therefore, aquaculture in Namibia could benefit from the inclusion of native mushrooms such as *T. schimperi* in fish feed formulations.

2.3 Assessing fish gastrointestinal microbiota composition

Diet plays a significant role in modulating the composition of the microbiota such that, functional ingredients are propositioned to influence the microbiota and confer benefits onto the host (Tapia-Paniagua et al., 2019). The principal mechanism of action of prebiotics is via its influence on the gut microbiota, therefore, interpreting the make-up of fish gut microbiota communities is highly significant to comprehend their dynamic roles and how they impact host physiology (Ghanbari et al., 2015; Guerreiro et al., 2018). The major goal of fish gastrointestinal microbiota studies is to provide a scientific foundation for innovating approaches for successfully manipulating these microbial communities to enhance host health and aquaculture productivity (Romero et al., 2014). To realize this objective, detailed understanding of the structure and interactions between gut microbiota and their host, especially taxonomic composition, genetic potential and maintenance within the gastrointestinal tract is necessary and could shed light on the function and dysfunction of the host (Wang et al., 2018). Fish gut microbiota have been studied and

characterized using culture dependent methods and more recently, culture independent methods (figure 7) (Romero et al., 2014).

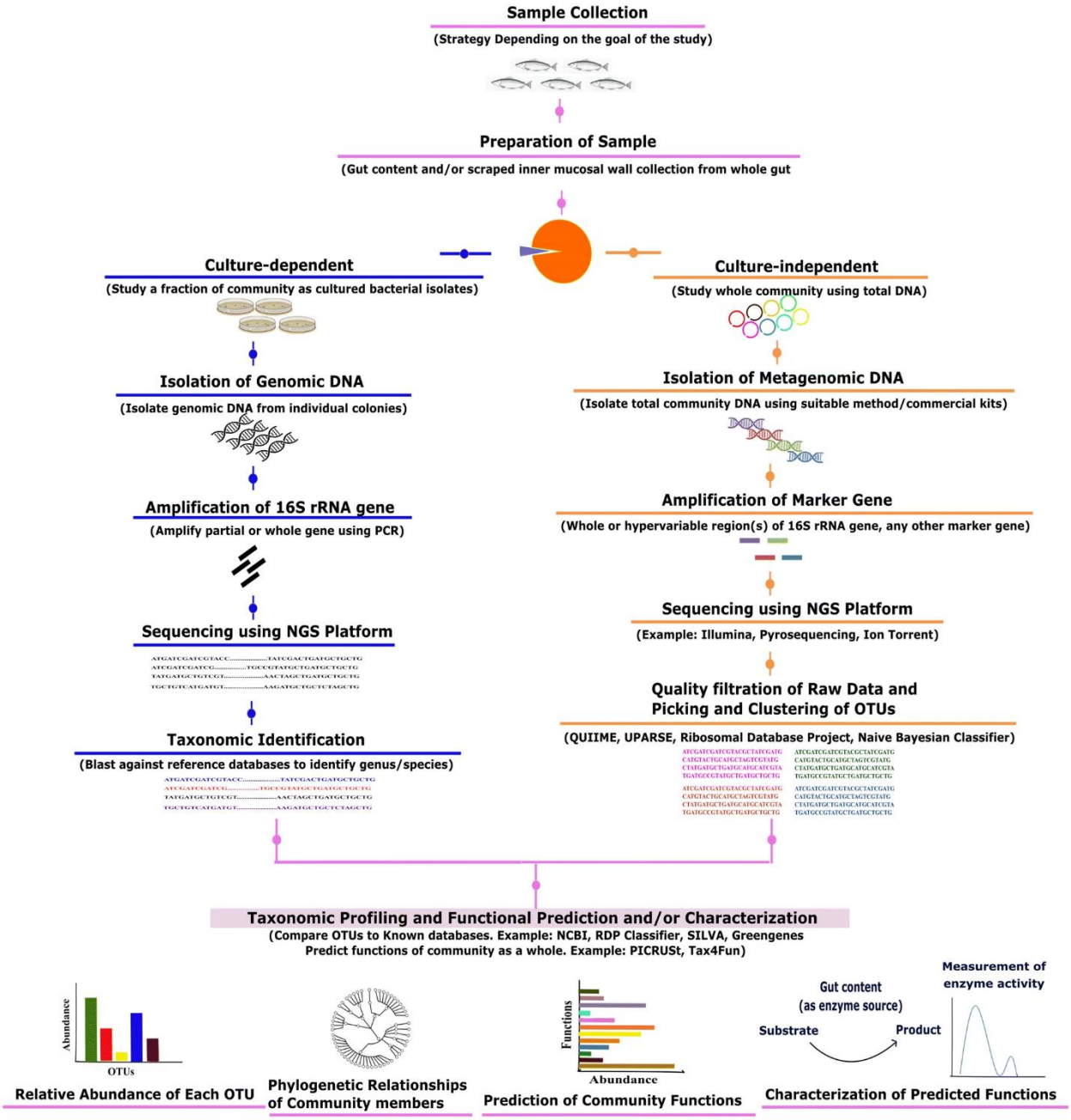

Figure 7: Diagrammatic illustration of the workflow for analyzing the fish gut microbiome for culture-dependent and culture independent methods. Source (Talwar et al., 2018).

2.3.1 Culture dependent methods

Culture dependent methods in studying fish gut microbiota involves, sampling the gut, spreading homogenates on general or selective media, incubation at appropriate temperatures, counting colonies to determine colony forming units, and finally identification of the microbes (Zhou et al., 2014). In older studies, biochemical tests and phenotypic observations were most relied upon for the identification of isolated microbes, however, due to the drawbacks such as lacking sufficient discrimination power, molecular methods are utilized recently as they give better and more accurate results (Romero et al., 2014; Zhou et al., 2014). These include, polymerase chain reaction (PCR), 16S rRNA sequencing analysis, randomly amplified polymorphic deoxyribonucleic acid (DNA) (RAPD) analysis, 16S rDNA restriction fragment length polymorphism (RFLP) (Zhou et al., 2014). The common molecular technique of bacterial identification in culture-dependent fish gut microbiota studies is 16S rRNA sequence analysis (see figure 7) and it been used to identify many bacterial species in different species of fish (Navarrete et al., 2009; Merrifield et al., 2014; Talwar et al., 2018; Wang et al., 2018).

It is widely known that only about 1% to 10% of all known microbes can be cultured in the laboratory. It is therefore no surprise that fish gut microbiota are of low cultivability. Furthermore, culture-based methods are time consuming, they lack accuracy and most incubation conditions do not reflect host environmental conditions (Romero et al., 2014; Zhou et al., 2014). Although culture-dependent techniques do not reveal an accurate depiction of the microbial communities in the gastrointestinal tract of the fish, they can be valuable if they are performed appropriately in combination with molecular identification techniques, (Romero et al., 2014; Zhou et al., 2014). For instance, culture-dependent

methods can still be relied upon when culture-independent methods are not readily available, or when the goal is to determine the number of culturable viable cells present in a sample for plate count studies. They may be able to point out differences due to minor dietary alterations in microbial communities (Ringø et al., 2016). It is recommended that future culture-based approaches appreciate the unique characteristics of fish and apply protocols that are representative of the host's environmental conditions, especially the selection of culture media and incubation conditions (Zhou et al., 2014).

2.3.2 Culture independent methods

The reliance on bacteria culture-based methods has significantly declined. Consequently, culture independent approaches have become the most appropriate and preferred method for microbial and metagenomics studies (Yukgehnaish et al., 2020). Culture independent methods highlighted the shortcomings of the culture dependent methods and they are effectively instrumental in advancing the understanding of complex ecosystems (Ringø et al., 2016). Without isolation, these approaches have aided the identification of microbes and revealed their phylogenetic affiliation unveiling the vast extent of microbial diversity (Romero et al., 2014). Culture independent methodologies include, molecular methods based on the sequence variability of 16s/18s/23s (rRNA) genes (Zhou et al., 2014; Ringø et al., 2016). As well as fluorescent-based methods that allows the simultaneous detection of different microbes using a set of fluorophores with different excitation and emission maxima for example fluorescent *in situ* hybridization (FISH), and electron microscopy (Zhou et al., 2014; Ringø et al., 2016).

In fish gut microbiota studies especially the ones analyzing microbial diversity, the 16s rRNA gene is the most targeted for bacterial taxonomy in most molecular based methods

(Ringø et al., 2016; Tarnecki et al., 2017). The 16s rRNA gene is used as a genetic marker and has been used to reconstruct prokaryotic phylogenies (Chaudhary et al., 2015). This is due to its ubiquitous distribution in bacterial populations and the presence of nine hypervariable regions V1-V9 that are conserved in all prokaryotes (Chaudhary et al., 2015; Johny et al., 2021). Approximating the diversity of fish gut microbiota via high throughput molecular sequencing using 16s rRNA genes may offer effective methods for evaluating microbial diversity and their mutations in respect with environmental conditions and farm management (Zarkasi et al., 2014). According to (Zhou et al., 2014), depending on the aim of the study, the following molecular methods have been used:

1. Clone libraries for identifying the microbiota composition.
2. Fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE) for analyzing microbial community structure and diversity.
3. Quantitative real-time polymerase chain reaction (qPCR) or FISH for determining the abundance of particular taxa or total microbial levels.
4. FISH and immunohistochemistry for assessing bacterial–host interactions at the mucosal brush border.

In most of these molecular approaches, community DNA is extracted, amplified using polymerase chain reaction (PCR) using specific primers for conserved regions of 16s rRNA followed by sequence analysis (Ringø et al., 2016). Recently, next generation sequencing (NGS) technology has been employed in the analysis of fish microbial communities' composition and functional properties (Ghanbari et al, 2015). These platforms produce significantly higher numbers of reads per run facilitating the attainment

of in-depth and accurate sequence data in a rapid and cost-effective way and therefore enable the detection of low abundance (rare) microbial communities (Ghanbari et al., 2015). While culture independent techniques avoid significant biases of culture-based methods, there are biases inherent to individual culture independent methods that could neglect rare bacterial taxa that could potentially play major roles in gut nutrition and disease (Zhou et al., 2014; Ringø et al., 2016). Principally, combining a few molecular based approaches may be indispensable for determining microbiota community composition for instance, clone libraries and PCR based profiling (Ringø et al., 2016). Romero et al., (2014) postulates that as these technologies become available (at low cost), our understanding and knowledge of the fish microbiota will improve significantly allowing identification of the rare biosphere and metabolic pathways.

Progress in DNA sequencing technology currently enables massive parallel sequencing of PCR amplicons or genomic samples (Zhou et al., 2014). This delivers adequate coverage including less abundant gut microbes (Zhou et al., 2014). Majority of the studies in fish microbiota frequently utilize two NGS technologies namely 454/Roche pyro sequencing and Illumina technologies (Ghanbari et al., 2015). These platforms are able to rapidly generate large volumes of sequence data in parallel cost-effectively (Ghanbari et al., 2015) . Individual sequencing technologies have their own set of biases and limitations, and the critical parameters to consider for NGS applications are: the length of the sequencing read, the numbers of sequencing reads, sequencing errors, the ability to include multiple samples, and the cost per sample (Zhou et al., 2014). Longer and more sequences reads, less sequencing errors, ability to run more samples at the same time and low cost are preferred. The first and most significant step in bioinformatics is to trim the sequences for

sufficient quality, although this reduces the number of useful sequences for phylogenetic analysis it is empirical to have high quality sequences (Zhou et al., 2014).

2.3.3 Metagenomics

Metagenomics has been defined as the direct genetic analysis of genomes contained with an environmental sample (Thomas et al., 2012). It reveals functional gene composition of microbial communities such as genetic information on potential novel biocatalysts or enzymes, genomic linkages between function and phylogeny for uncultured organisms, and evolutionary profiles of community function and structure (Thomas et al., 2012). Metagenomics analysis can either be function-based which consists of constructing metagenomic libraries containing fragments of community DNA and screening for a specific phenotype (Johny et al., 2021). Otherwise, it can be sequence-based whereby PCR primers are designed to amplify genes of interest from metagenomic DNA followed by sequencing amplicons or pool clone libraries to identify specific genes or phylogenetic anchors of interest for example 16s rRNA (Johny et al., 2021).

Illumina sequencing technology is perhaps the most commonly used NGS approach in fish gut microbiota metagenomics studies and the principle behind this platform is sequencing-by-synthesis chemistry (figure 8) (Ghanbari et al, 2015). First, DNA fragments fixed to compliment adaptors on a flow cell surface are amplified to yield clusters (Ghanbari, 2015; Johny et al., 2021). Followed by the addition of the reaction mixture consisting of fluorescently labelled reversible terminator nucleotides along with DNA polymerase and sequencing primers which initiates the sequencing (Metzker, 2010; Ghanbari et al., 2015; Johny et al., 2021). Lastly, the position of the nucleotide is then recorded using a fluorescence scanner (Metzker, 2010; Ghanbari et al., 2015; Johny et al.,

2021). In amplicon data processing, the preliminary step is to demultiplex reads, merge paired-end reads, remove barcode and primer sequences, and a quality control step to remove low-quality sequences (Johny et al., 2021). Then, the reads are clustered into operational taxonomic units (OTUs) or ASVs based on sequence similarity for further bioinformatics analysis such as inferring taxonomic composition (Johny et al., 2021) .

In ASVs the sequence variants are distinguished by at least a single nucleotide change, as opposed to OTUs most previously used in sequence analysis where reads are clustered based on 97% similarity (Callahan et al., 2017). In fact, they argued that ASVs should be used in the place of OTUs in marker gene data analysis. ASVs give higher resolution, are intrinsically biologically meaningful, are reference bias free and can be used again, replicated and easily understood (Callahan et al., 2017). Therefore, despite the limitations associated with them, ASVs significantly addresses analysis associated concerns and allows for more extensive and replicable analyses compared to OTUs (Callahan et al., 2017).

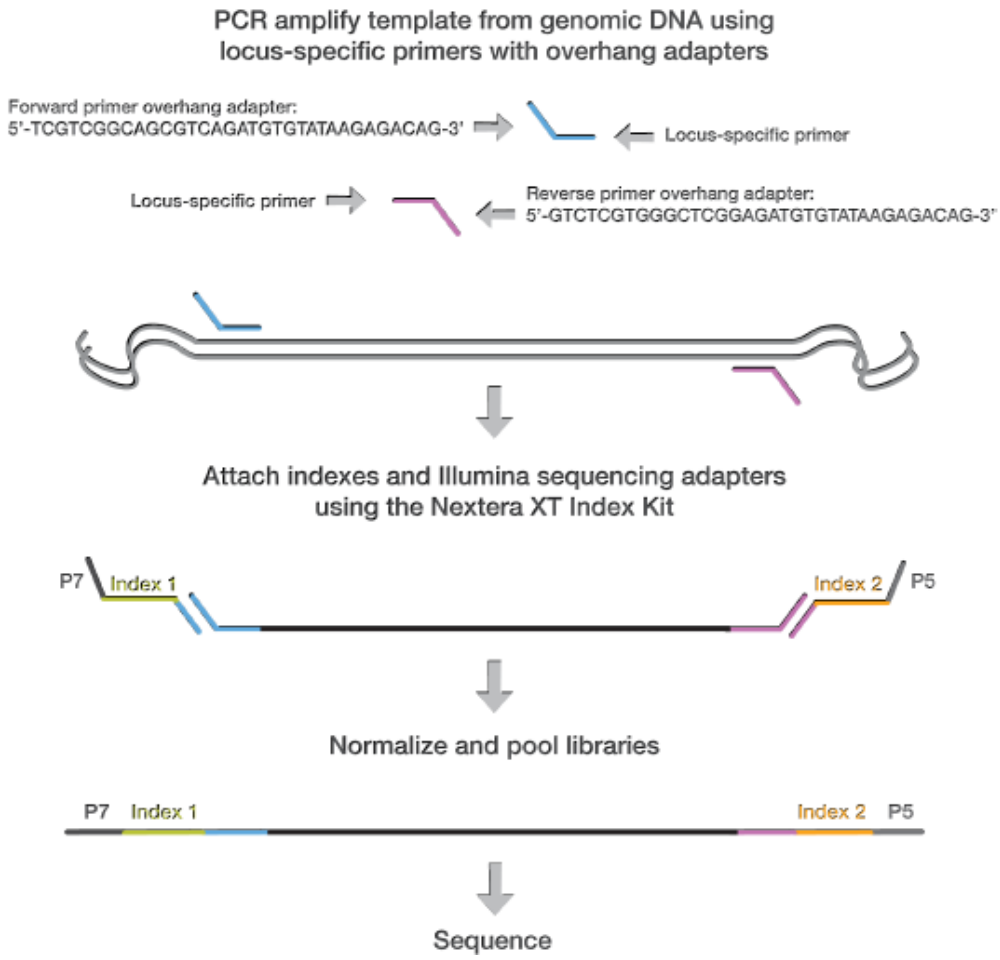

Figure 8: 16s V3-V4 amplicon work flow. Source: (Illumina, 2013).

Among the Illumina platforms, HiSeq sequencers yield larger sequence data per run (up to 1500 Gb), at the lowest cost per base, with the shortest reads; as compared to the MiSeq platforms which generates 44–50 million paired-end reads, with higher costs per sequenced base and longer sequence reads (particularly used in amplicon sequencing projects (Ghanbari et al., 2015) . Furthermore, recently released NextSeq and NovaSeq perform at an intermediate scale in terms of output, read length and costs per base compared to HiSeq and MiSeq and may be valuable for large sequencing projects (Ghanbari et al., 2015; Johny et al., 2021). Using Illumina sequencing for 16S rRNA

surveys takes advantage of larger number of reads and low cost, while including a quality control assembly step to reduce sequencing errors (Zhou et al., 2014).

Aquaculture is an ideal media for the proliferation of microorganisms due to large amounts of water used, nutrient sources such as feed, fertilizers, prebiotics, probiotics, and chemicals such as antibiotics, products to reduce ammonia, pH buffers as well as organic matter; giving aquaculture perhaps the most intricate and diverse microbiological interaction networks (Martínez-Porchas and Vargas-Albores, 2017; Ortiz-Estrada et al., 2019). Currently, the developments in NGS technologies have revolutionized fish gut microbiota studies by delivering faster results as well as novel, deep resolution insights into the composition and function of the microbiome (Yukgehnaish et al., 2020; Johny et al., 2021) . Furthermore, the factors that shape gut microbiota and the physiological functions of microbiota are the two major themes of fish gut microbiome research (Johny et al., 2021). Most aquaculture microbiota metagenomics research focus on exposing and comprehending the symbiotic and antagonistic interactions between the microbiota and eukaryotes, resulting in insightful knowledge of those ties by relating information revealed by extracted DNA to host or environment specific host species (Yukgehnaish et al., 2020).

Metagenomics research is of outmost importance in aquaculture, and thanks to NGS and new bioinformatics technologies it is now possible to study the diversity of intracellular bacteria and at the same time reveal relevant genomic information from such communities (Martínez-Porchas and Vargas-Albores, 2017). Metagenomics presents further proof needed to understand and elucidate the extent of microbial diversity in aquaculture facilities via the analysis of hyper variable regions of 16S rRNA for prokaryotes and 18S for eukaryotes (Martínez-Porchas and Vargas-Albores, 2017). In addition, 16s rRNA

gene-based metagenomics can be used to predict taxonomical and functional profiles and explain metabolic capabilities of microbiota communities (Ortiz-Estrada et al., 2019). Functional metagenomics can help explain microbe metabolism in aquaculture. For example, by studying the genes and operons encoding for proteins involved in sensing, uptake and utilization of particular metabolites; the capacity and biochemical pathways of given microbial communities to recycle nutrients could be understood with potential for applications in bioremediation or recirculation processes (Martínez-Porchas and Vargas-Albores, 2017).

Furthermore, metagenomics studies provide the opportunity to detect and identify novel pathogenic bacterial and viral strains before they become a problem (Martínez-Porchas and Vargas-Albores, 2017). They can also be applied in bio floc technologies to help clear up the full mechanism of this technology (Martínez-Porchas & Vargas-albores, 2017). In addition, metagenomics studies can be used to detect antimicrobial resistance and search for novel probiotics and monitoring microbial evolution of communities (Martínez-Porchas & Vargas-albores, 2017). Other applications include discovery of novel bioactive molecules such as antibiotics and enzymes (Martínez-Porchas & Vargas-albores, 2017). Unfortunately, the large amounts of noisy and partial data generated in metagenomics presents a challenge for biologists and bioinformaticians (Martínez-Porchas & Vargas-albores, 2017). Nevertheless, the development of powerful and robust bioinformatics tools has made it possible to interpret these results (Johny et al., 2021) . Above all, the data generated in metagenomics studies could be useful in improving fish feed formulations or developing probiotics or prebiotics for application in aquaculture (Johny et al., 2021).

CHAPTER 3: RESEARCH METHODS

3.1. Research design

This study was based on the application of the effect of inulin and mushroom powder on fish growth performance and feed utilization parameters (weight gain rate, specific growth rate, and feed conversion ratio). While the application of metagenomics analyses was applied in the assessment of inulin and mushrooms on the gastro intestinal microbial diversity of *O. mossambicus*.

3.2. Study area

The study was conducted at HIAC, MFMR located at the Hardap Dam Game Reserve about 26 kilometres northwest out of Mariental, Hardap region, Namibia (24°29’44” S, 17°51’50” E).

3.3. Procedures

3.3.1 Experimental diets

The basal diet which entails Aqua Plus 2mm Tilapia Starter Feed pellets (figure 9 A) comprising 40% protein and 5% fat was purchased from Avi Products, South Africa. Organic Inulin powder (figure 9 B) by Now Foods was purchased from Germany. Moreover, Omajowa mushrooms (*T. schimperi*) (figure 9 C) were obtained from local vendors in Okahandja, Namibia. The mushrooms were brush-cleaned, shredded into smaller pieces and air dried for two days. The dried mushroom pieces were then homogenized into fine powder using a Waring laboratory blender (Waring Commercial, Torrington, USA).

Figure 9: basal diet (source: this work), B: Inulin powder (source: this work), C: Omajowa mushrooms. Source: (Amadhila, 2019).

Three experimental diets; Inulin, Inulin+ Mushroom and Control were made following procedures developed by Li, Tuan, et al. (2019) with minor modifications. The inulin and mushroom powder were added at one percent dry weight of fish feed to de-ionised water to make a concentration of 25% per diet as described in table 1 and added to the basal diet by spraying the solution of inulin and pouring the suspension of inulin + mushrooms on the commercial fish feed pellets and mixing homogeneously by hand to ensure even distribution onto the pellets. The feeds were then spread evenly onto clean plastic sheets and left overnight to dry in an air-conditioned room. After drying they were packed away into separate buckets and kept at -20 degrees Celsius until the start of the feeding trials.

Table 1: Experimental diets formulation

Experimental diet	**Basal diet (g)**	**Prebiotic at 1% dry weight (g)**	**De-ionised water (mL)**
Inulin (I)	3000	30	120

Inulin + Mushroom (IM)	3000	15 (I) + 15 (M)	120
Control (C)	3000	0	120

3.3.2 Fish preparation and feeding trials

O. mossambicus fingerlings reared at HIAC, MFMR were obtained and randomly assigned to each experimental diet (Control, Inulin and Inulin + mushroom). Two hundred and seventy (thirty per tank) fingerlings were randomly distributed into 9 (three replicates of three experimental diets) tanks (H- 95 cm and D- 100 cm) (figure 10). Each contained **0.448 m^3** of municipal water with adequate continuous aeration (CFW Fans Side Channel Blower Model ZXB-510) in a water recirculation system and a water heater to keep the temperature of the water at optimum temperature between 25 to 29 degrees Celsius (Fitzsimmons, 2014). To acclimatize the fingerlings to experimental conditions, the fish were fed control diet until apparent satiation four times daily (10:00, 12:00, 14:00 and 16:00) for a week.

Figure 10: Hatchery tanks (source: this work)

The feeding trials in this study were set up as previously described by Gabriel et al. (2019) with minor modifications. After acclimatization, initial fish mass of the fish per tank was collectively recorded and fed experimental diets four times daily (10:00, 12:00, 14:00 and 16:00) for six weeks. Daily fish feed was calculated to provide 5% of the fish body weight, however, it was adjusted accordingly depending on feed consumption to keep it close to apparent satiation. Daily fish feed consumption was recorded. All fish from each tank were weighed every two weeks in order to adjust the calculated daily fish feed. Fish were not fed on the day of sampling. Throughout the study, continuous aeration was maintained. Water quality parameters; temperature and dissolved oxygen (DO) were monitored and recorded daily using Hach HQ 40D machine and luminescent dissolved oxygen (LDO) probe. During the course of the experiment, the average water temperature was 25.69 ±

0.06 °C with a minimum of 25.32 °C and a maximum of 25.68 °C; and the average DO was 6.42 ± 0.12 mg/L with a minimum of 6.12 mg/L and a maximum of 6.65 mg/L. Water was exchanged every two weeks and mortalities were removed and recorded daily.

3.3.3 Growth performance and feed utilization

All the fish was starved for twenty-four hours and their final body weight (BW) was recorded. Together with initial recordings were used to calculate growth performance and feed utilization parameters as described by Li, Tuan, et al. (2019) at the end of the feeding trials as follows:

$$\text{Weight gain rate (\%)} = \frac{(final\ BW\ (g) - initial\ BW(g))}{initial\ BW} \times 100$$

$$\text{Specific growth rate (\%/day)} = \frac{(In\ final\ BW\ (g) - In\ initial\ BW\ (g))}{feeding\ days} \times 100$$

$$\text{Feed conversion ratio} = \frac{total\ feed\ consumption\ (g)}{final\ BW\ (g) - initial\ BW\ (g)}$$

$$\text{Survival rate (\%)} = \frac{final\ number\ of\ fish}{initial\ number\ of\ fish} \times 100$$

3.3.4 Proximate composition

This experiment was performed using 3 fish from each tank/replicate with gastro intestinal tract removed kept in Ziploc bags at -20 degrees Celsius at the end of the feeding trials. They were transported to the Ministry of Fisheries Laboratory at Onavivi Inland Fisheries and Aquaculture Centre, Outapi (-17° 44' 76.36" S, 14° 78' 26.8" E) and The University of Namibia, Windhoek (-22° 36' 23.99"S, 17° 03' 16.20" E) for further proximate composition analyses.

Moisture content was determined using the aeAdam PMB 202 Moisture Analyser (figure 11 C) following the manufacturer's protocol. Approximately two grams of fish tissue was spread evenly onto the analyser plate, during operation. The analyser dehydrated the tissue sample at 110 degrees Celsius for a period ranging from 18-33 minutes until all moisture has escaped from the sample. The analyser has a built-in balance and calculator to determine the moisture content expressed in percentage using the following formula:

$$\text{Moisture content (\%)} = \frac{initial\ sample\ mass\ (g) - final\ sample\ mass\ (g)}{initial\ sample\ mass} \times 100$$

Ash content was determined by igniting approximately two grams of wet tissue samples at 560 °C for 5 hours in an ashing furnace (Carbolite AAF 1100, country of origine) (figure 11 D) in heat-stable crucibles (Gabriel et al., 2019). The mass of the sample together with crucible was recorded before and after burning to determine ash content expressed as percentage as follows:

$$\text{Ash content (\%)} = \frac{final\ sample\ mass(g)}{initial\ sample\ mass\ (g)} \times 100$$

Crude lipid content was determined by ether extraction system FOSS Soxtec 2043 (figure 11 A & B) following manufacturer's laboratory protocol guide using petroleum ether as the solvent, (Gabriel et al., 2019). The initial mass of the sample and the final mass of the residue obtained were used to determine lipid content expressed in percentage as follows:

$$\text{\% lipid} = \frac{residue\ mass\ (g)}{initial\ sample\ mass\ (g)} \times 100$$

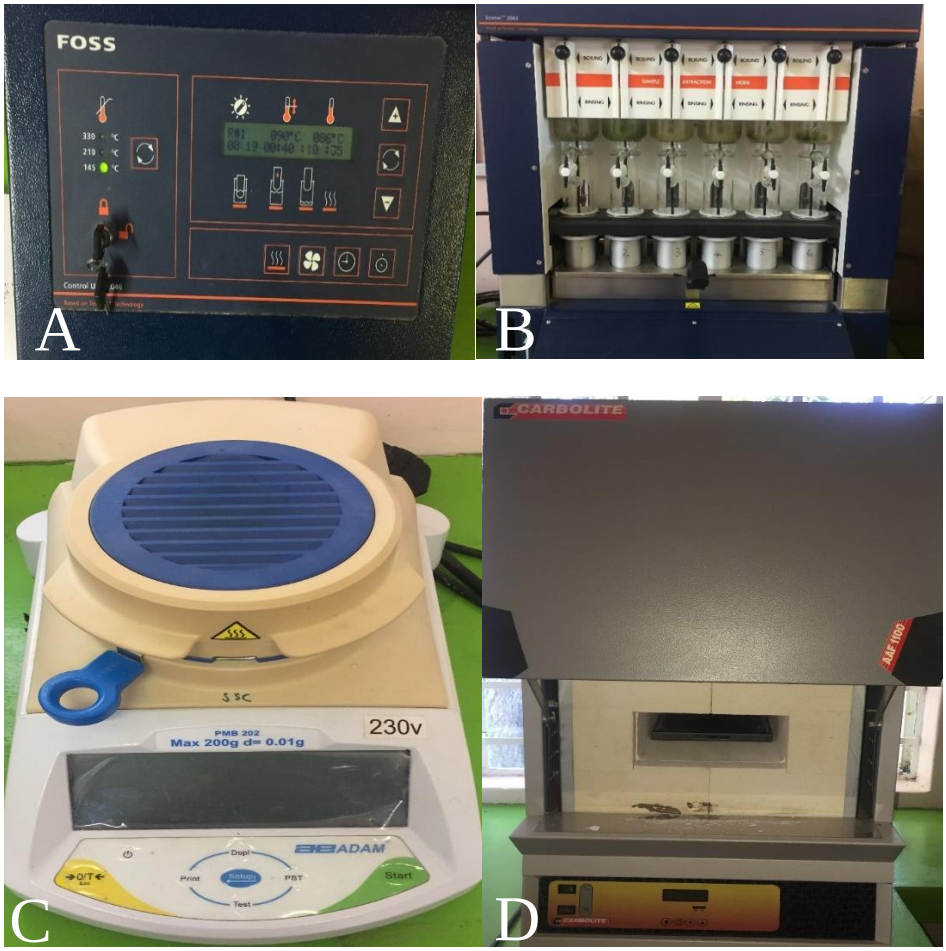

Figure 11: FOSS Soxtec 2043 ether extraction system (A&B), C: aeADAM moisture analyser and D: Carbolite AAF 100 ashing furnace/oven. (Source: this work).

For protein content determination, samples were sent to Analytical Laboratory Services, Windhoek 22° 52’ 49” S, 17° 07’ 62” E. Protein content was determined from six fish samples, two from each of the three experimental groups using the Kedjahl methods (Association of Analytical Chemists, 2000).

3.3.5 Gastrointestinal microbiota modulation

3.3.5.1 Sampling

At the beginning of the feeding trials and after every two weeks, two fish from each replicate were sacrificed and the gastro intestinal tract contents were removed (figure 12) and kept at -20 ˚C. This was done to monitor the evolution of the gastro intestinal microbiota population. Fish were euthanized by immersing them in ice water, a common practice within the Ministry of Fisheries to kill and immobilize fish in a humane way. After eight weeks, the final sampling was done as described above, gastro intestinal tract contents kept at -20 °C were transported to The University of Namibia laboratory and kept at -80 °C until further analysis.

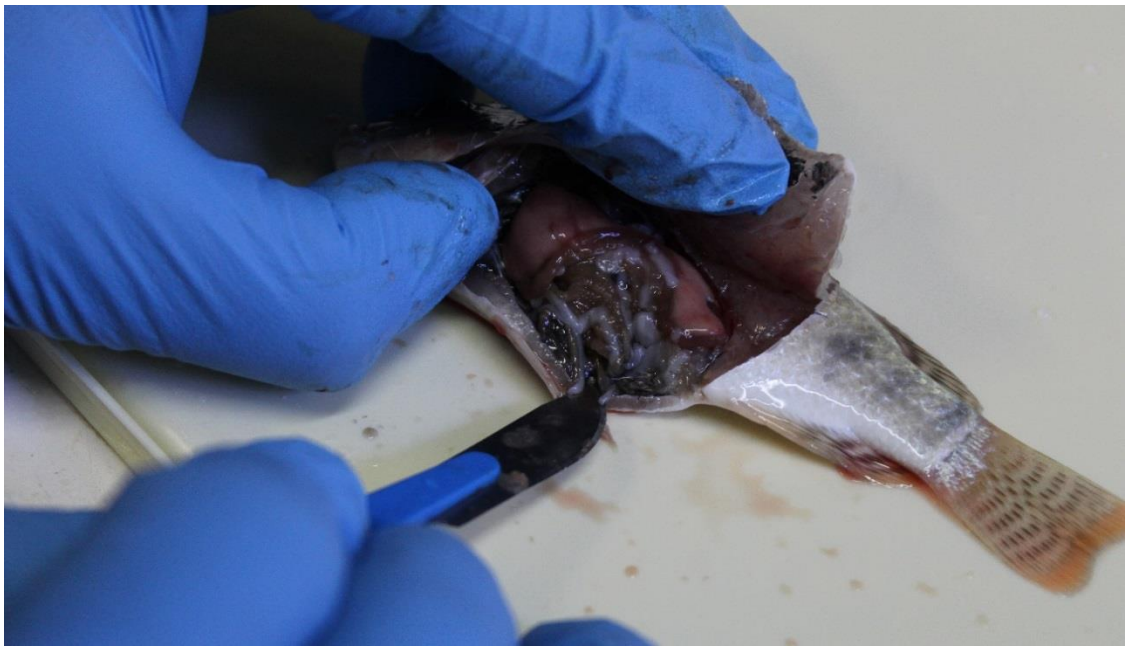

Figure 12: Dissected fish for gastrointestinal removal (source: this work)

3.3.5.2 Culture dependent method

Anaerobic total plate count was conducted on six intestinal samples (day 0: control group (C1); day 29: inulin group (I29) and inulin + mushroom group (IM29) inulin + mushroom group (IM29); and day 57: inulin group (I57), inulin + mushroom group (IM57) and control group (C57) in triplicate. Preparation of samples for the anaerobic plate count were done as previously described by (Jatobá et al., 2011; Koh et al., 2016) with minor modifications. Intestinal samples were homogenized using sterile mortar and pestle, then serially diluted by 1:10 in distilled water in 1.5 ml Eppendorf tubes and all the dilutions were plated onto Plate Count Agar (PCA) using the spread plate method and incubated at room temperature (25 °C) under anaerobic condition using an anoxic tank. After incubation, only the plates with 30-300 colonies were counted (Rawling et al., 2009). To calculate CFU per gram, the formula below was used and then the number of CFU per gram was transformed into log10 in IBM SPSS version 26.

$$CFU\ per\ gram = no.of\ CFUs \times \frac{1}{final\ dilution\ factor}$$

3.3.5.3 Culture independent method

DNA extraction

DNA was extracted from six intestinal samples (C1, I29, IM29, I57, IM57, C57) using the Soltis Lab CTAB DNA Extraction Protocol with minor modifications (Doyle & Doyle, 1987; Cullings, 1992). Eluted DNA was quantified and checked for quality (appendix 1A & 1B) using Nanodrop 2000c (ThermoFisher Scientific, country). And the samples were sent to Inqaba Biotechnologies in South Africa for 16s rRNA metagenomics analysis.

Metagenomics sequencing

The purified genomic DNA was characterized using the standard procedure established by Inqaba Biotechnologies (South Africa) for high throughput 16s rRNA metagenomics sequencing on Illumina Miseq sequencing platform. The primers used were '341F CCTACGGGNGGCWGCAG' and '785R GACTACHVGGGTATCTAATCC' Thijs et al. (2017) which targeted the V3-V4 region of the 16s rRNA gene. The generation of 16S rRNA V3-V4 region amplicons was done as described in the 16s Metagenomics Sequencing Library Preparation guide (Illumina, 2013).

Sequence analysis

Paired-end demultiplexed sequences containing quality information were received from the sequencing centre Inqaba Biotechnologies in FASTQ format. Paired-end sequences were imported in Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2021.4 installed in a virtual machine (Virtual box) as artifacts and all sequence analysis were carried out using plugins available in QIIME2 environment (Bolyen et al., 2019). Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin, a reference free algorithm was used to; trim bases with a median Phred33 quality score below 28, denoise, remove chimeric sequences and singletons, join paired-end reads, dereplicate and cluster reads into ASVs that were ≥ 99% similar to each other (Callahan et al., 2016). ASVs outputs from DADA2 were aligned with mafft, masked with mask and a rooted phylogenetic tree was built using fasttree to enable downstream diversity metrics analysis (Stackebrandt and Goodfellow, 1991; Price et al., 2010; Katoh and Standley, 2013). The diversity plugin was used to determine the core alpha diversity metrics (Shannon's index, and observed features (ASVs)) and core beta diversity metrics (Jaccards's distance, Bray-Curtis distance,

Unweighted and weighted UniFrac distance) at a rarefied sampling depth of 14600 sequences. Taxonomy was assigned using a pre-trained Greengenes 99% OTUs 16S rRNA full length sequences (Mcdonald et al., 2012; Bokulich et al., 2018; Robeson II et al., 2020) to generate taxa bar plots. Differential abundance was analysed using cluster correlation balances in gneiss (Morton et al., 2017). The graphs and plots generated were in the QIIME2 zipped visualization (.qzv) format and were visualized on the QIIME2 view web interface.

3.4 Statistical analyses

Three experimental diets, Inulin (basal diet + 1% inulin), Inulin+ Mushroom (basal diet + 0.5% inulin + 0.5% *T. schimperi* powder) and Control (basal diet only) were used in this study. All measurements in the experiments for growth performance and feed utilization, proximate composition and total anaerobic bacterial count were carried out in triplicate. Except for the determination of protein content which was done in replicate due to the limitation of funds (sourced from own pocket) to pay for the labporatory test. Moreover, only six DNA samples were sent for metagenomics sequencing, this was also due to the limitation of funds due to the high cost of sequencing per sample.

Data were analyzed using IBM SPSS Statistics version 27 (2020) at 95% confidence interval. The results were expressed as mean ± standard error (SE). Shapiro-Wilk test was used to test whether the data was normally or non-normally distributed. The results showed no evidence of non-normality of the data for the variables WGR ($W(9) = 0.91$, $P = 0.33$), SGR ($W(9) = 0.93$, $P = 0.46$), FCR ($W(9) = 0.86$, $P = 0.10$), SR ($W(9) = 0.85$, $P = 0.07$), moisture content ($W(9) = 0.95$, $P = 0.67$), ash content ($W(9) = 0.97$, $P = 0.87$) and log10 CFU per gram ($W(18) = 0.91$, $P = 0.08$). On the other hand, Shapiro-Wilk test

showed that the data for the variables fat content (W (9) = 0.77, P = 0.009), and protein content (W (6) = 0.64, P = 0.001) departed from normality.

In addition to normality, further assumptions for analysis of variance (ANOVA) namely, homogeneity of variance and independent samples were tested (Sawyer, 2009). Levene's test was used to test the data for homogeneity of variance. The Levene's test rejected the null hypotheses of equal population variances for the variables FCR (F (2,6) = 6.40, P = 0.03) and log 10 CFU per gram (F (5,12) = 3.16, P = 0.048); and it could not be computed for the protein content data due to all absolute deviations being constant in each cell. The Levene's test failed to reject the null hypotheses of equal population variances for the variables WGR (F (2,6) = 1.61, P = 0.28), SGR (F (2,6) = 1.33, P = 0.33), SR (F (2,6) = 4.31, P = 0.07), moisture content (F (2,6) = 2.94, P = 0.13), fat content (F (2,6) = 4.90, P = 0.06) and ash content (F (2,6) = 3.42, P = 0.10).

Since the experiment was a randomized design, WGR, SGR, SR, moisture content and ash content data met all the assumptions of ANOVA and therefore, one way ANOVA was performed to compare the effect of the experimental diets on WGR, SGR, SR moisture and ash content. While, Kruskal-Wallis Mann-Whitney test was used to compare the effect of the experimental diets on, FCR, fat content, protein content and log 10 CFU per gram because they violated at least one assumption for ANOVA. Kruskal-Wallis and Permutational Analysis of Variance (PERMANOVA) tests were used to test the significant differences in the alpha and beta diversity plots respectively in the QIIME2 environment.

3.5 Ethics

The proposal was approved by the University of Namibia Centre of Postgraduate Studies committee and ethical clearance from University of Namibia, Research and Ethics Committee was obtained. Permission to use data and facilities from the Ministry of Fisheries was obtained (Appendix 2). Fish were euthanized with ice cold water during handling and sampling. After all the procedures, fish were recycled following procedures at the University of Namibia.

CHAPTER 4: RESULTS

4.1 Growth performance and feed utilization

In this study, the results of the experiment carried out on the growth performance and feed utilization parameters (Table 2) show the differences between the three experimental diets. The Kruskal-Wallis test showed that the experimental prebiotic treatments had no significant effect on the FCR ($H(2) = 1.88$, $P = 0.39$) of *O. mossambicus* fingerlings in the study. One-way ANOVA revealed significant differences in mean WGR ($F(2,6) = 7.64$, $P = 0.02$), SGR ($F(2,6) = 7.83$, $P = 0.02$), and SR ($F(2,6) = 5.29$, $P = 0.05$) between the experimental diets. Furthermore, post hoc Tukey HSD test (Table 3) revealed that the inulin experimental diet had a significantly lower WGR ($P = 0.02$) and SGR ($P = 0.02$) compared to the control diet. However, no significant differences in mean WGR ($P > 0.05$) and SGR ($P > 0.05$) between the inulin diet and inulin + mushroom diet. As well as no significant difference in mean WGR ($P > 0.05$) and SGR ($P > 0.05$) between the inulin + mushroom diet and control diet. On the other hand, the Tukey HSD test revealed no significant differences between the groups in mean SR ($P > 0.05$), this is despite the one-way ANOVA giving a statistically significant result ($F(2,6) = 5.29$, $P = 0.05$).

Table 2: Means of growth performance and feed utilization parameters at $\alpha = 0.05$

Diet	SGR (%)	WGR (%)	FCR	SR (%)
Inulin	1.80 ± 0.07^{e}	117.25 ± 6.20^{e}	2.34 ± 0.14^{e}	95.83 ± 4.17^{e}
Inulin + mushroom	1.89 ± 0.02^{e}	124.91 ± 2.19^{e}	2.52 ± 0.11^{e}	94.44 ± 1.39^{e}
Control	2.15 ± 0.09^{e}	152.59 ± 9.61^{e}	1.87 ± 0.43^{e}	78.89 ± 5.56^{e}

Table 3: One-way ANOVA post hoc Tukey HSD test results at α = 0.05

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment group	(J) Treatment group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Weight gain ratio in %	Inulin	Inulin+Mushroom	-7.66000	9.51169	.714	-36.8445	21.5245
		Control	-35.33667*	9.51169	.023	-64.5211	-6.1522
	Inulin+Mushroom	Inulin	7.66000	9.51169	.714	-21.5245	36.8445
		Control	-27.67667	9.51169	.061	-56.8611	1.5078
	Control	Inulin	35.33667*	9.51169	.023	6.1522	64.5211
		Inulin+Mushroom	27.67667	9.51169	.061	-1.5078	56.8611
Specific growth rate in %/day	Inulin	Inulin+Mushroom	-.08667	.09306	.642	-.3722	.1989
		Control	-.35333*	.09306	.021	-.6389	-.0678
	Inulin+Mushroom	Inulin	.08667	.09306	.642	-.1989	.3722
		Control	-.26667	.09306	.064	-.5522	.0189
	Control	Inulin	.35333*	.09306	.021	.0678	.6389
		Inulin+Mushroom	.26667	.09306	.064	-.0189	.5522
Survival rate in %	Inulin	Inulin+Mushroom	1.39000	5.78276	.969	-16.3531	19.1331
		Control	16.94667	5.78276	.059	-.7964	34.6898

	Inulin+Mushroom	Inulin	-1.39000	5.78276	.969	-19.1331	16.3531
		Control	15.55667	5.78276	.080	-2.1864	33.2998
	Control	Inulin	-16.94667	5.78276	.059	-34.6898	.7964
		Inulin+Mushroom	-15.55667	5.78276	.080	-33.2998	2.1864

*. The mean difference is significant at the 0.05 level.

4.2 Proximate composition

To determine the effect of inulin or inulin + mushroom on nutrient deposition in the studied fish, proximate composition of fish such as moisture (figure 13), fat (figure 14), ash (figure 15) and protein are analyzed (Tiengtam et al., 2015). The results of the proximate composition analysis of *O. mossambicus* fingerlings in the study are presented in table 4 and appendix 3. In this study, Kruskal-Wallis test revealed no significant differences in fat content ($H(2) = 2.76$, $P = 0.25$) and protein content ($H(2) = 1.25$, $P = 0.54$) between the experimental diets. Similarly, one way ANOVA showed no statistically significant differences in mean moisture ($F(2,6) = 0.87$, $P = 0.47$) and ash content ($F(2,6) = 0.35$, $P = 0.72$) between groups.

Table 4: Mean proximate composition analyses of *O. mossambicus* at $\alpha = 0.05$

Diet	Moisture (%)	Fat content (%)	Ash content (%)	Protein content (%)
Inulin	74.73 ± 0.75^{e}	1.10 ± 0.33^{e}	0.94 ± 0.02^{e}	19.50 ± 0.50^{e}
Inulin + mushroom	75.73 ± 0.75^{e}	0.69 ± 0.13^{e}	0.94 ± 0.04^{e}	20.00 ± 0.00^{e}
Control	73.70 ± 1.69^{e}	1.77 ± 0.68^{e}	0.91 ± 0.003^{e}	19.50 ± 0.50^{e}

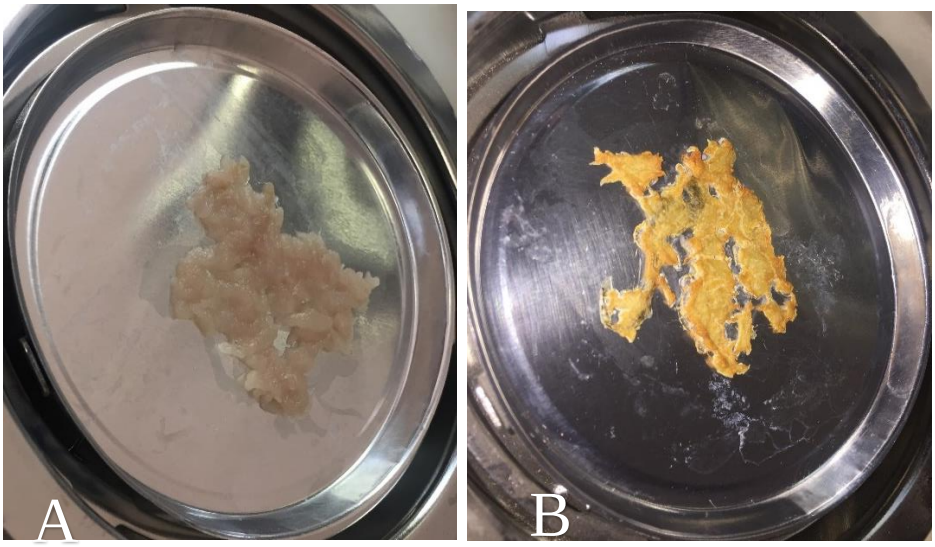

Figure 13: Moisture content before (A) and after (B) (source: this work)

Figure 14: Fat content (source: this work).

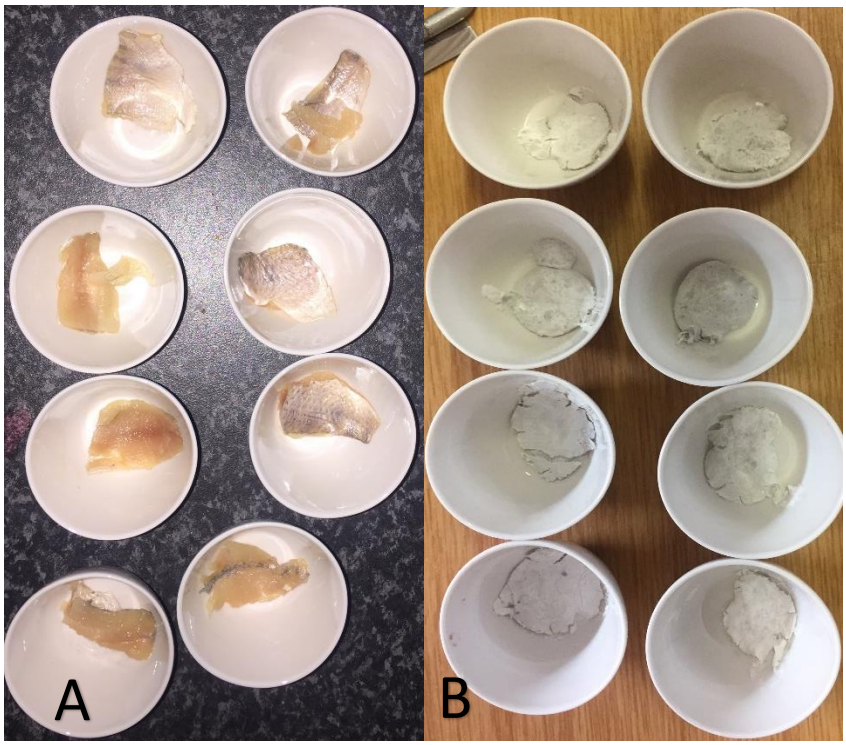

Figure 15: Ash content before (A) and after (B) (source: this work).

4.3 Gastrointestinal microbiota modulation

4.3.1 Culture dependent method

The results of the total viable anaerobic bacterial count in this study are expressed as mean log 10 CFU/g in different experimental diets (Table 5). The Kruskal_Wallis test showed that the experimental diets had some statistically significant effect on the log 10 CFU per gram (H (5) = 16.22, P = 0.006) as the study progressed from day 0 to day 57. The experimental diets reduced the total viable anaerobic bacteria in the intestinal samples. Post hoc Mann-Whitney test (table 6) using the Bonferroni adjusted alpha level 0.003 (0.05/15) showed that the log 10 CFU per gram of IM57 (mdn = 2.00) was significantly lower than that of C1 (mdn = 17.00) P = 0.001. No further significant differences were observed (Table 6).

Table 5: Mean log 10 CFU/g in experimental diets on different days of sampling at α = 0.05

Sample	Mean Log10CFU/g
C1	4.15 ± 0.01 [e]
I29	4.08 ± 0.02 [e]
IM29	4.06 ± 0.02 [e]
I57	3.76 ± 0.02 [e]
IM57	3.54 ± 0.05 [e]
C57	3.87 ± 0.02 [e]

Table 6: Post hoc Mann-Whitney pairwise comparisons of treatment on day at α = 0.05 and Bonferroni adjusted α = 0.003

Pairwise Comparisons of Treatment on day

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
IM57-I57	3.000	4.357	.689	.491	1.000
IM57-C57	-6.000	4.357	-1.377	.168	1.000
IM57-IM29	9.833	4.357	2.257	.024	.360
IM57-I29	11.167	4.357	2.563	.010	.156
IM57-C1	15.000	4.357	3.443	.001	.009

I57-C57	-3.000	4.357	-.689	.491	1.000
I57-IM29	6.833	4.357	1.568	.117	1.000
I57-I29	8.167	4.357	1.875	.061	.913
I57-C1	12.000	4.357	2.754	.006	.088
C57-IM29	3.833	4.357	.880	.379	1.000
C57-I29	5.167	4.357	1.186	.236	1.000
C57-C1	9.000	4.357	2.066	.039	.583
IM29-I29	1.333	4.357	.306	.760	1.000
IM29-C1	5.167	4.357	1.186	.236	1.000
I29-C1	3.833	4.357	.880	.379	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .050.

a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

4.3.2 Culture independent method

Demultiplexed sequence reads were received from Inqaba Biotechnology after sequencing on Illumina Miseq platform with primers targeting the V3-V4 regions of the 16S rRNA gene. From the six samples sequenced, a total of 387353 forward and 387353 reverse read sequences were obtained. They ranged from 54478 to 74029 reads with an average of

64558.83 in both forward and reverse reads. The quality of the reads was determined using the Phred33 score (Cock et al., 2010). The quality of the forward reads (appendix 4A) was good with poor quality at the end from approximately position 292. On the other hand, the quality of the reverse reads (appendix 4B) was good however poor quality started at approximately position 252, this is characteristic of reverse reads, which tend to have lower quality than their counterparts (Kwon et al., 2013). After quality filtering 387353 reads, 284201 good quality reads were retained, after de-noising 279351 remained, 253864 were merged and only 137838 were non-chimeric (see appendix 5).

The alpha diversity of the samples in this study were based on the following alpha diversity metrics, observed ASVs (observed features) and Shannon entropy (richness and evenness). A sampling depth of 14600 reads was sufficient to capture the richness and evenness in all the samples (appendix 6A-B). The results (Table 7 and appendix 7A-D) show that sampling date and treatment had an impact on the within sample diversity of the bacterial communities in the samples. The box plots of the Shannon's entropy (appendix 7A) and observed features (appendix 7C) show that there was a decrease in within sample diversity from the first day of the trial to the end of the feeding trials. Hence, the sampling date had decreasing effect on the diversity of the bacterial communities within the sample.

Table 7: Alpha diversity metrics Observed features and Shannon entropy

Sample	Sampling day	Experimental diet	Observed ASVs	Shannon entropy
C1	Day 0	Basal	705	9.18

129	Day 29	Inulin	589	8.93
IM29	Day 29	Inulin + mushroom	359	8.32
I57	Day 57	Inulin	478	8.56
IM57	Day 57	Inulin + mushroom	329	7.29
C57	Day 57	Basal	233	6.97

The box plots of the Shannon's entropy (appendix 7B) and observed features (appendix 7D) show that the experimental diets had an effect on the within sample diversity of the bacterial communities. The inulin diet showed higher observed features (478 ASVs) and richer and more even distribution (Shannon entropy = 8.56) of bacterial communities, followed by the control diet (329 ASVs; Shannon entropy = 7.29) and the Inulin + mushroom diet (233 ASVs; Shannon entropy = 6.97). The Kruskal Wallis test was conducted to test the statistical significance of the differences in diversity observed in the box plots. The test results showed that there were no statistically significant differences in the observed ASVs based on sampling date ($H(2) = 3.10$, $P = 0.21$) and based on treatment $H(2) = 1.14$, $P = 0.56$). Moreover, there were no statistically significant differences in Shannon entropy based on treatment $H(2) = 3.09$, $P = 0.21$) or sampling date $H(2) = 1.14$, $P = 0.21$).

Principal Coordinate Analysis (PCoA) by means of ordination was used to determine the changes that occurred in the gut bacterial communities of the *O. mossambicus* between

samples due to the administration of prebiotic and mushroom (figures 16 & 17). The Bray-Curtis beta diversity metric assessed how dissimilar the samples are based on microbial composition; while Jaccard distance determined how similar communities are by measuring the distance between samples (Kers & Saccenti, 2021). Moreover, the UniFrac distances used fractions of the branches (unweighted) and the relative abundances of sequences (weighted) to determine how far the members of a community are using phylogenetic distances (Kers & Saccenti, 2021).

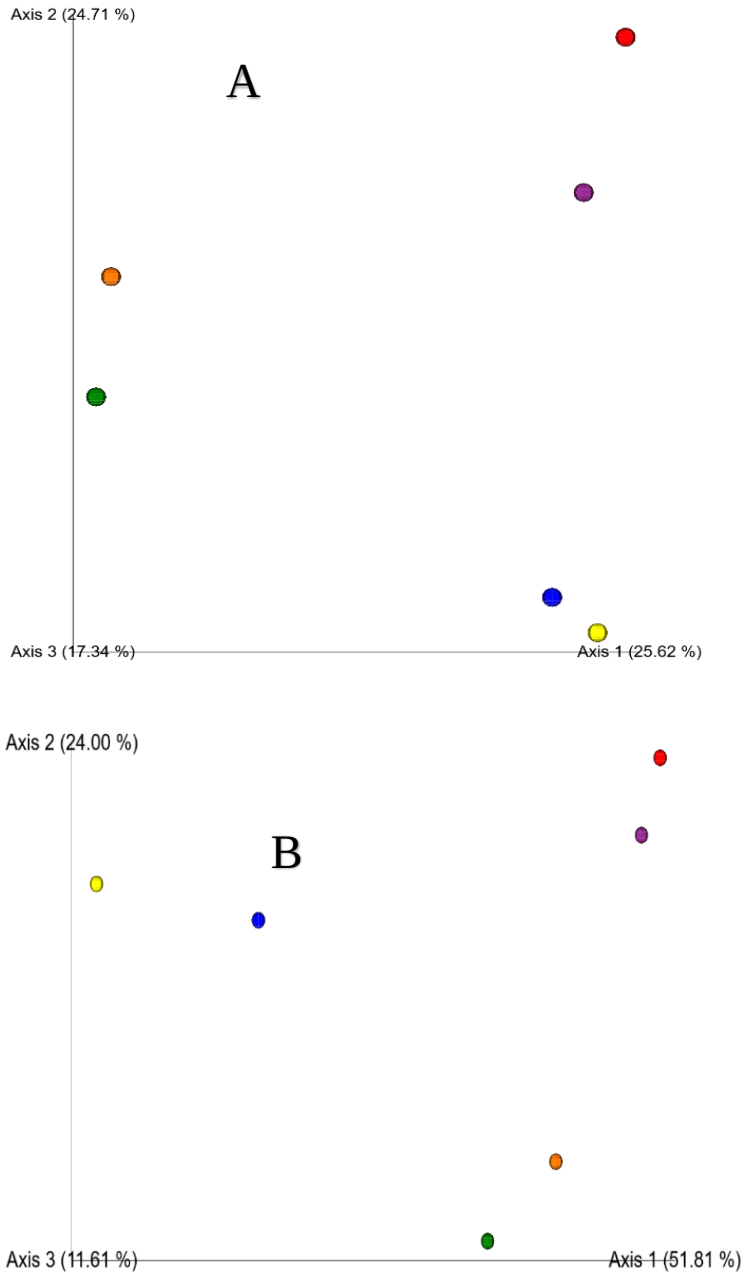

Figure 16: PCoA of Unweighted Unifrac (A) showing how far the members are from each other based on fractions of the branches and weighted Unifrac (B) based on the relative abundances of the sequences (key: red: Control_1, blue: Control_57, orange: Inulin_29, green: Inulin_57, purple: Inulin+Mushroom_29, and yellow: Inulin+nushroom_57).

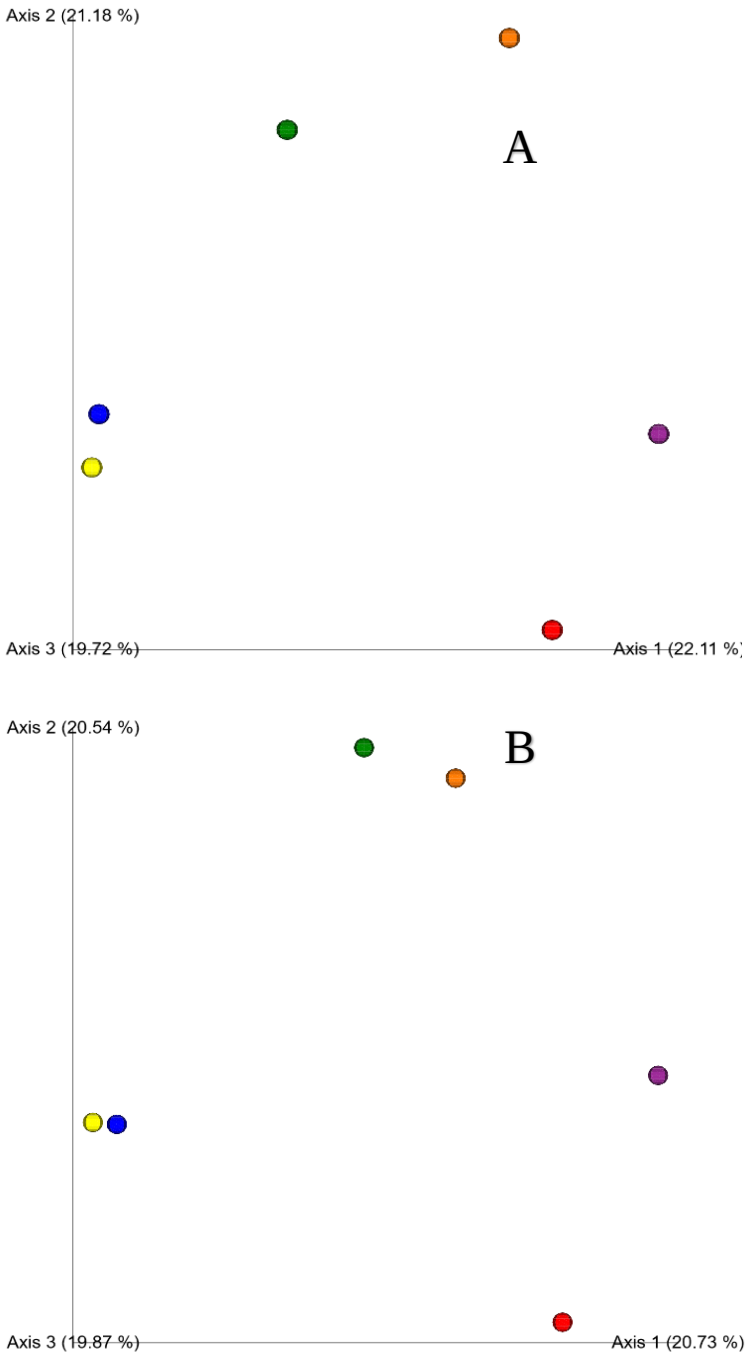

Figure 17: PCoA analysis of Bray-Curtis showing how dissimilar (A) and Jaccard (B) showing how similar the communities are between the samples (key: red: C1, blue: C57, orange: I29, green: I57, purple: IM29, and yellow: IM57)

The result obtained from all the PCoA plots shows the following trends, C1 and IM27 clustered together, C57 and IM57 clustered together and the I27 and I57 clustered together in all the different beta diversity metrics measured in this study. This shows that samples C1 and IM29, C57 and IM57, and I29 and I57 had similar diversity of gut bacterial communities. However, the three resultant clusters showed evidence of differing diversity compared to each other. Moreover, the samples from the inulin treatment group clustered together and away from the other treatments indicating that inulin had a great influence in the diversity of the samples. The group containing the control and the Inulin + mushroom clustered together indicating that these groups were not so different from each other. PERMANOVA of six samples and 3 groups under 999 permutations was used to test the significant differences in the PCoA distances between the samples. The results of the PERMANOVA test (table 8) showed no significant differences ($P > 0.05$) in the distances between the samples.

Table 8: PERMANOVA analysis based of beta-diversity metrics (unweighted unifrac, weighted unifrac, Bray-Curtis and Jaccards's distance) of the sequenced samples ($n = 6$, 999 permutations and $\alpha = 0.05$)

Beta-diversity metric	Pseudo-F	P
Unweighted unifrac	1.09	0.53
Weighted unifrac	0.84	0.13
Bray-Curtis	1.00	0.39
Jaccards's distance	1.00	0.35

The result obtained from the taxonomic classification showed variation between the six samples in this study, the taxa bar plots in below show the relative abundance of the bacterial communities in the samples based on phylum (figures 18 and 19) and genus (figures 20 and 21) level of taxonomy. The common most abundant phyla in all these samples were *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*. Moreover, about 5.22% of the phyla was unknown or unclassified. The most abundant genera varied per sample: in C1 the most abundant genera were *Streptococcus*, *Prevotella*, *Lactobacillus* and *Oscillospira*; in I27 were, *Streptococcus*, *Clostridium*, *Corynebacterium*, *Bacteroides* and *Archeolesplasma*; in IM27 were *Streptococcus*, *Prevotella* and *Clostridium*; while in C57 were *Rubrobacter* and *Enterobacteriaceae*; in I57 were *Rubrobacter*, *Streptococcus* and *Clostridium*; and in I57 were *Gemmata*, *Bacillus*, *Hyphomicrobium* and *Rubrobacter*. Similarly,about 48.76% of the features were unknown or unclassified. Overall, the phyla *Actinobacteria* and *Proteobactereria* were relatively increased over time in all treatments, while *Firmicutes* and *Bacteriodetes* were reduced.

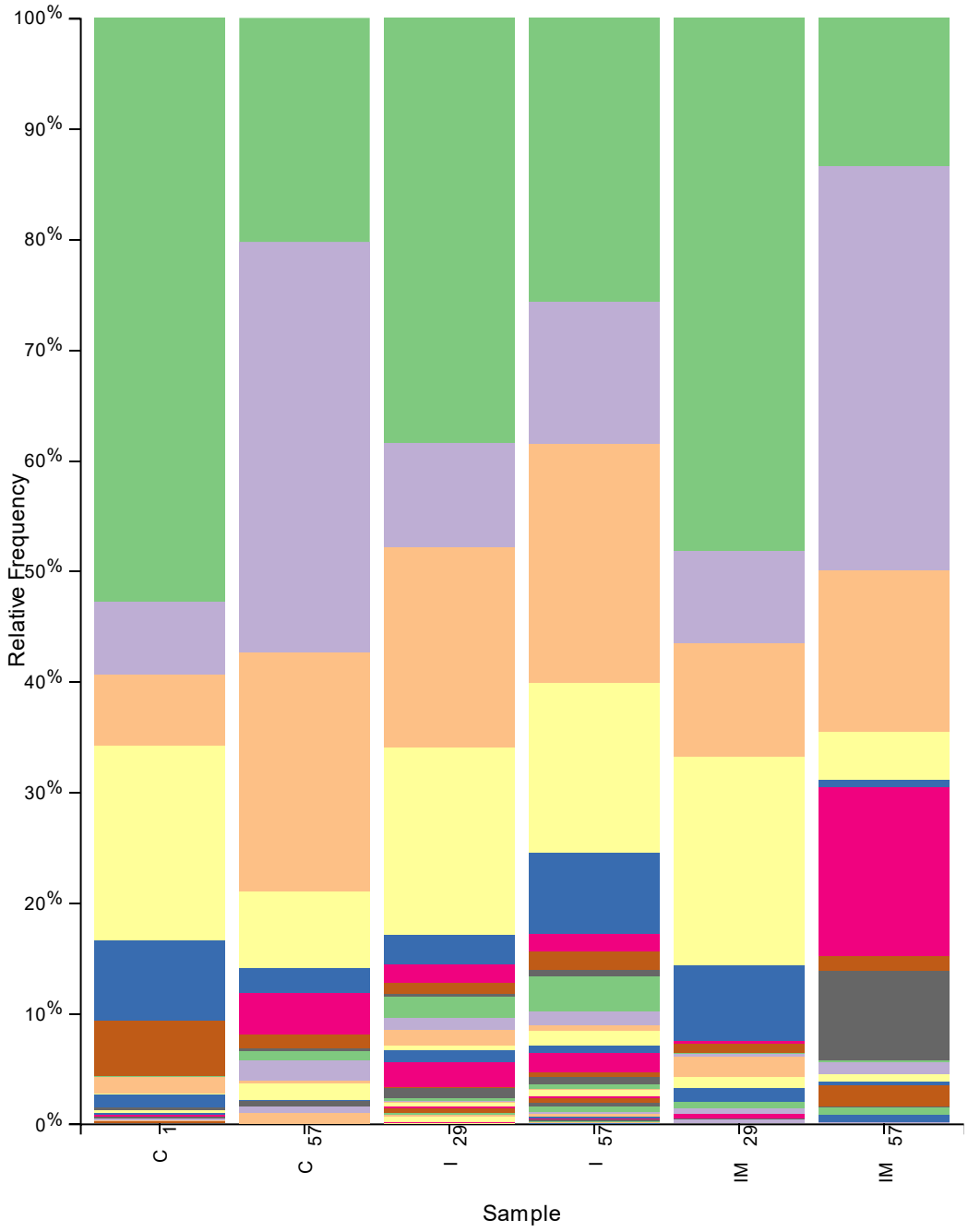

Figure 18: Taxa bar plot of relative abundance of bacteria of the samples at phylum level

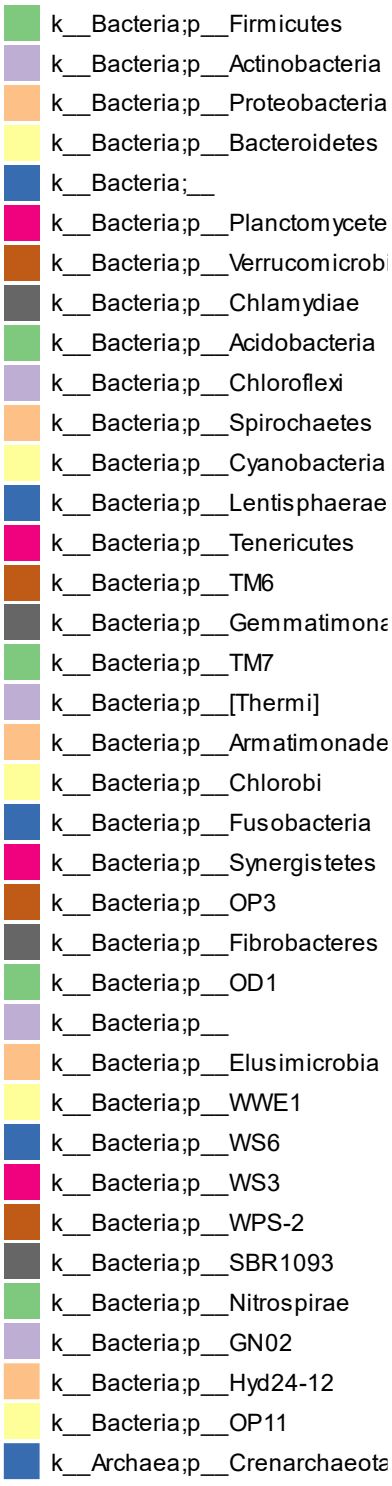

Figure 19: Key for taxa bar plot of relative abundance of bacteria of the samples at phylum level (figure 18).

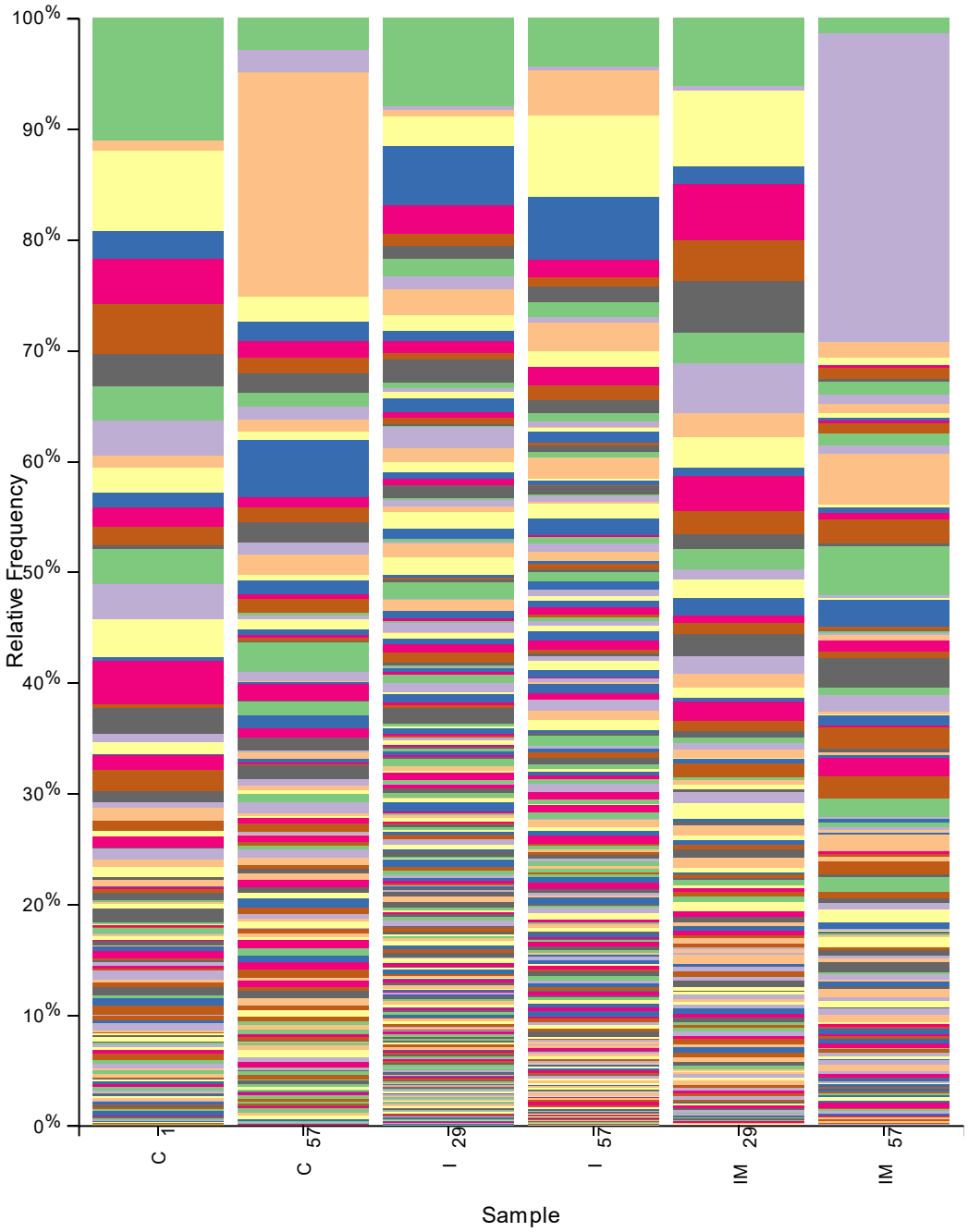

Figure 20: Taxa bar plot of relative abundance of bacteria of the samples at genus level

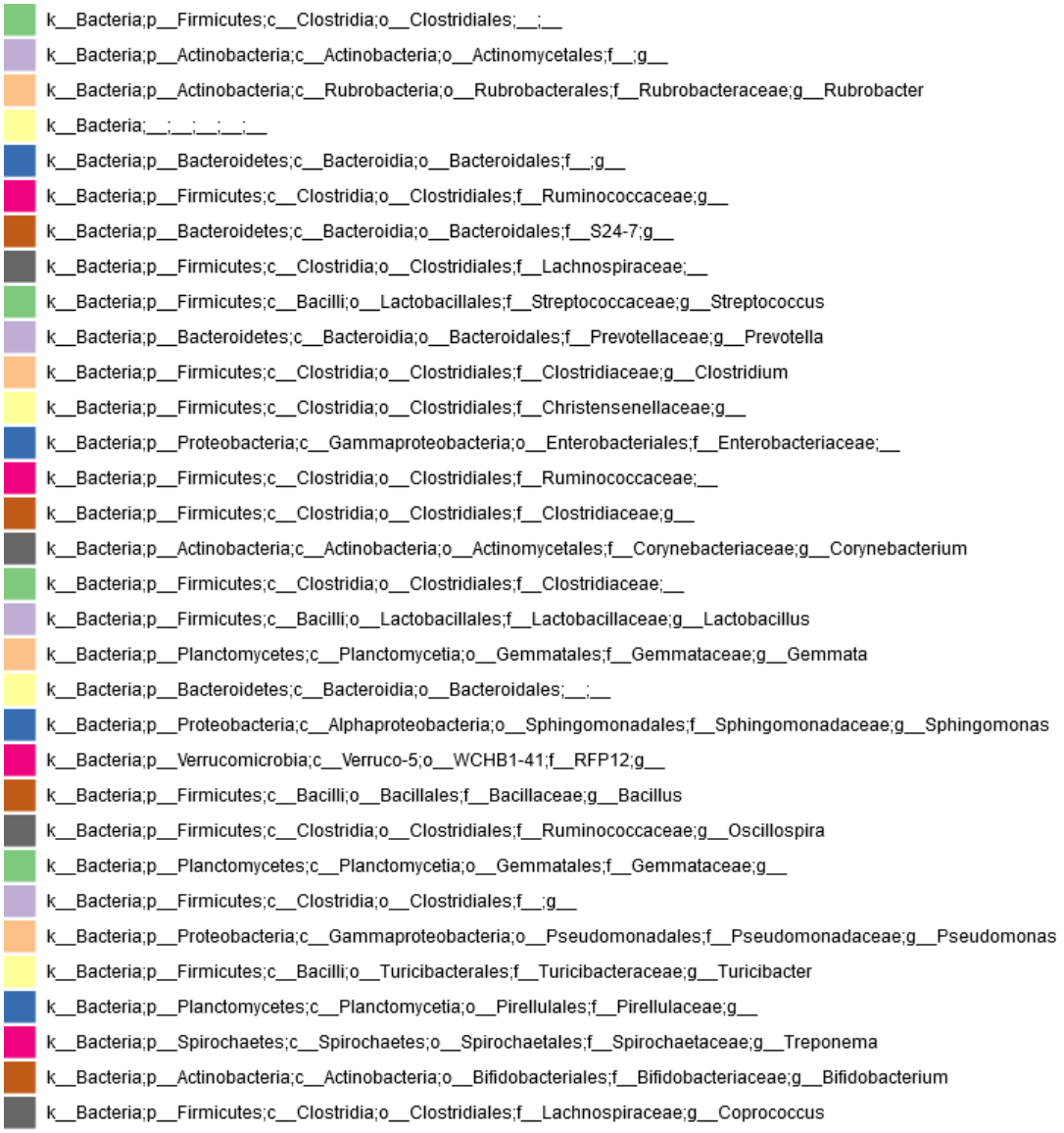

Figure 21: Key for taxa bar plot of relative abundance of bacteria of the samples at genus level (figure 20).

Additional sequence analysis was done by Inqaba Biotechnologies at 97% similarity and clustering into OTUs; taxonomic classification was done through the Ribosomal Database Project (RDP) 16s database v16 (appendix 8A-F & 9).

The heatmap (figure 22) of the differential abundances generated using Gneiss shows the clustering of the taxa that increased or reduced in abundance between the samples, the

features in red show increased abundance while the ones in blue show decreased abundance as shown on the key.

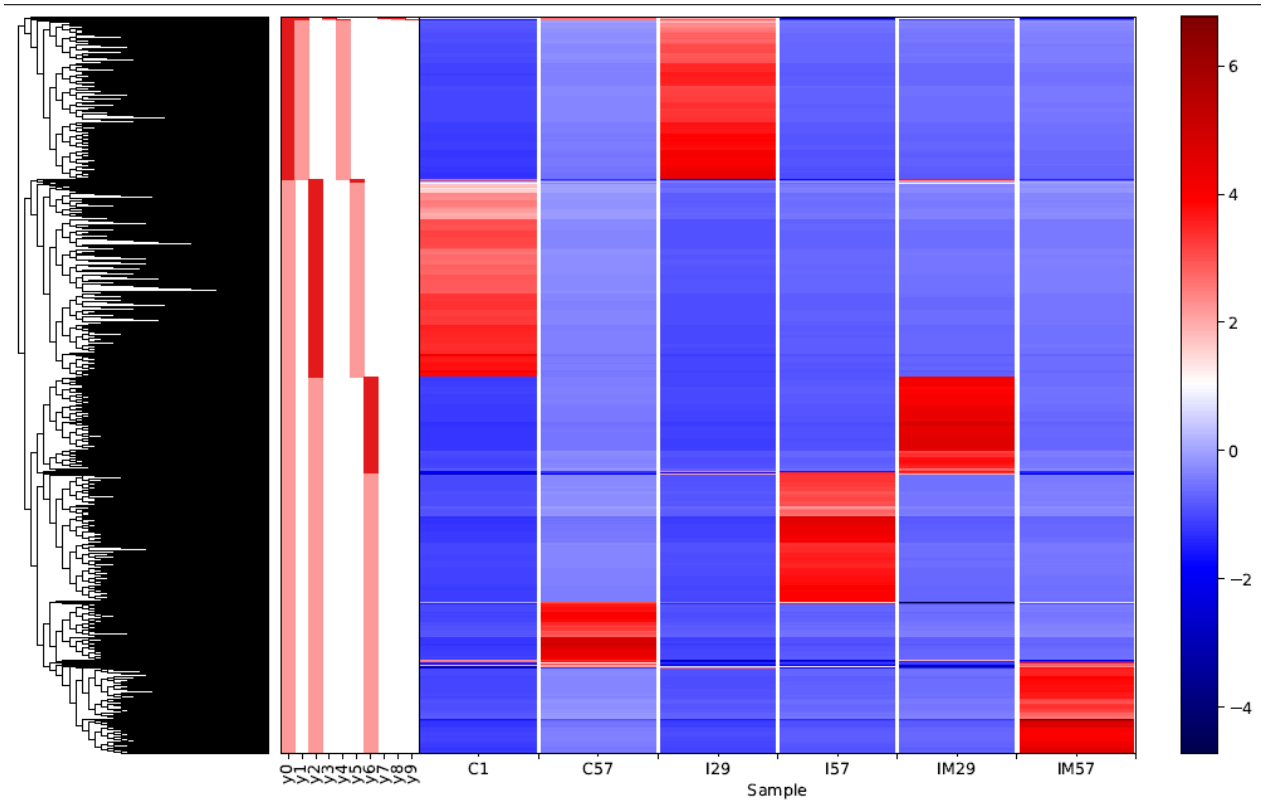

Figure 22: Dendrogram heatmap showing the differential abundance as a result of feeding with experimental feeds.

CHAPTER 5: DISCUSSION

It has been validated that the gut microbiota of fish represents a crucial component that could contribute to the health of a host; and its modulation through the administration of prebiotics, probiotics, synbiotics, and SCFAs has become the focal point of most fish microbiota studies (reference gut microbiota modulation and prebiotics in aqua). Prebiotics have been studied extensively in aquaculture to improve growth, health and overall well-being of fish (Ringo et al., 2010). However, the effect of prebiotics on *O. mossambicus* have seldom been studied in Namibia and beyond. The Nile tilapia is mostly studied probably due to its faster growth rates that has led to its increased global production (Prabu et al., 2019). This study is one of the few to have investigated the effect of inulin and *T. schimperi* mushrooms on the gut microbiota modulation of *O. mossambicus* in Namibia.

In this study, the result obtained from the administration of inulin showed a reduction in growth performance parameters such as WGR and SGR in comparison to the control group. Conversely, there were no significant differences in growth performance parameters such as FCR and SR between the experimental diets. Therefore, the dietary administration of 1% inulin in this study had no positive effect on the growth performance of *O. mossambicus*. Similarly, shrimp (*Fenneropenaeus indicus*) fed inulin enriched *Artemia nauplii* showed a lack of growth performance enhancement in a study by (Hoseinifar et al., 2010). Additionally, Eshaghzadeh et al. (2015) reported that there was no significant effect on the growth performance of Common carp (*Cyprinus carpio*) fed with inulin at 1% inclusion level (10 g inulin per kg) for seven weeks. Yuji-Sado et al. (2015) also reported that there was no observable effect on the growth performance of

Nile tilapia (*O. niloticus*) when fed with 0.2, 0.4 and 0.6 % dietary MOS for 60 days. Likewise, Hoseinifar et al. (2016) reported no significant effect on growth performance of Common carp larvae fed 0.5 and 1% sc-FOS for seven weeks. The reduction or no significant effect of inulin and other prebiotics on growth performance may be attributed to other factors in the experimental environment or the condition of the fish itself and not necessarily the versus effect of the prebiotic (Yousefian & Amiri, 2009).

On the contrary, Yones et al. (2020) reported that inulin supplementation at 2.5 g/kg in Nile Tilapia enhanced growth performance and feed utilization. Ibrahem et al. (2010) also reported improved growth performance of Nile tilapia fed with 5 g/kg inulin for two months. Similarly, Tiengtam et al. (2015) reported that juvenile Nile tilapia fed inulin (5 and 10 g/kg) and Jerusalem artichoke tuber (5 and 10 g/kg) diets exhibited better growth performance compared to the control. Various methods of prebiotic administration, dosage levels, fermentability and differences in gut morphology and intestinal microbiota of fish are some of the factors contributing to differing results from the application of prebiotics in aquaculture (Hoseinifar et al., 2016).

Furthermore, it was demonstrated that the administration of inulin + *T. schimperi* mushroom (0.5% + 0.5%) had no significant impact on the growth performance and feed utilization parameters of *O. mossambicus.* Similarly, Muin et al. (2014) reported no effect in replacing rice bran with *Pleurotus florida* mushroom stalk waste at 16 and 32% dietary inclusion on the growth performance in Nile tilapia fingerlings. Moreover, Dobšíková et al. (2013) also found no significant effect on growth performance in Common carp of micronized β-1.3/1.6-D-glucan derived from oyster mushroom *P. ostreatus* regardless of the inclusion level. Conversely, *Agaricus bisporus*, white bottom mushroom powder

(WBMP) fed at 0.5, 1 and 2% for 8 weeks to Common carp fingerlings resulted in significantly improved growth performance (Zou et al., 2016; Hoseinifar et al., 2019). Equally, Van Doan et al. (2017) reported notably improved growth performance in Nile tilapia fed *Cordyceps militaris* spent mushroom substrate at 5, 10, 20 and 40 g/kg dietary inclusion for 8 weeks. Besides, Selim et al., (2015) also showed that MOS combined with β-glucans exhibited enhanced growth performance of Nile tilapia at 1.5 and 3 g/kg inclusion level after 30 days. The differences in effects on growth performance may be attributed to the different mushroom species, inclusion levels, methods of inclusion and fish species (Zou et al., 2016; Hoseinifar et al., 2019).

There are no documented effects of *T. schimperi* mushroom powder in combination with inulin powder on the growth performance of *O. mossambicus* or any aquaculture species in Namibia. Nevertheless, Amadhila (2019) reported the prebiotic effect of polysaccharides extracted from *T. schimperi* mushrooms. Other mushrooms species have been studied for applications as prebiotics in aquaculture and have also found varying effects on growth performance and feed utilization parameters (Mohd Din et al., 2012; Ahmed et al., 2017; Hoseinifar et al., 2019). Polysaccharides, peptide/protein derivates and proteoglycans of mushrooms are essential prebiotics that play significant roles in immunomodulation and anti-tumor activities (Singdevsachan et al., 2016).

The improved growth performance might be linked to the alteration effect of gastrointestinal microbiota towards the advantageous microflora such as lactic acid bacteria that leads to enhanced physiological conditions of the gastrointestinal tract and nutrient digestibility (Zou et al., 2016). In detail, beneficial bacteria in the intestines breakdown nondigestible fibers, the by-products of this metabolism such as SCFAs,

vitamins and enzymes are absorbed and further utilized by the host along the intestinal tract (Haygood & Jha, 2018). Leading to improved nutrient digestion, absorption and utilization which translates into enhanced growth performance of the fish (Haygood & Jha, 2018).

The present study carried out an analysis of the proximate composition parameters (moisture, ash, lipid and protein). Consequently, the results showed that there was no significant difference across all treatment groups in whole body composition parameters. The dietary supplementation of inulin and *T. schimperi* powder did not affect the proximate composition of *O. mossambicus*. Similarly, there was no effect on whole body composition parameters (moisture, ash, lipid and protein) in, Nile tilapia fed with inulin (2.5 and 5 g/kg) and Jerusalem artichoke (5 and 10 g/kg) (Tiengtam et al., 2015). As well as in hybrid catfish (*Pangasianodon gigas* x *Pangasianodon hypophthalmus*) fed 0.6% XOS, GOS, FOS and MOS (Hahor et al., 2019). On the other hand, Selim et al. (2015) reported higher protein and fat content in Nile tilapia fed a combination of β-glucan and MOS. Additionally, Mohd Din et al., (2012) reported higher lipid and lower moisture content in Nile tilapia supplemented with mushroom stalk meal of *Pleurotus sajorcaju* at 0.5, 1.0 and 1.5% inclusion. Whole body composition analyses determine the effect of prebiotics on nutrient deposition in fish because the dietary effect of prebiotics on weight gain results from nutrient contents such as water (moisture), protein, lipid and ash (Tiengtam et al., 2015).

The results obtained from the culture-dependent method showed that incorporating both inulin and *T. schimperi* mushroom powder in fish feed portends the capability to reduce the total anaerobic bacteria viable count significantly in *O. mossambicus* fingerlings

compared to inulin alone and the control group. These results are similar to those of (Koh et al., 2016), who reported that there was a significant reduction in the total viable bacterial counts in expelled feces of Nile tilapia fed with organic acid blend (0.5 and 1%) and oxytetracycline (0.5%) when compared to the control diet. In addition, viable cultivable heterotrophic bacterial count was significantly decreased in Nile tilapia and marine shrimp (*Litopenaeus vannamei*) fed probiotic *Lactobacillus plantarum* supplemented diet in comparison to the control diet (Jatobá et al., 2011).

On the contrary, this study further showed that the inulin experimental diet had no significant effect on the anaerobic viable counts in comparison to the control group. Similarly, Hoseinifar et al. (2016), reported that there was no significant difference in total viable counts of intestinal samples between Common carp larvae fed scFOS supplemented diets at levels (0.5 and 1%) and those fed 0% scFOS (control diet). As well, intestinal heterotrophic bacteria were not affected by dietary GOS in Caspian roach (*Rutilus rutilus*) fry (Hoseinifar et al., 2013). The reduction in viable counts in fish fed with prebiotics can be related to the beneficial effects of prebiotics on the gastrointestinal microbial communities which leads to increased abundance of advantageous microbiota (Hoseinifar et al., 2013). Which leads to the reduction of non-beneficial microbiota through competitive exclusion and the production of inhibitory substances like bacteriocins, antimicrobial compounds, lactic acid, acetic acid and hydrogen peroxide (Llewellyn et al., 2014; Guerreiro et al., 2018).

In culture independent methods, the inulin experimental diet showed the highest within sample diversity followed by inulin + mushrooms, while control samples showed the least diversity at the end of the experiment (see table 7). Although the Kruskal Wallis test

showed that the observed differences were not statistically significant (P value > 0.05), this test has little power when dealing with smaller sample sizes (Sullivan, 2018). As a matter of fact, no matter the differences observed in the samples, the Kruskal Wallis test will consistently yield a P value > 0.05 when the total sample size of seven or less (Motulsky, 2021). Based on the results (see table 7 and appendix 7 A-D) this study showed that inulin and inulin + mushrooms experimental diets resulted in the higher alpha diversity compared to the control. Similarly, *Nibea coibor* fed combinations of inulin, GOS and D-sorbitol resulted in higher alpha diversity in comparison to the control (Li, Tuan, et al., 2019). Additionally, González-félix et al. (2018) reported higher alpha diversity of microbes when a yeast based prebiotic GroBiotic®-A was combined with probiotic *Bacillus subtilis*. On the contrary, reduced alpha diversity in gilt head seabream was observed when inulin was added to the basal diet (Cerezuela et al., 2013). As well, Tapia-paniagua et al. (2011) and Cerezuela et al. (2013) both reported reductions in alpha diversity in fish fed with combinations of inulin with *Bacillus subtilis* (probiotic) and *Debaryomyces hansenii* (yeast) respectively. Notably, some studies reported no significant differences between control and prebiotic/synbiotic combinations (Lochmann et al., 2011; Tapia-Paniagua et al., 2019; Z. Li et al., 2019; Wang et al., 2020).

Equally important, this study detected a decrease in alpha diversity as the feeding trials progressed (see table 7). Tapia-paniagua et al. (2011) also found that the alpha diversity after feeding fish prebiotic experimental feeds for two weeks was higher than the diversity at 4 weeks. This correlates with our results where alpha diversity reduced from day 1 of sampling to day 27 (4 weeks after feeding experimental diets) to day 57 (8 weeks after feeding experimental diets) (see table 7). It is possible that the duration of the

prebiotic/probiotic administration may have an impact on the diversity of the microbiota and is definitely an area that could be investigated further. The differences observed in within sample diversity analyses could be attributed to the administration of prebiotics proven to impact the diversity of the microbial communities in fish (Ringø et al., 2010; Song et al., 2014; Akhter et al., 2015).

In beta diversity analysis, this study reported that fish fed the inulin treatment showed higher diversity compared the control and inulin + mushroom treatments, furthermore, sampling date had a significant effect on the diversity. The PERMANOVA test showed that the difference in the distances were not significant. According to Kelly et al. (2015), the power of the PERMANOVA test for samples below 22 per group can be skewed, bringing up once again the limitation of the small sample size (6) of this study. Therefore, we focus on the PCoA plots results which clearly depict separation in the distances between the samples. Correspondingly, Zhou et al. (2020) reported significant separation between the control diet and inulin diet fed to white shrimp, indicating that inulin greatly influenced the overall diversity of intestinal microbiota. Moreover, addition of inulin increased the diversity of the of the gut microbiota of Nile tilapia (Wang et al., 2021). On the contrary, Cerezuela et al. (2013) reported some reductions in intestinal microbiota beta diversity in Arctic charr following the inclusion of inulin in experimental diets.

Overall, both alpha and beta diversity indices in this study showed that inulin had a great impact on the diversity of the gut microbiota of *O. mossambicus* in comparison to the basal diet and the inulin + mushroom combination. Contradictions in the results from prebiotic inclusion in fish from study to study amplifies the dynamic bacterial interactions in different environments (Yukgehnaish et al., 2020). Microbial diversity in fish gut

microbiota contributes immensely to the proper functioning of a host organism (Talwar et al., 2018).

Moreover, it was observed that the gut microbiota of most fish consists of *Proteobacteria, Firmicutes, Fusobacteria* and *Cyanobacteria* which are the true representation of the core microbiota of most fish (Gaikwad et al., 2017). It has been established that *Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria, Cyanobacteria, Bacteroidetes* and *Planctomycetes* form part of the core gut microbiota of tilapia (Baldo et al., 2015; Gaikwad et al., 2017; Bereded et al., 2020, 2021). The composition of the major phyla in this study was similar in all treatments and only differed by one or two different phyla. The dominant phyla were *Firmicutes, Actinobacteria, Proteobacteria* and *Bacteroidetes*. *Firmicutes* and *Bacteroidetes* were reduced over time in all experimental feeds while *Proteobacteria* and *Actinobacteria* were increased. Li et al. (2014) reported similar results where *Proteobacteria* and *Firmicutes* were the most abundant phyla in fish with varying feeding habits. On the other hand, the abundance of *Firmicutes* increased after feeding the shrimp with 0.4% inulin (Zhou et al., 2020). Although *Fusobacteria* is one of the major phyla in tilapia gut core microbiota, it was notably less abundant in the current study. These major phyla have empirical roles in host metabolism. For example, *Proteobacteria* help maintain the balance of the anaerobic environment in the gut (Moon et al., 2018). *Firmicutes* break down dietary fibers and assist in modulating the uptake of fat (Semova et al., 2013; Gharechahi & Salekdeh, 2018). While *Fusobacteria* help modulate the immune system and act as anti-inflammatory (Terova et al., 2016).

In this study, the genera level of taxonomy showed variable abundances between the six samples. *Streptococcus* was highly abundant in Control_1, Inulin+mushroom_27,

Inulin_27 and Inulin_57; while *Prevotella* was found in Control_1 and Inulin+mushroom_27; and *Clostridium* was found in Inulin+mushroom_27, Inulin_27 and Inulin_57. Ray et al. (2017) reported different highly abundant genera in Nile tilapia fed Previda compared to the current study. Evidently, Rubrobacter increased in abundance as it was highly abundant in all the treatment groups on the last day of sampling (see appendix 8 A-B). Some ASVs were not assigned a taxonomy, probably due to one of two reasons. They are novel bacteria or these sequences do not have assigned taxonomy in the Greengenes database (Balvočiūtė & Huson, 2017).

The administration of prebiotics in aquaculture has yielded varying results, some studies have reported positive or negative effects, while others found no significant effects on the growth performance, proximate composition and gut microbiota diversity. These differences can be attributed to differences in fish species, prebiotics, mushroom species, methods and levels of inclusion and other factors. The prebiotics (inulin and *T. schimperi* mushroom powder) in this study were incorporated in the feed by dissolving the prebiotics in distilled water and sprayed on all feed pellets as described by (Li, Tuan, et al., 2019) and (González-félix et al., 2018). Some studies have included the prebiotics in the feed formulation before extrusion (Tiengtam et al., 2015; Van Doan et al., 2018; Gabriel et al., 2019).

In the final analysis, feeding *O. mossambicus* fingerlings with inulin and *T. schimperi* mushroom powder incorporated fish feed had no significant effects on growth performance, feed utilization and proximate composition analysis in this study. This study has laid ground to the knowledge of the effects of dietary prebiotics on growth performance in aquaculture in Namibia. Furthermore, the incorporation of inulin and *T.*

schimperi into the basal diet resulted in the reduction of total anaerobic viable counts and increased diversity of the gut microbiota of *O. mossambicus* fingerlings. The reduction in anaerobic bacterial counts and increase in fish gut microbiota diversity shows that prebiotics portends the capability to decrease the presence of non-beneficial microbes and enhance beneficial microbes that contribute to improved growth performance and improved health status of fish.

CHAPTER 6: CONCLUSION

This study hypothesized that the supplementation of inulin and *T. schimperi* mushroom could improve the growth performance and modulate the gastrointestinal microbiota of *O. mossambicus* fingerlings. This study indicated that inulin and *T. schimperi* mushroom powder had no significant effect on the growth performance and feed utilization. However, it was observed that the experimental treatments containing inulin and *T. schimperi* powder significantly reduced the anaerobic bacterial count; as well as increased the alpha and beta diversity of the gut microbiota of *O. mossambicus* fingerlings. *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* phyla were highly abundant in gastrointestinal tract of *O. mossambicus* fingerlings following prebiotic administration. They form part of the core microbiota of fish and serve critical roles in the maintenance of gut health in fish. At genus level, *Streptococcus, Prevotella*, *Clostridium* and *Rubrobacter* were highly abundant.

The application of prebiotics in aquaculture have been confirmed to improve growth performance and modulate the gut microbiota of fish. Although the current study reported no impact on growth performance, it showed that indeed prebiotics inulin and *T. schimperi* mushrooms can modulate the gut microbiota of *O. mossambicus* fingerlings. The differences in prebiotics' application results are due to many factors that affect growth performance and gut microbiota diversity of fish. This study offers insight into the potential of supplementing fish feeds with functional ingredients to improve performance and health. Therefore, more studies on improving performance of farmed species in Namibia using different types of functional feed additives are needed.

CHAPTER 7: RECOMMENDATIONS

As aquaculture develops in globally and in Namibia, studies focusing on improving the growth and overall health of farmed fish culminating in improved production are of outmost importance. There is a need to perform a study that will investigate different functional feed additives and prebiotics such as mushrooms and MOS in aquaculture diets. Moreover, there is a need to carry out more studies on different prebiotic percentage inclusion levels and methods of incorporation such as incorporating prebiotics in the feed formulation before pelleting. As well as survival challenge studies against common pathogens (bacteria and parasites) and stress along prebiotic administration to better optimize the beneficial effects. Furthermore, there is a need to focus on other aspects of prebiotic effects such as SCFAs production, immunostimulation, biochemistry and hematology which are necessary to provide a better understanding on the mechanism of action of prebiotics on numerous biologically active constituents available in the fish. Additionally, there is a need to perform more studies that will determine the effect of prebiotics on other freshwater farmed species in Namibia such as Hybrid Nile tilapia, Red-breast tilapia, Three-spot tilapia and African catfish. Likewise, there is a need for government, universities, research institutes, policy makers as well as non-governmental bodies to release adequate funding that could boost more effort on how to increase the sampling size for more experiments that will culminate into more interesting results. What is more, sampling the surrounding environment can provide further insight into the various diversity and ecology of complex microbial communities in aquaculture. Lastly, further analysis to identify ASVs with unassigned taxonomy in other databases are recommended as there is potential to find novel bacteria.

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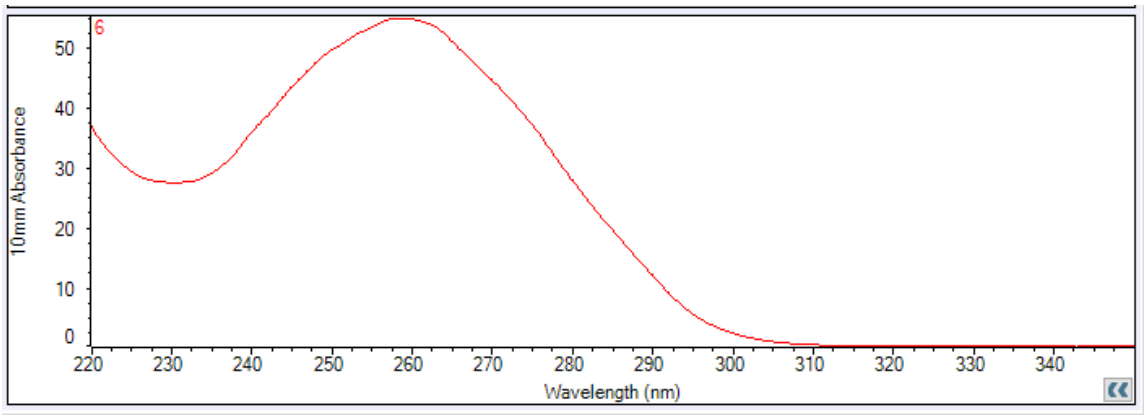
APPENDICES

Appendix 1A: DNA Concentration and A260/280 ratio.

Sample	Concentration (ng/µL)	Nanodrop 260/280 ratio
C1	35	1.56
I29	37.1	1.51
IM29	48.8	1.27
I57	476.5	1.97
IM57	49.3	1.49
C1	2743.9	1.96

Appendix 1B: Nanodrop graph of sample C1 (Nanodrop 2000c spectrophotometer).



Appendix 2: Approval to use MFMR facilities and data for research

REPUBLIC OF NAMIBIA

MINISTRY OF FISHERIES AND MARINE RESOURCES

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INTERNAL MEMORANDUM

To: Dr. Moses Maurihungirire
EXECUTIVE DIRECTOR

Cc: Mrs. Hilaria Namoloh
CHIEF DEVELOPMENT OFFICER

From: Ms. Kaulo Salushando
FISHERIES RESEARCH TECHNICIAN

Date: 16 September 2019

RE: REQUEST TO USE MINISTERIAL FACILITIES AND DATA FOR RESEARCH

This memo seeks for your approval on the above-mentioned request.

I am currently on study leave pursuing my Master's Degree in Microbiology specialising in Food Microbiology at the University of Namibia. My thesis will focus on prebiotics as additives in fish feed as they confer many benefits to fish such as improved growth and feed utilization, improved health, modulation of intestinal microbiota and improved immune response.

The aim is to investigate the effects of prebiotics in terms of improved growth response and modulation of gastrointestinal microbiota. The research will be supervised by Assistant Professor Ahmad Cheikhyoussef at the University of Namibia Multidisciplinary Research Centre and co-supervised by Ms Kerthu Hangula, Senior Fisheries Biologist at Hardap IAC. I am writing to request for permission to use ministerial facilities and data in order to carry out this research.

A proposal is being drafted for approval by the University of Namibia research

Appendix 2: Approval to use MFMR facilities and data for research continues…

committee and a copy of the 1st draft of the proposal is attached for further information.
Final draft of the proposal will also be submitted for ministerial approval.

Recommended/~~Not Recommended~~
probiotic has become an impotant part of the aquaculture industry. Therefore, we are looking forward to see the outcome of this study.

18/9/2019
Date

Mrs. Kenthu Hangula
ACTING CHIEF FISHERIES BIOLOGIST

Recommended/~~Not Recommended~~

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.....

29/09/23
Date

Mr. Johannes Hamukwaya
DEPUTY DIRECTOR

Recommended/Not ~~Recommended~~

.....

.....

23.9.19
Date

Mr. Rudolph Cloete
DIRECTOR

Recommended/Not Recommended
The request ~~is~~ is at the discretion of the Executive Director. No need for MTC recommendation

25.09.19
Date

Mrs. Elizabeth Swartz
CHAIRPERSON OF THE MINISTERIAL TRAINING COMMITTEE

Approved/~~Not Approved~~

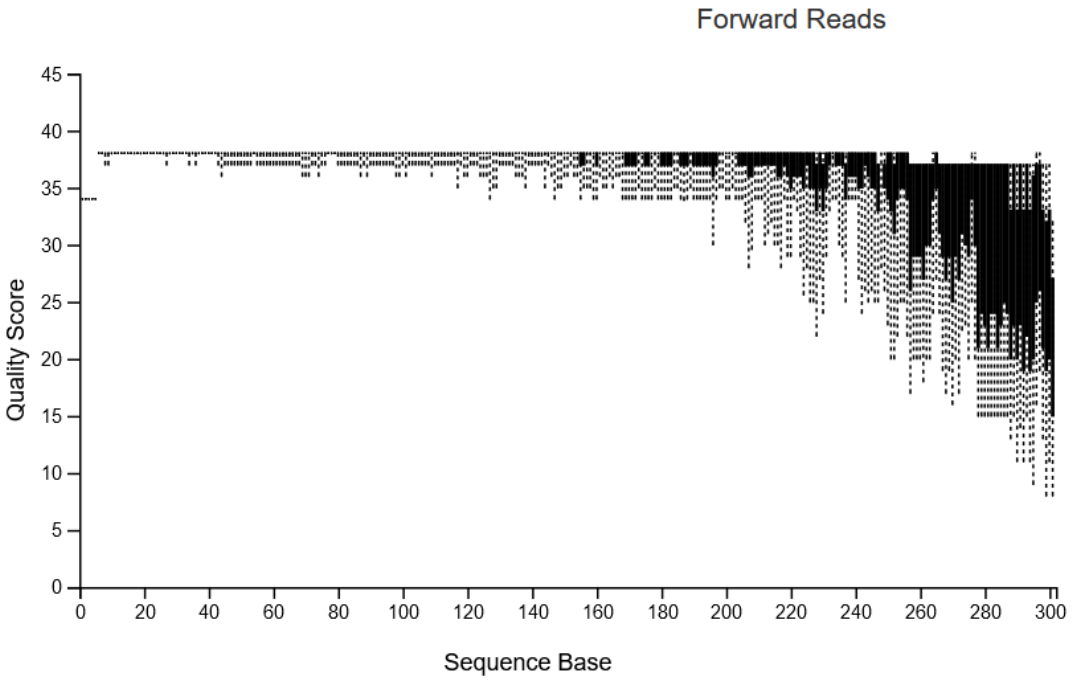
Dr. Moses Maurihungirire
EXECUTIVE DIRECTOR

MINISTRY OF FISH... RESOURCES

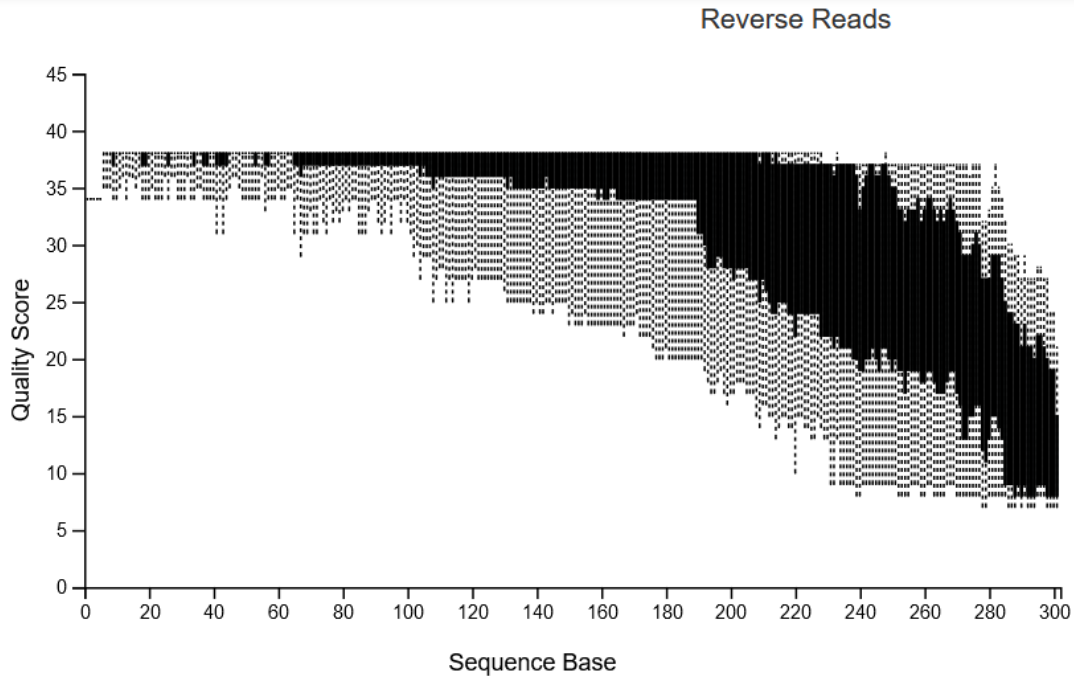
Appendix 3: Proximate composition raw data

Treatment pond no	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Inulin 1	72,9		0,96	0,9
Inulin 2	74	20	1,74	0,95
Inulin 3	75,5	19	0,61	0,96
Inulin+Mushroom 4	74,85	20	0,97	1
Inulin+Mushroom 5	75,15	20	0,55	0,95
Inulin+Mushroom 6	77,2		0,56	0,87
Control 7	72,35		1,24	0,92
Control 8	71,7	19	3,12	0,91
Control 9	77,05	20	0,95	0,91

Appendix 4A: Phred33 quality score plot of forward reads (generated in QIIME2).



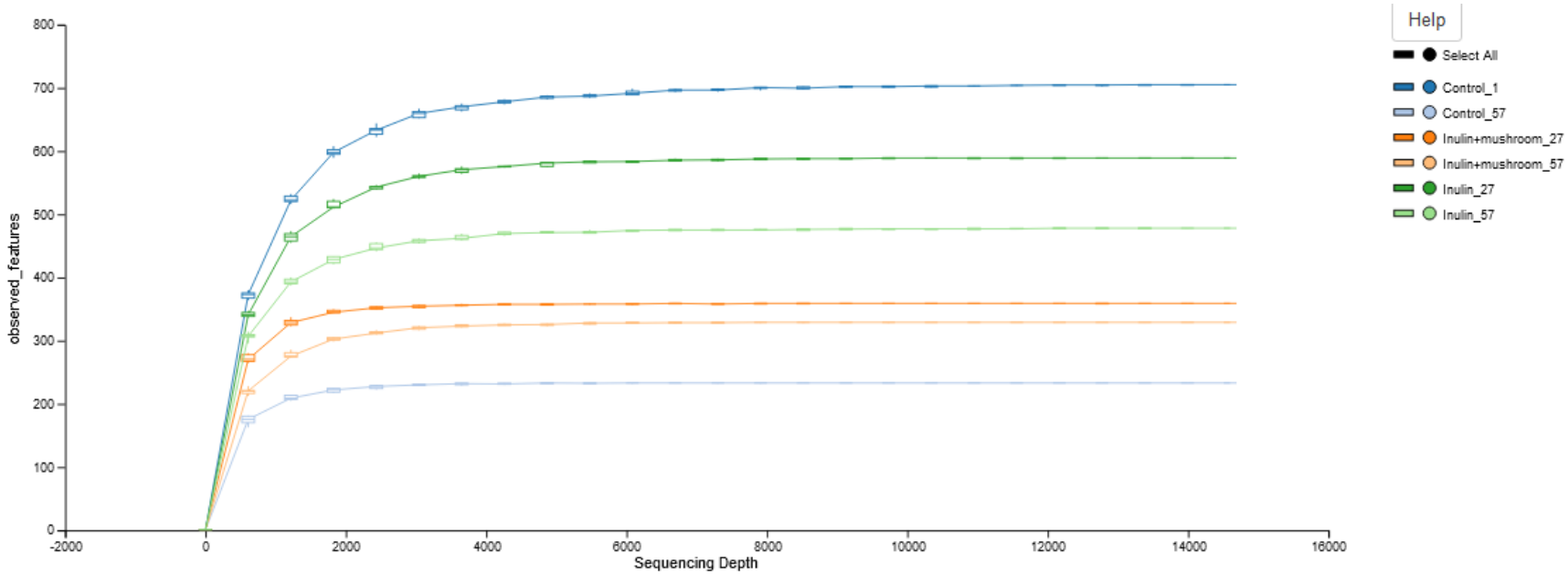
Appendix 4B: Phred33 quality score plot of reverse reads (generated in QIIME2).



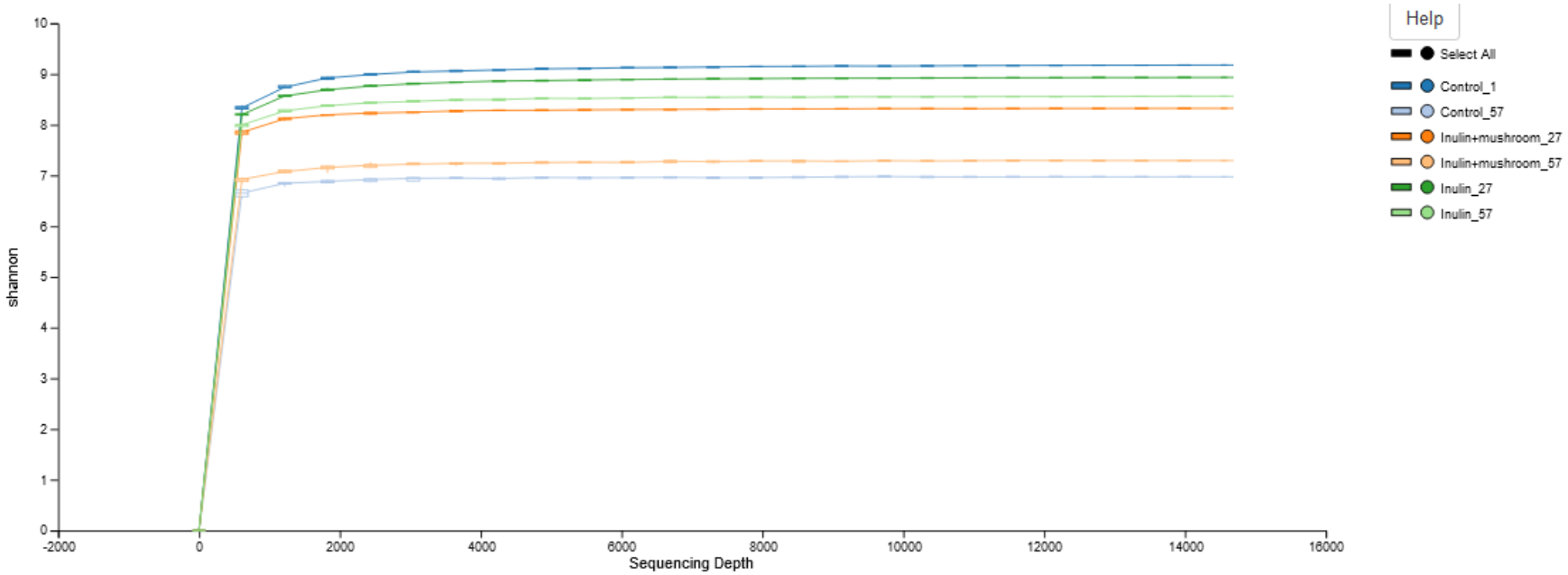
Appendix 5: Summary of denoising statistics from DADA2 output per sample (generated in QIIME2).

Sample	Raw sequences	Filtered	Denoised	Merged	Non-chimeric
C1	74029	55416	54544	46631	25366
I29	70630	51749	50492	45162	28831
IM29	54478	39065	38629	36487	24551
I57	58140	43585	42624	39210	23797
IM57	70498	50948	50062	44958	20657
C57	59578	43438	43000	41416	14636
Total	**387353**	**284201**	**279351**	**253864**	**137838**

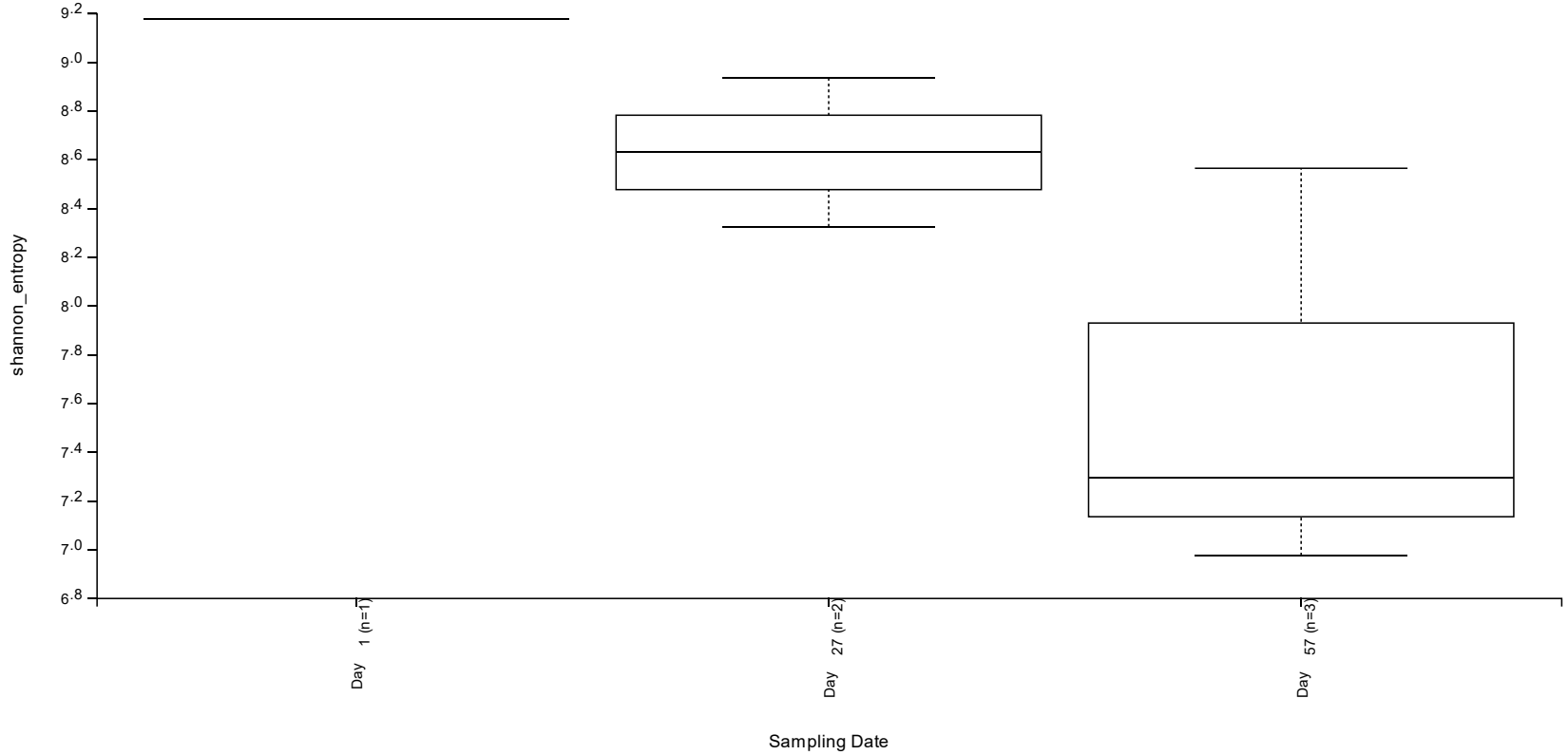
Appendix 6A: Alpha rarefaction curves of the observed features (ASVs) per sample **(generated in QIIME2).**



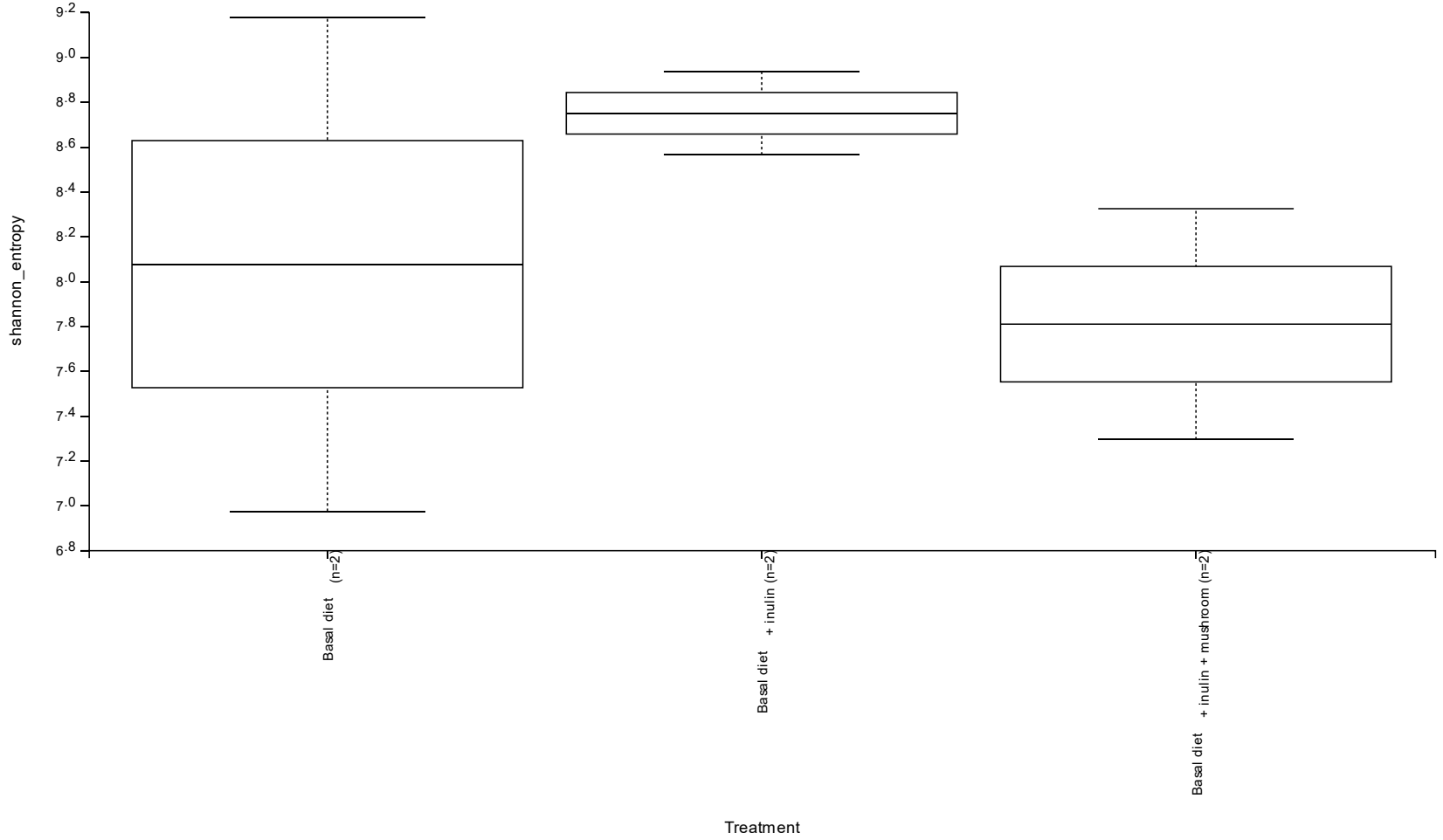
Appendix 6B: Alpha rarefaction curves of Shannon entropy per sample (generated in QIIME2)



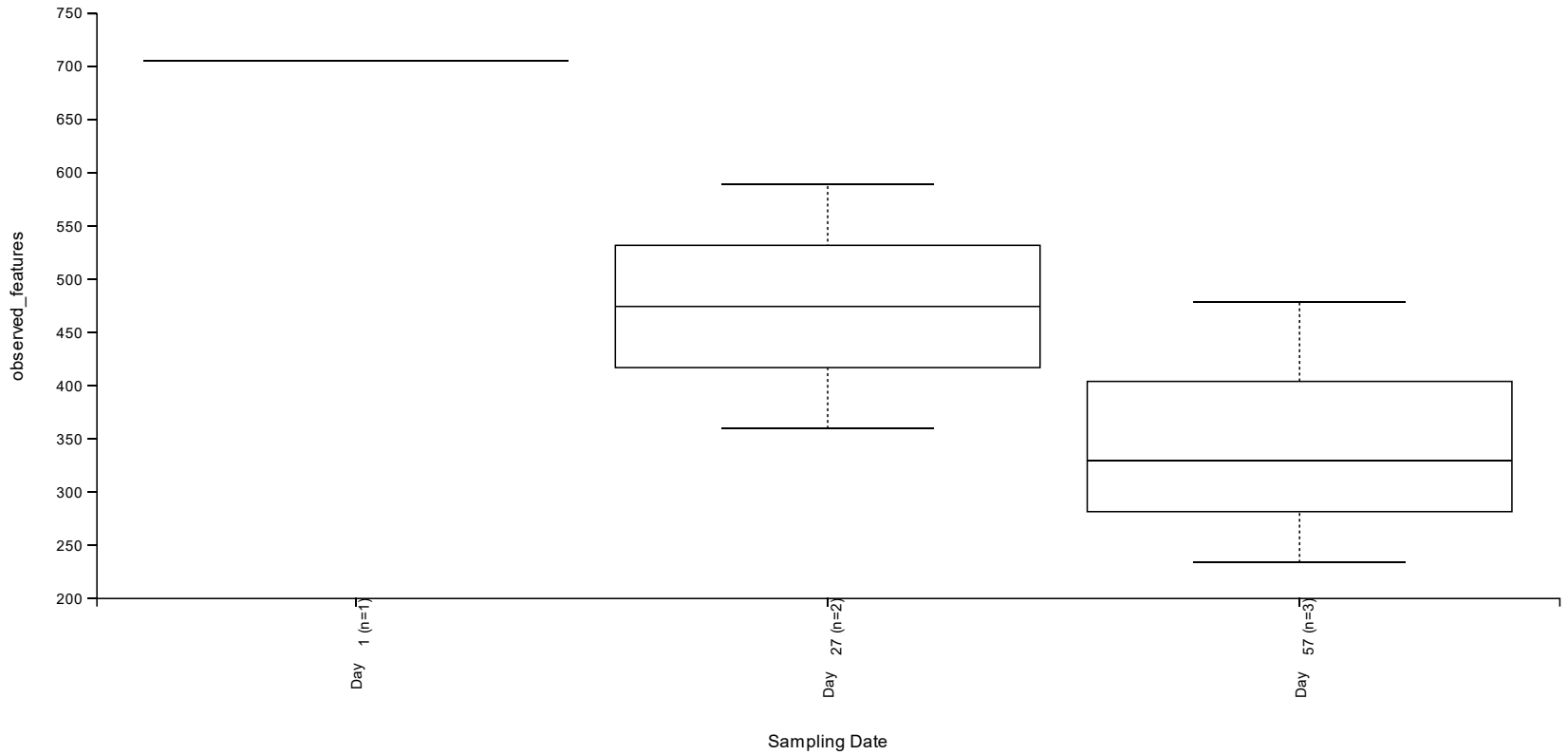
Appendix 7A: Box plot of Shannon entropy, showing how sampling date impacted richness and evenness of microbiota within samples (generated in QIIME2).



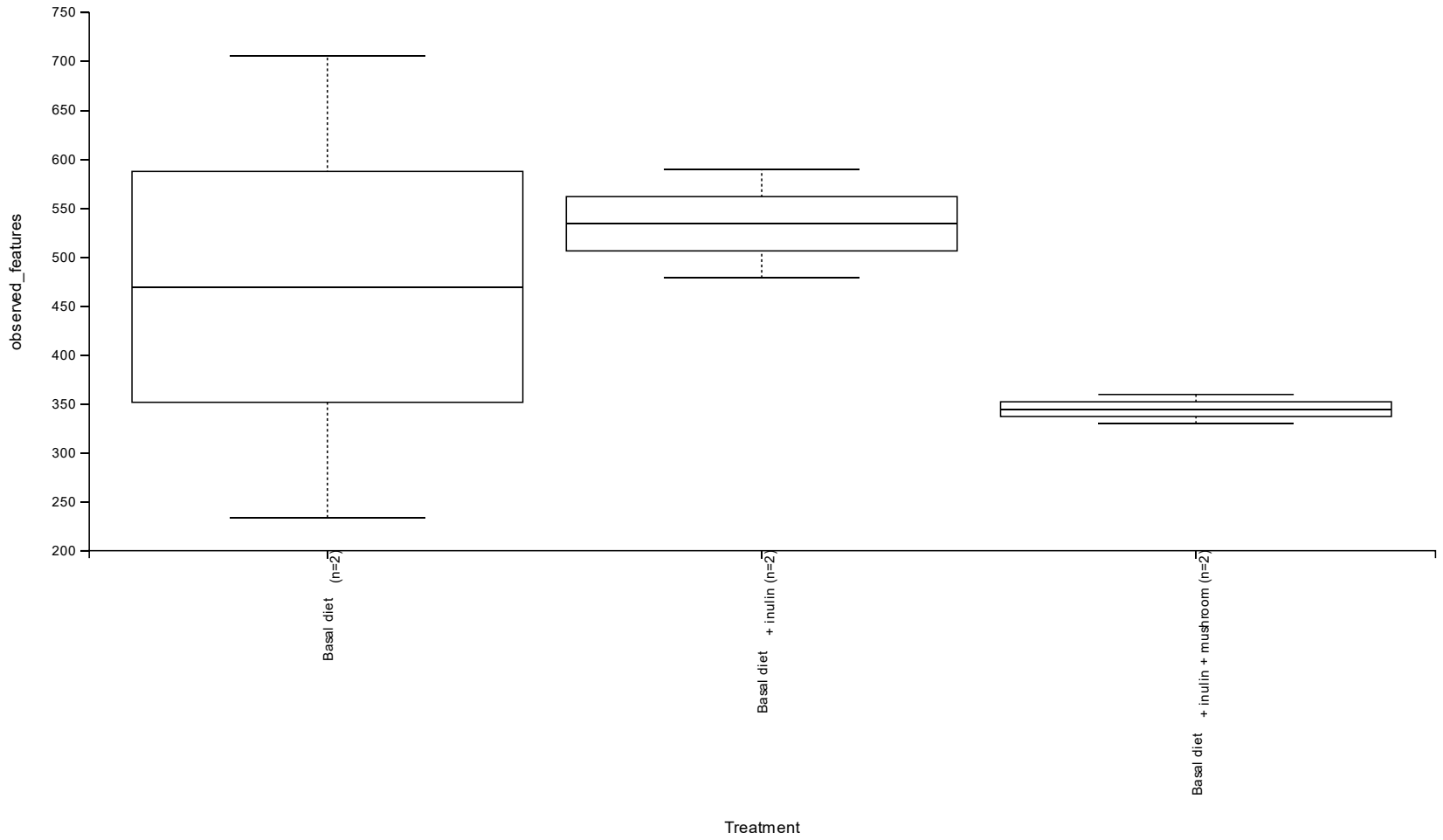
Appendix 7B: Box plot of Shannon entropy, showing how experimental treatments impacted richness and evenness of microbiota within samples (generated in QIIME2).



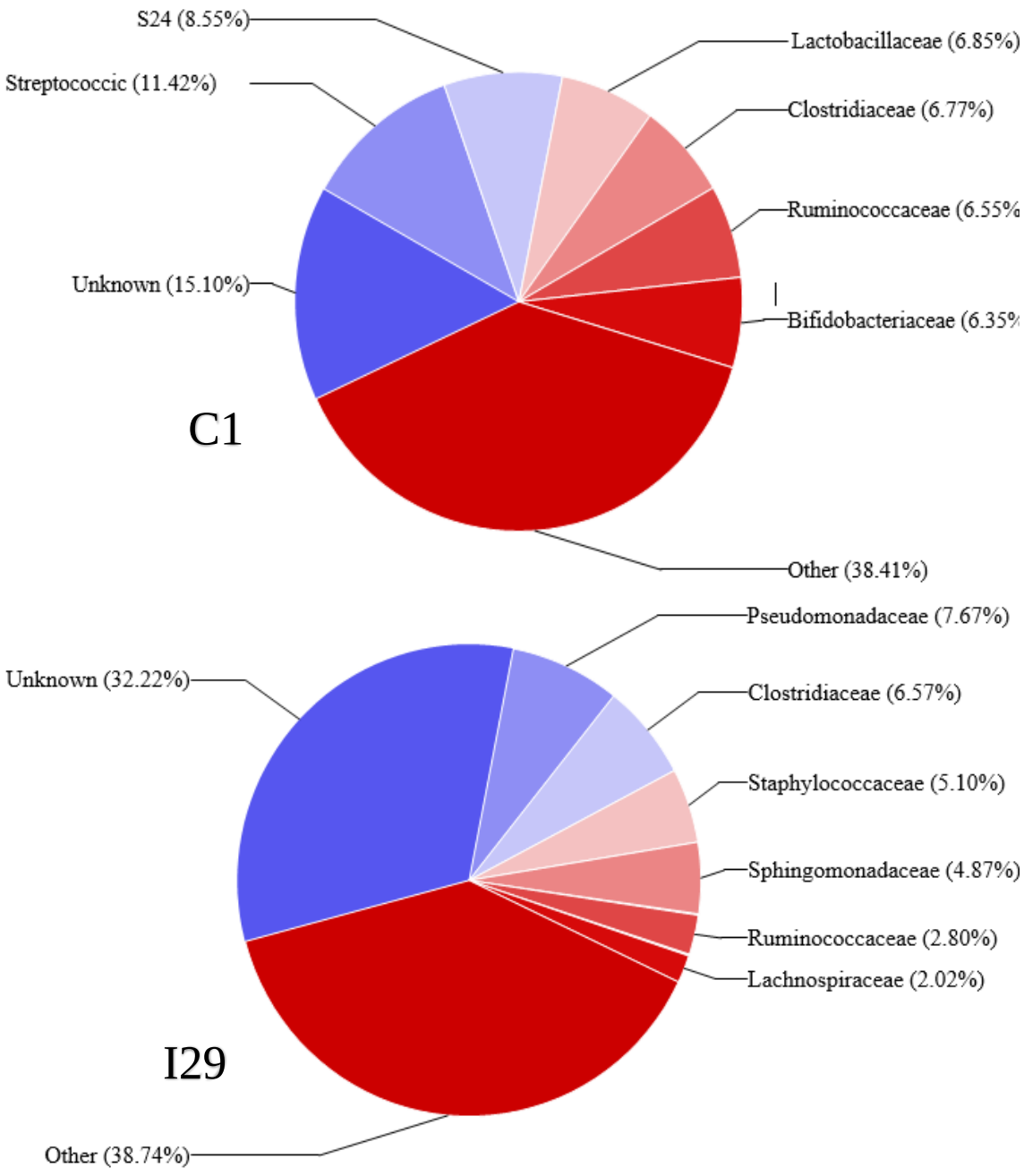
Appendix 7C: Box plot of observed features (ASVs), showing the number ASVs as influenced by sampling date (generated in QIIME2).



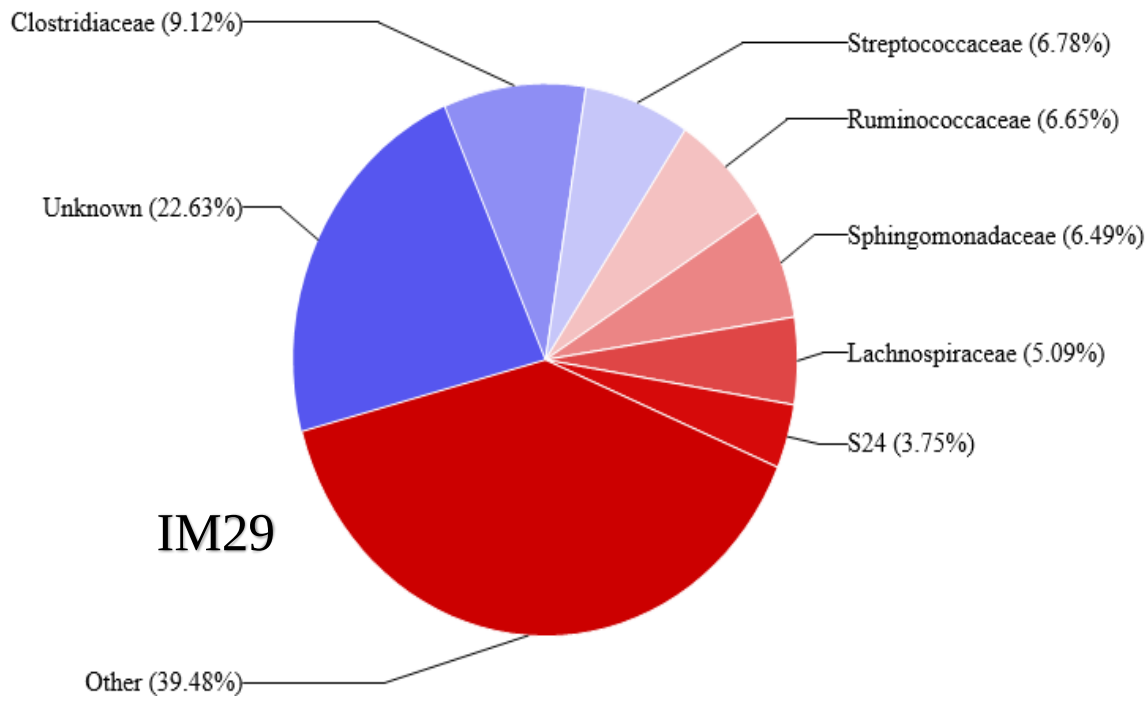
Appendix 7D: Box plot of observed features (ASVs), showing the number ASVs as influenced by the type of prebiotic treatment (generated in QIIME2).

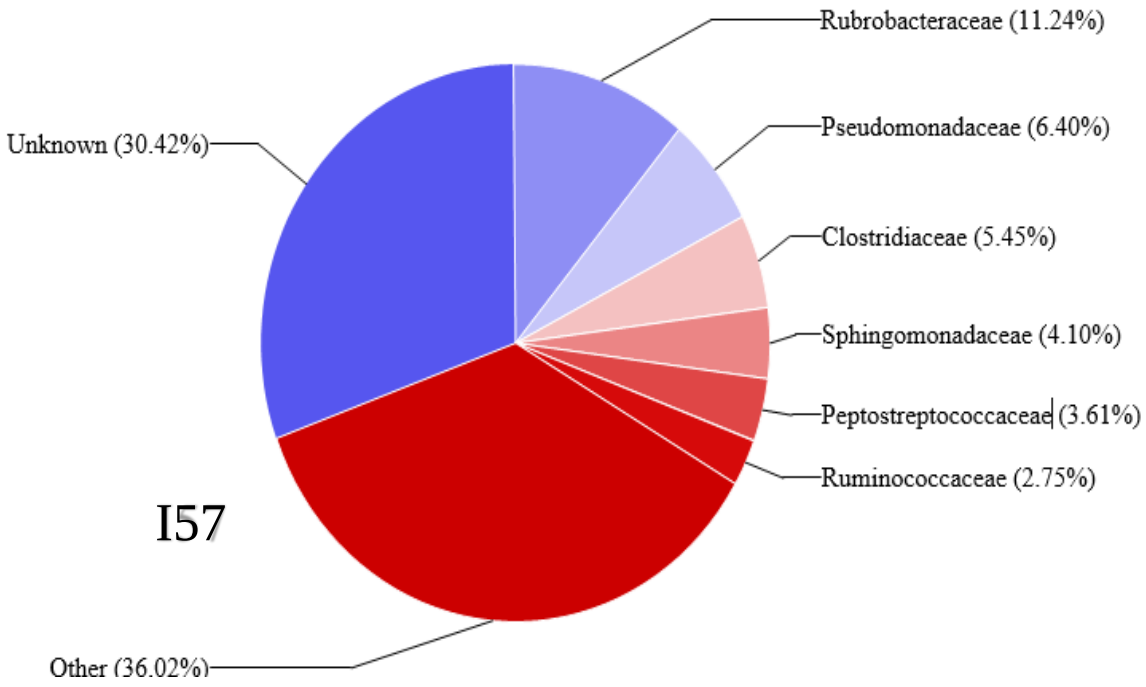


Appendix 8A: Relative abundances of samples C1 and I29 at family level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech analysis).

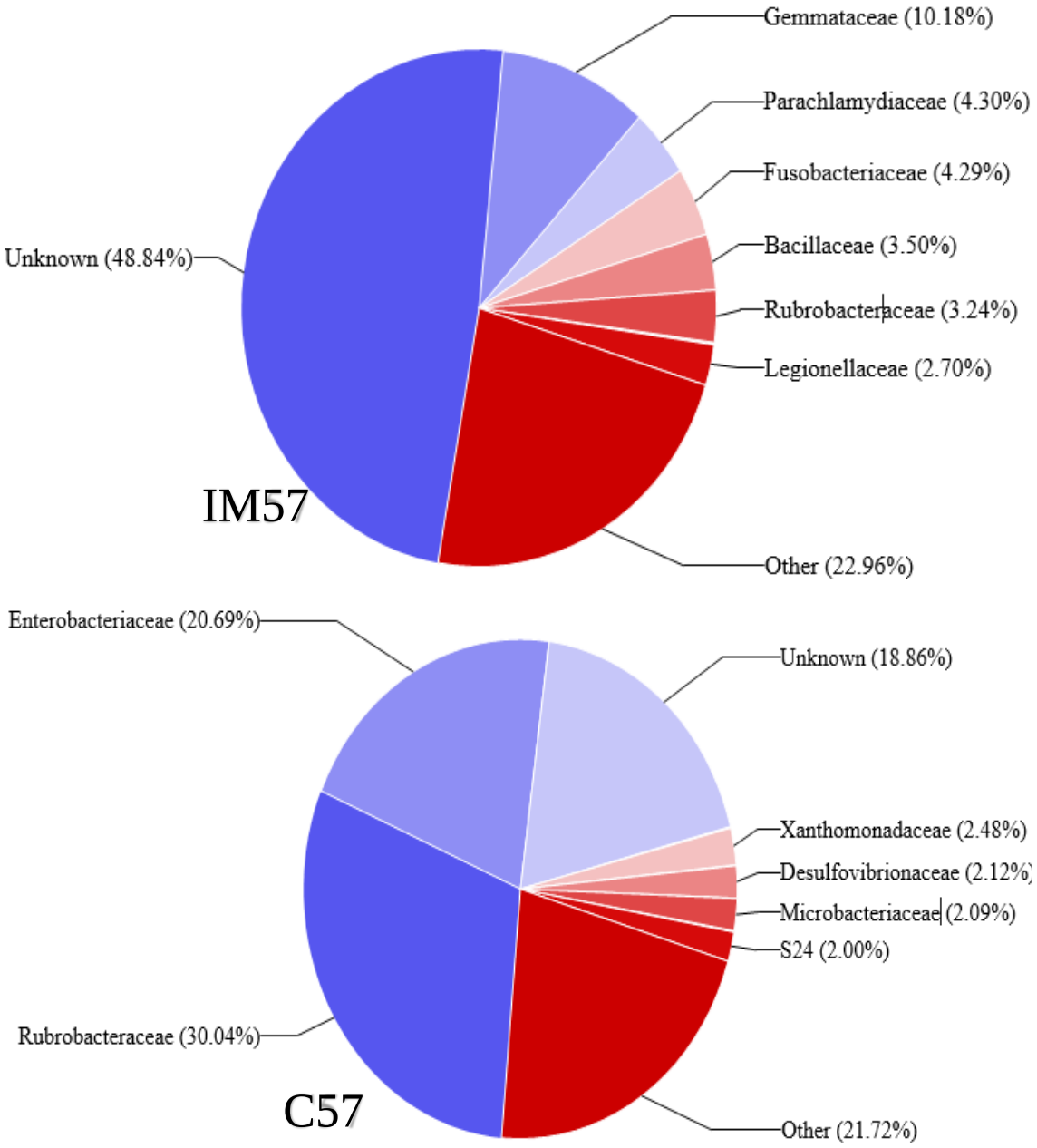


Appendix 8B: Relative abundances of samples IM29 and I57 at family level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech analysis).

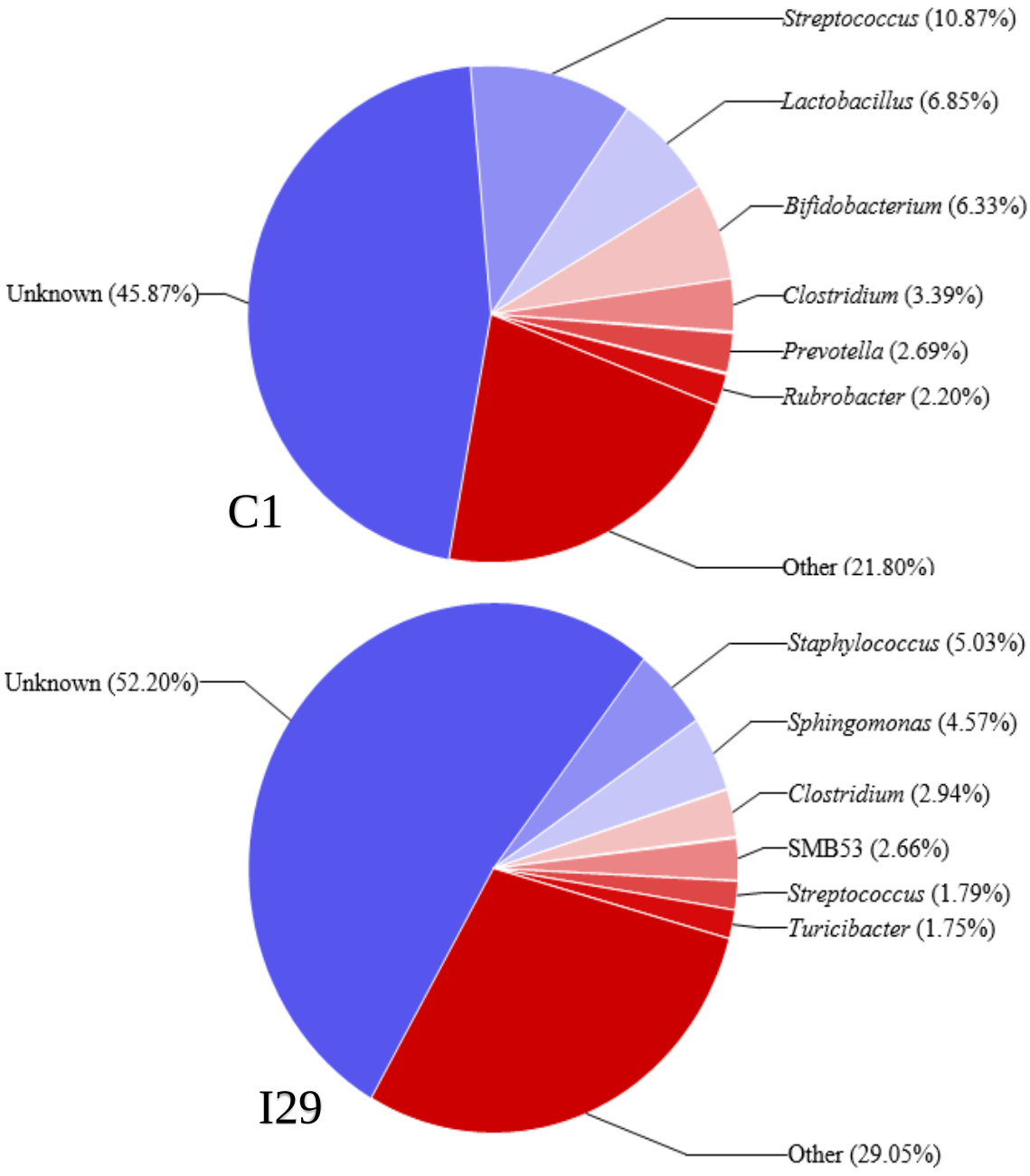




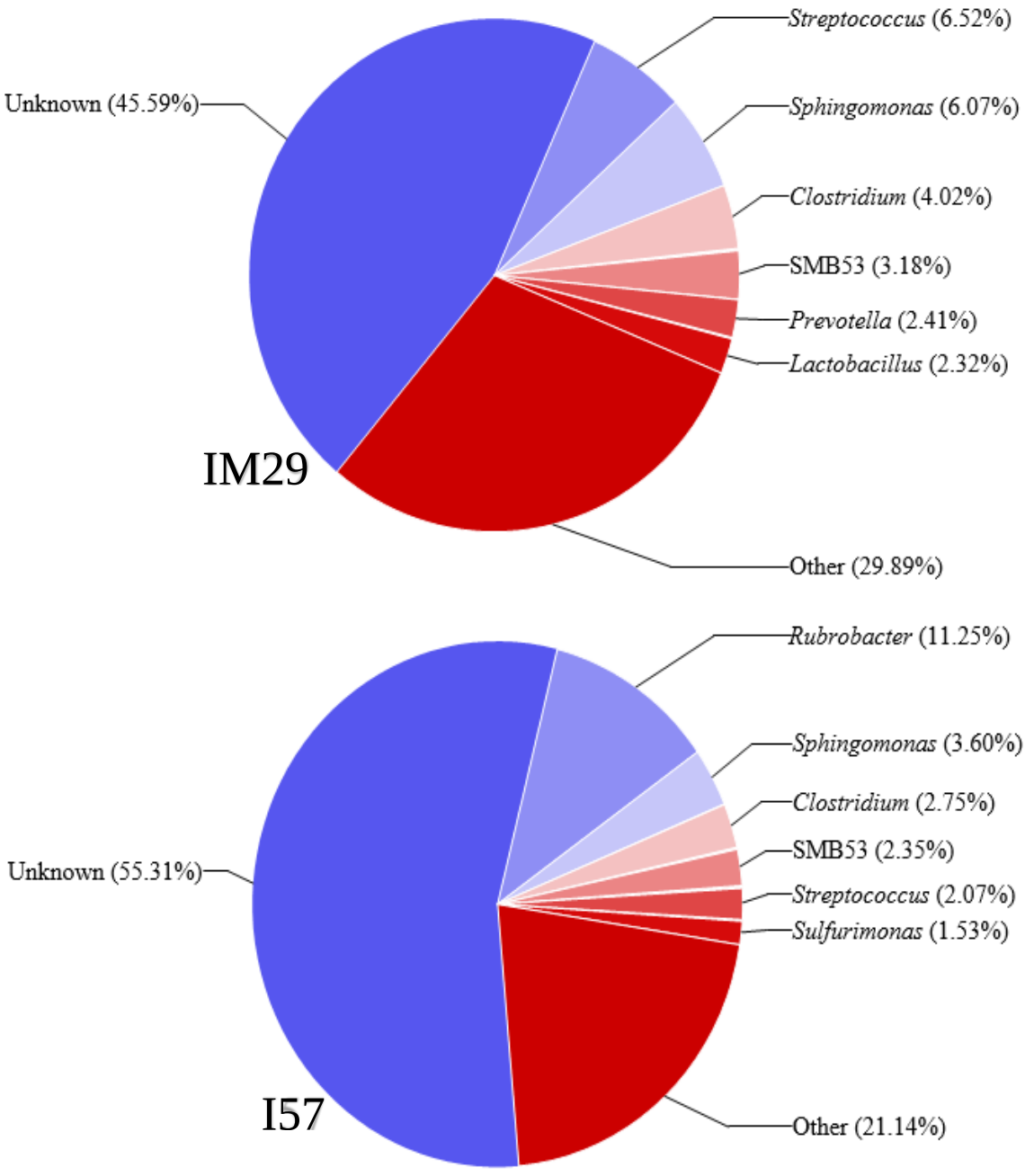
Appendix 8C: Relative abundances of samples IM57 and C57 at family level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech analysis).



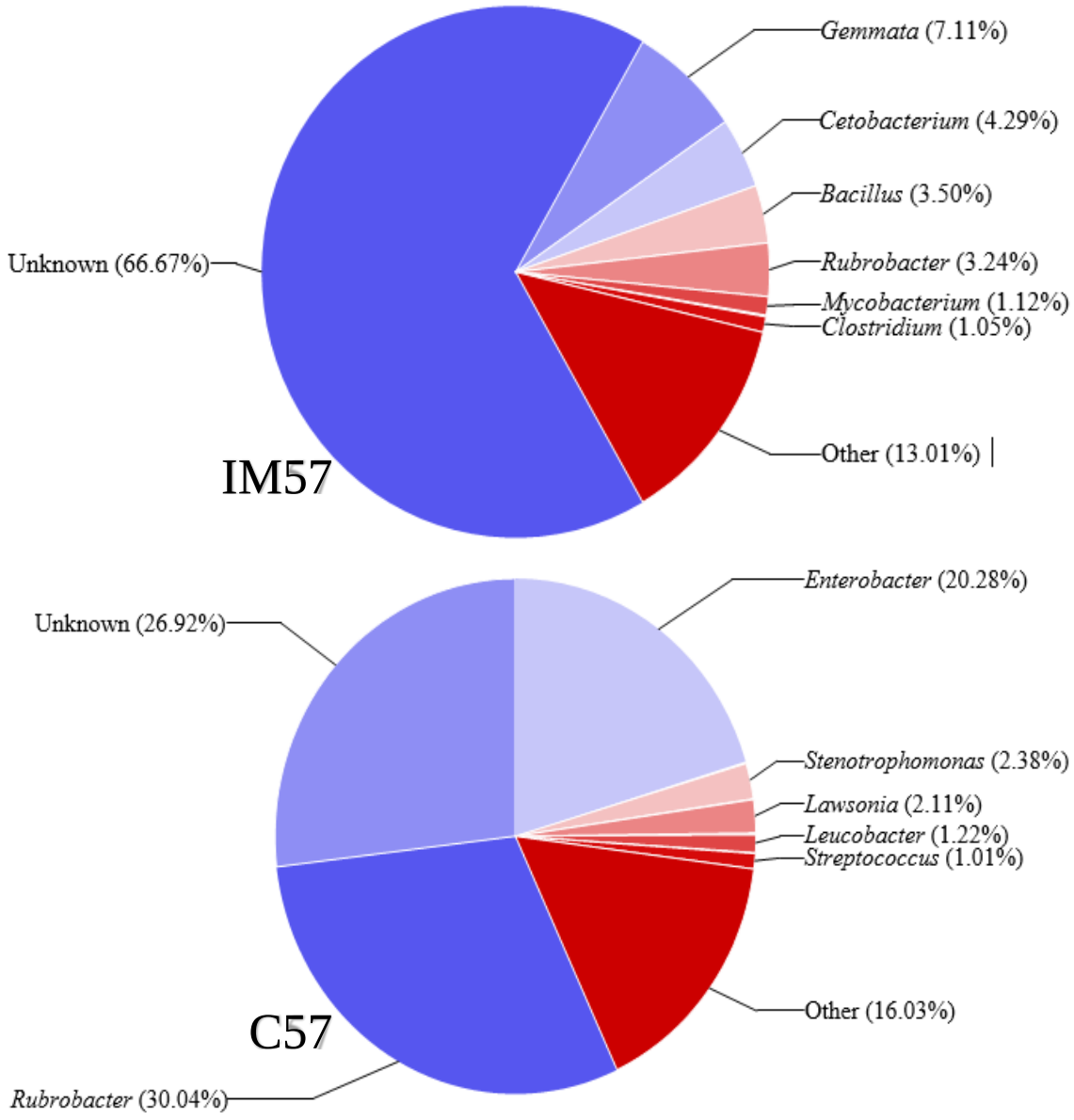
Appendix 8D: Relative abundances of samples C1 and I29 at genus level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech).



Appendix 8E: Relative abundances of samples IM29 and I57 at genus level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech).



Appendix 8F: Relative abundances of samples IM57 and C57 at genus level based on 97% similarity taxonomic classification in RDP database (source: Inqaba Biotech).



Appendix 9: Species classification from the RDP database

C1		**I29**		**IM29**		**I57**		**IM57**		**C57**	
Species	**%**	**Species**	**%**	**Species**	**%**	**Species**	**%**	**Species**	**%**	**Species**	**%**
Unknown_	32.32	Unknown_	39.5	Unknown_	27.19	Unknown_	41.93	Unknown_	57.19	*Rubrobacter_*	30.06
Unknown	16.02	Unknown	13.51	Unknown	20.22	Unknown	13.83	Unknown	10.49	Unknown_	20.98
/Streptococcus_luteciae	8.11	*Sphingomonas_*	4.47	*Streptococcus_luteciae*	4.91	*Rubrobacter_*	11.25	*Gemmata_*	7.12	Unknown	13.63
Bifidobacterium_pseudolongum	5.31	*SMB53_*	2.66	*Sphingomonas_*	4.89	*Sphingomonas_*	3.53	*Cetobacterium_somerae*	4.29	*Enterobacter_cloacae*	13.51
Lactobacillus_reuteri	3.83	*Staphylococcus_*	2.63	*SMB53_*	3.18	*SMB53_*	2.35	*Rubrobacter_*	3.24	*Stenotrophomonas_*	2.38
Streptococcus_alactolyticus	2.41	*Turicibacter_*	1.75	*Prevotella_*	2.4	*Clostridium_*	1.54	*Bacillus_cereus*	2.82	*Lawsonia_*	2.12
*Rubrobacter_*	2.2	*Clostridium_*	1.63	*Treponema_*	1.4	*Sulfurimonas_*	1.09	*Mycobacterium_*	1.02	*Leucobacter_*	1.22
*SMB53_*	1.94	*Corynebacterium_*	1.54	*Lactobacillus_reuteri*	1.36	*Arcobacter_cryaerophilus*	1.08	*Sphingomonas_*	1.01	*Gemmata_*	0.91

*Megasphaera_*	1.89	*Staphylococcus_epidermidis*	1.47	*Corynebacterium_*	1.26	*Corynebacterium_*	0.96	*Rhodobacter_*	0.91	*Clavibacter_*	0.76
*Prevotella_*	1.83	*Arcobacter_cryaerophilus*	1.19	*Oscillospira_*	1.17	*Streptococcus_infantis*	0.87	*Candidatus_*	0.89	*Sphingomonas_*	0.74
*Lactobacillus_*	1.53	*Finegoldia_*	1.15	*Bifidobacterium_pseudolongum*	1.1	*Paludibacter_*	0.73	*Aquicella_*	0.68	*Micrococcus_*	0.68
*Oscillospira_*	1.49	*Streptococcus_luteciae*	1.08	*Sphingomonas_wittichii*	1.09	*Tissierella_Soehngenia_*	0.69	*Hyphomicrobium_*	0.58	*Bacillus_cereus*	0.65
*Turicibacter_*	1.23	*Rubrobacter_*	0.97	*Turicibacter_*	1.06	*Streptococcus_luteciae*	0.66	*Tatlockia_*	0.56	*Prevotella_*	0.62
Clostridium_celatum	1.1	*Peptoniphilus_*	0.88	*Succinivibrio_*	1.04	*Acholeplasma_*	0.57	*Streptococcus_luteciae*	0.52	*Paenibacillus_chondroitinus*	0.6
*Treponema_*	1.03	*Coprococcus_*	0.87	*Rothia_mucilaginosa*	0.98	*Unknown_sordellii*	0.55	*Clavibacter_*	0.51	*Streptococcus_luteciae*	0.56
Bacillus_cereus	1.02	*Aerococcus_*	0.75	*Cloacibacterium_*	0.98	*Staphylococcus_*	0.52	*Bacillus_*	0.5	*Lactobacillus_reuteri*	0.54
Lactobacillus_vaginalis	1	*Bacteroides_*	0.73	*Parabacteroides_*	0.95	*Kaistobacter_*	0.5	*Planctomyces_*	0.37	*Faecalibacterium_*	0.51
*Sphingomonas_*	0.92	*Acholeplasma_*	0.73	*Lactobacillus_*	0.92	*Propionibacterium_acnes*	0.49	*Streptococcus_infantis*	0.3	*Megasphaera_*	0.49
*Parabacteroides_*	0.71	*Prevotella_*	0.71	*Fibrobacter_*	0.86	*Pseudomonas_*	0.47	*Prevotella_*	0.26	*Rhodobacter_*	0.48

Clostridium_butyricum	0.68	*Propionibacterium_acnes*	0.58	*Staphylococcus_*	0.82	*RFN20_*	0.47	*A17_*	0.25	*Mycobacterium_*	0.47
*Coprococcus_*	0.67	*Porphyromonas_*	0.5	*Clostridium_*	0.79	*Facklamia_*	0.46	*Clostridium_*	0.24	*Corynebacterium_*	0.44
*Paenibacillus_*	0.61	*Paraprevotella_*	0.46	*Clostridium_perfringens*	0.75	*Coprococcus_*	0.44	*Clostridium_butyricum*	0.23	*Turicibacter_*	0.31
*Succinivibrio_*	0.56	*Chryseobacterium_*	0.45	*Propionibacterium_acnes*	0.74	*Arcobacter_*	0.44	*Lactobacillus_*	0.22	*Methylobacterium_*	0.26
*YRC22_*	0.54	*Skermanella_*	0.44	*Coprococcus_*	0.7	*Candidatus_*	0.41	*Rhodoplanes_*	0.22	*Fimbriimonas_*	0.26
Paenibacillus_chondroitinus	0.36	*Anaerococcus_*	0.42	*Streptococcus_alactolyticus*	0.61	*Flavisolibacter_*	0.41	*Chryseobacterium_*	0.19	*Lactobacillus_*	0.25
*Erwinia_*	0.34	*Solibacillus_*	0.41	*CF231_*	0.6	*Cellulosibacter_alkalithermophilus*	0.39	*SMB53_*	0.19	*Corynebacterium_simulans*	0.24
Prevotella_ruminicola	0.34	*Streptococcus_infantis*	0.4	*Clostridium_celatum*	0.59	*Pseudonocardia_*	0.38	*Parachlamydia_*	0.18	*Phycicoccus_*	0.24
*Phascolarctobacterium_*	0.33	*1_*	0.39	*Clostridium_butyricum*	0.57	*Lactobacillus_*	0.37	*Elusimicrobium_*	0.16	*Roseomonas_mucosa*	0.24
*Lactococcus_*	0.32	*Unknown_sordellii*	0.36	*Streptococcus_infantis*	0.56	*Chryseobacterium_*	0.37	*Staphylococcus_epidermidis*	0.15	*Staphylococcus_epidermidis*	0.23
*Sutterella_*	0.29	*Candidatus_*	0.36	*Phycicoccus_*	0.56	*Lactococcus_*	0.36	*Lactobacillus_vaginalis*	0.15	*Kaistobacter_*	0.23

*Fibrobacter_*	0.28	*Clostridium_celatum*	0.35	*YRC22_*	0.54	*Staphylococcus_epidermidis*	0.36	*Stenotrophomonas_*	0.14	*Phormidium_*	0.23
*Corynebacterium_*	0.27	*Geodermatophilus_*	0.35	*Clostridium_ruminantium*	0.54	*Lactobacillus_helveticus*	0.36	*Phycicoccus_*	0.14	*Hydrogenophaga_*	0.22
Clostridium_perfringens	0.27	*Treponema_*	0.34	*Rhodococcus_*	0.52	*Streptococcus_*	0.31	*Rhizobium_*	0.13	*Plesiomonas_shigelloides*	0.22
*Ruminococcus_*	0.26	*WAL_1855D_*	0.34	*Pseudomonas_stutzeri*	0.51	*Clostridium_ruminantium*	0.31	*Pediococcus_*	0.13	*Streptococcus_alactolyticus*	0.22
*Epulopiscium_*	0.25	*Bacteroides_nordii*	0.33	*Escherichia_coli*	0.5	*Prevotella_*	0.3	*Caldilinea_*	0.13	*B_*	0.22
Prevotella_melaninogenica	0.23	*Bacillus_*	0.33	*Pseudomonas_veronii*	0.45	*Epulopiscium_*	0.29	*Acinetobacter_*	0.13	*Ruminococcus_*	0.17
Clostridium_ruminantium	0.22	*RFN20_*	0.32	*Clavibacter_*	0.45	*Rhodobacter_*	0.29	*Enterobacter_cloacae*	0.12	*Opitutus_*	0.16
Methylobacterium_komagatae	0.21	*Aquabacterium_*	0.31	*Novosphingobium_*	0.42	*Oscillospira_*	0.29	*Cryocola_*	0.12	*Pimelobacter_*	0.16
Roseicyclus_mahoneyensis	0.2	*Cloacibacterium_*	0.31	*Phascolarctobacterium_*	0.4	*Paraprevotella_*	0.29	*Bacillus_anthracis*	0.11	*Clostridium_bowmanii*	0.16
*Blautia_*	0.19	*Moraxella_*	0.3	*Bacillus_cereus*	0.4	*Sulfurimonas_autotrophica*	0.28	*Pseudoxanthomonas_indica*	0.11	*Methylobacterium_komagatae*	0.15
Lysinibacillus_boronitolerans	0.19	*Clostridium_perfringens*	0.3	*Anaerococcus_*	0.38	*Acinetobacter_lwoffii*	0.27	*Clostridium_celatum*	0.11	*Streptococcus_infantis*	0.13

Salmonella_enterica	0.18	*Acinetobacter_schindleri*	0.3	*Pseudomonas_*	0.37	*Desulfuromonas_*	0.26	*Pirellula_*	0.11	*Phascolarctobacterium_*	0.13
*Faecalibacterium_*	0.18	*Flavisolibacter_*	0.29	*Blautia_*	0.34	*Desulfococcus_*	0.26	*Psychrobacter_pulmonis*	0.11	*CF231_*	0.13
*Hydrogenophaga_*	0.17	*Campylobacter_ureolyticus*	0.29	*B_*	0.34	*Acinetobacter_*	0.26	*Isosphaera_*	0.09	*Williamsia_*	0.12
*Desulfovibrio_*	0.17	*Tissierella_Soehngenia_*	0.28	*Phenylobacterium_*	0.31	*Escherichia_coli*	0.24	*YRC22_*	0.09	*Cetobacterium_somerae*	0.12
*Cryocola_*	0.16	*Sulfurimonas_*	0.28	*Clostridium_sardiniense*	0.31	*Nostoc_muscorum*	0.23	*Coprococcus_*	0.09	*Clostridium_perfringens*	0.12
Unknown_difficile	0.16	*Desulfuromonas_*	0.27	*Aerococcus_*	0.29	*Streptococcus_alactolyticus*	0.23	*Oscillospira_*	0.09	*Paracoccus_*	0.12
*Erythromicrobium_*	0.16	*Sphaerochaeta_*	0.26	*Bacteroides_*	0.29	*Syntrophus_*	0.22	*Prevotella_melaninogenica*	0.09	*Acinetobacter_*	0.12
Lactobacillus_ruminis	0.15	*Clostridium_ruminantium*	0.26	*WAL_1855D_*	0.29	*Halomicronema_hongdechloris*	0.22	*Turicibacter_*	0.09	*Caulobacter_*	0.11
*Anaerococcus_*	0.15	*Stenotrophomonas_*	0.26	*Unknown_difficile*	0.28	*Oxobacter_*	0.21	*Micrococcus_*	0.08	*Streptococcus_*	0.1
*Staphylococcus_*	0.15	*Streptococcus_*	0.25	*Butyricicoccus_pullicaecorum*	0.28	*Acetobacterium_*	0.2	*Bifidobacterium_animalis*	0.08	*Arthrobacter_oxydans*	0.1
Bifidobacterium_animalis	0.14	*Acholeplasma_brassicae*	0.25	*Methylobacterium_komagatae*	0.27	*Haemophilus_influenzae*	0.19	*Paenibacillus_chondroitinus*	0.08	*Klebsiella_*	0.1

*Campylobacter_*	0.14	*Acutodesmus_obliquus*	0.23	*Streptomyces_*	0.27	*Enterobacter_cloacae*	0.19	*Bdellovibrio_*	0.08	*Demequina_*	0.1
Shigella_sonnei	0.13	*Mycoplana_*	0.21	*Streptococcus_agalactiae*	0.27	*Phycicoccus_*	0.19	*Ruminococcus_albus*	0.08	*Pseudomonas_*	0.09
Streptococcus_infantis	0.12	*Clostridium_sardiniense*	0.2	*Allobaculum_*	0.26	*vadinHB04_*	0.18	*Ruminococcus_*	0.08	*Leptothrix_*	0.09
Clostridium_sardiniense	0.12	*WCHB1_*	0.2	*Blautia_producta*	0.26	*Turicibacter_*	0.17	*Propionibacterium_acnes*	0.07	*Gemmatimonas_*	0.09
Clostridium_metallolevans	0.12	*Rhodoplanes_*	0.18	*Bifidobacterium_animalis*	0.26	*Stenotrophomonas_*	0.17	*Bifidobacterium_pseudolongum*	0.07	*Luteimonas_*	0.08
*Kocuria_*	0.12	*Pantoea_agglomerans*	0.18	*Rubellimicrobium_*	0.25	*Cloacibacterium_*	0.17	*Coxiella_*	0.07	*Actinotalea_*	0.08
Bacteroides_fragilis	0.12	*Ruminococcus_*	0.18	*Chryseobacterium_*	0.23	*Blastococcus_aggregatus*	0.17	*Rhodococcus_*	0.07	*Nocardioides_dilutus*	0.08
*Roseburia_*	0.11	*Clavibacter_*	0.17	*Shuttleworthia_*	0.23	*Bifidobacterium_pseudolongum*	0.16	*Actinomycetospora_*	0.07	*Treponema_*	0.08
Oscillospira_guilliermondii	0.11	*Gallicola_*	0.17	*Lactococcus_*	0.22	*Ensifer_adhaerens*	0.15	*Epulopiscium_*	0.07	*Staphylococcus_*	0.08
*Clostridium_*	0.11	*Planctomyces_*	0.17	*Hymenobacter_*	0.22	*Chthoniobacter_*	0.15	*Legionella_*	0.06	*Haliangium_*	0.07
*Sneathia_*	0.1	*Kaistobacter_*	0.17	*Tepidimonas_*	0.22	*Clostridium_butyricum*	0.15	*Bradyrhizobium_*	0.06	*Paracoccus_marcusii*	0.07

Unknown_gnavus	0.1	*Oscillospira_*	0.15	*Jeotgalicoccus_*	0.22	*Leptothrix_*	0.15	*Leptothrix_*	0.06	*Enterobacter_hormaechei*	0.07
*Sphaerochaeta_*	0.1	*Ruminococcus_flavefaciens*	0.15	*Bacillus_flexus*	0.21	*Rhodoplanes_*	0.14	*Mycobacterium_gordonae*	0.06	*Cellulomonas_*	0.07
Coprococcus_catus	0.1	*Proteiniclasticum_*	0.15	*Oxobacter_*	0.21	*vadinCA02_*	0.13	*Lactobacillus_delbrueckii*	0.06	*Schlegelella_*	0.07
*Rhodoferax_*	0.1	*Rubellimicrobium_*	0.15	*Stenotrophomonas_*	0.2	*Clostridium_neonatale*	0.12	*Methylosinus_*	0.06	*Clostridium_butyricum*	0.06
Enterococcus_sulfureus	0.1	*Acidovorax_wohlfahrtii*	0.15	*Acinetobacter_*	0.19	*Desulfitobacter_*	0.12	*Streptococcus_*	0.06	*Brachybacterium_*	0.05
Clostridium_hathewayi	0.09	*Propionibacterium_granulosum*	0.15	*Staphylococcus_epidermidis*	0.19	*Desulfatibacillum_alkenivorans*	0.12	*Megasphaera_*	0.06	*Ralstonia_*	0.05
*Pyramidobacter_*	0.09	*Agromyces_*	0.15	*Wolinella_succinogenes*	0.19	*Gemmata_*	0.12	*Butyricicoccus_pullicaecorum*	0.05	*Mesorhizobium_*	0.05
*Mycobacterium_*	0.09	*Pseudonocardia_*	0.14	*Paludibacter_*	0.17	*Paracoccus_marcusii*	0.12	*Streptococcus_alactolyticus*	0.05	*Brevibacterium_*	0.05
*Georgenia_*	0.09	*Modestobacter_*	0.14	*Alloiococcus_otitis*	0.17	*Clostridium_perfringens*	0.12	*Pseudoclavibacter_bifida*	0.05	*Erythrobacter_*	0.05
Victivallis_vadensis	0.09	*Roseburia_faecis*	0.14	*Micrococcus_*	0.16	*Sphaerochaeta_*	0.12	*Caloramator_*	0.05	*Lactococcus_*	0.05
Prevotella_pallens	0.09	*Syntrophus_*	0.14	*Streptococcus_*	0.16	*Clostridium_botulinum*	0.11	*Myroides_odoratimimus*	0.04	*Georgenia_*	0.04

Akkermansia_muciniphila	0.09	*Cystobacter_*	0.14	*Kocuria_palustris*	0.15	*Macrococcus_*	0.11	*Brevibacillus_*	0.04	*Clostridium_*	0.04
*Allobaculum_*	0.08	*Lactobacillus_iners*	0.13	*Sutterella_*	0.15	*Thiomicrospira_frisia*	0.11	*Providencia_*	0.04	*Methylotenera_mobilis*	0.04
Ruminococcus_flavefaciens	0.08	*Desulfamplus_magnetomortis*	0.13	*Cryocola_*	0.14	*Skermanella_*	0.1	*Paludibacter_*	0.04	*Novosphingobium_*	0.03
*Schwartzia_*	0.08	*Blautia_producta*	0.13	*Comamonas_*	0.14	*Geobacter_*	0.1	*Gordonia_*	0.04	*Sphingobium_*	0.03
Pseudoclavibacter_bifida	0.08	*PSB_*	0.13	*Arthrobacter_*	0.14	*Unknown_difficile*	0.1	*Paracoccus_marcusii*	0.04	*Candidatus_*	0.03
*Nocardia_*	0.07	*Opitutus_*	0.13	*Enterococcus_*	0.14	*Actinomyces_*	0.1	*Lysinibacillus_boronitolerans*	0.04	*Balneimonas_*	0.03
Lactobacillus_iners	0.07	*Actinomadura_atramentaria*	0.12	*Bacillus_*	0.13	*Roseococcus_*	0.1	*Pseudomonas_*	0.04	*Alicyclobacillus_acidocaldarius*	0.03
Bifidobacterium_adolescentis	0.07	*Unknown_difficile*	0.12	*Lysinibacillus_boronitolerans*	0.13	*Microbispora_rosea*	0.1	*Methylobacterium_organophilum*	0.04	*Inquilinus_*	0.03
*Clavibacter_*	0.07	*Enterobacter_turicensis*	0.12	*Flavobacterium_*	0.13	*Pimelobacter_*	0.09	*Staphylococcus_*	0.04	*Flavobacterium_gelidilacus*	0.03
*Streptococcus_*	0.06	*Dialister_*	0.12	*Sulfurospirillum_*	0.13	*Rhizobium_*	0.09	*Gardnerella_*	0.04	*Planctomyces_*	0.03
*Leptothrix_*	0.06	*Blautia_*	0.12	*Serratia_marcescens*	0.11	*Clavibacter_*	0.09	*Paenibacillus_*	0.04	*Lysobacter_*	0.02

*Dorea_*	0.06	*Anaerovorax_*	0.12	*Bifidobacterium_longum*	0.1	*4_*	0.08	*Unknown_bifermentans*	0.04	*Rhodoplanes_*	0.02
*Methylobacterium_*	0.06	*vadinHB04_*	0.12	*Actinomyces_*	0.1	*Geodermatophilus_*	0.08	*Serratia_marcescens*	0.04	*Lactobacillus_iners*	0.02
*Pseudobutyrivibrio_*	0.06	*Hydrogenophaga_*	0.12	*Erwinia_*	0.1	*PSB_*	0.07	*Anaerostipes_*	0.03	*Coprococcus_*	0.02
*Sarcina_*	0.06	*Helicobacter_apodemus*	0.12	*Enhydrobacter_*	0.08	*Pleomorphomonas_oryzae*	0.07	*Acidovorax_*	0.03	*Luteolibacter_*	0.02
Leuconostoc_mesenteroides	0.05	*Micrococcus_*	0.11	*Dorea_*	0.08	*Sphingomonas_asaccharolytica*	0.07	*Corynebacterium_*	0.02	*Cryocola_*	0.02
*Paludibacter_*	0.05	*Sharpea_*	0.11	*Sphingomonas_asaccharolytica*	0.07	*Dethiosulfovibrio_peptidovorans*	0.07	*Sphingobium_*	0.02	*Nocardioides_furvisabuli*	0.02
*Micrococcus_*	0.05	*CF231_*	0.11	*Adlercreutzia_*	0.07	*Micrococcus_*	0.07	*Staphylococcus_aureus*	0.02	*Weissella_*	0.02
Rubrivivax_gelatinosus	0.05	*Eggerthella_lenta*	0.11	*Labrys_*	0.06	*Leucobacter_*	0.07	*Sphingomonas_asaccharolytica*	0.02	*Caloramator_*	0.01
*Bacteroides_*	0.04	*Alicyclobacillus_*	0.11	*Ralstonia_*	0.06	*Thermovum_composti*	0.07	*Clostridium_perfringens*	0.02	*Rummeliibacillus_*	0.01
*Rhodobacter_*	0.04	*Pseudomonas_stutzeri*	0.11	*Paenibacillus_*	0.05	*Rheinheimera_*	0.06	*Bacillus_foraminis*	0.02	*Paracoccus_kawasakiensis*	0.01
Clostridium_neonatale	0.04	*Rhodobacter_*	0.11	*Bacillus_thermoamylovorans*	0.05	*Paenibacillus_chondroitinus*	0.06	*Bacillus_selenatarsenatis*	0.02	*Acinetobacter_lwoffii*	0.01

Arcobacter_cryaerophilus	0.04	*Acinetobacter_lwoffii*	0.1	*Janthinobacterium_*	0.05	*Blvii28_*	0.06	*Allobaculum_*	0.02	*Pseudomonas_alcaligenes*	0.01
Atopobium_vaginae	0.04	*Paracoccus_marcusii*	0.1	*Lactococcus_garvieae*	0.04	*Mycobacterium_*	0.06	*Alishewanella_*	0.02	*Desulfuromonas_*	0.01
Collinsella_aerofaciens	0.04	*Leucobacter_*	0.1	*Bifidobacterium_*	0.03	*Clostridium_sticklandii*	0.05	*Bacteroides_*	0.02	*Clostridium_celatum*	0.01
*Acinetobacter_*	0.04	*W22_*	0.1	*Lactobacillus_vaginalis*	0.03	*Providencia_*	0.05	*Oxobacter_*	0.02		
Ruminococcus_albus	0.04	*Staphylococcus_equorum*	0.1	*Brachybacterium_*	0.03	*Syntrophomonas_*	0.05	*Brevibacillus_laterosporus*	0.02		
*Stenotrophomonas_*	0.04	*Paludibacter_*	0.09	*Alistipes_massiliensis*	0.02	*Dactylosporangium_*	0.05	*Pantoea_agglomerans*	0.01		
*Aerococcus_*	0.04	*Dehalobacterium_*	0.09			*Planomicrobium_*	0.05	*Prevotella_copri*	0.01		
*Anaerovibrio_*	0.03	*Sphingobium_*	0.09			*Inquilinus_*	0.05	*Kaistobacter_*	0.01		
*Enterococcus_*	0.03	*Devosia_*	0.09			*Comamonas_*	0.05				
Clostridium_hungatei	0.03	*Unknown_gnavus*	0.09			*Hydrogenophaga_*	0.04				
Bacteroides_uniformis	0.03	*Sphingomonas_asaccharolytica*	0.08			*Tepidimonas_*	0.04				

Staphylococcus_equorum	0.03	*Sulfurospirillum_*	0.08			*Simplicispira_*	0.04				
*Streptosporangium_*	0.03	*Oceanobacillus_chironomi*	0.08			*Kytococcus_*	0.04				
*Fusobacterium_*	0.03	*Cellulomonas_*	0.08			*wall_spirochete*	0.04				
*Bradyrhizobium_*	0.03	*Cellvibrio_*	0.08			*Janthinobacterium_*	0.04				
Vestibaculum_illigatum	0.03	*Escherichia_coli*	0.08			*Burkholderia_*	0.03				
Unknown_torques	0.03	*Butyrivibrio_*	0.08			*Actinomycetales_Ellin122*	0.03				
Rothia_mucilaginosa	0.03	*Bacillus_flexus*	0.08			*Allobaculum_*	0.03				
*Kaistobacter_*	0.03	*Catellatospora_*	0.07			*Aeromicrobium_*	0.03				
Lactobacillus_helveticus	0.03	*Nesterenkonia_*	0.07			*Rubrivivax_gelatinosus*	0.03				
*Chryseobacterium_*	0.03	*Janthinobacterium_*	0.07			*Haemophilus_*	0.03				
*Acidovorax_*	0.02	*Nitrospira_calida*	0.07			*Staphylococcus_haemolyticus*	0.02				

*Brachybacterium_*	0.02	*Lactobacillus_*	0.07			*Aerococcus_*	0.02				
*Novosphingobium_*	0.02	*Kribbella_*	0.07			*Garciella_*	0.02				
Butyricicoccus_pullicaecorum	0.02	*Erwinia_*	0.07			*Klebsiella_*	0.02				
*Bacillus_*	0.02	*Jeotgalicoccus_*	0.07			*Brevibacterium_paucivorans*	0.02				
Bacillus_anthracis	0.02	*Ewingella_americana*	0.07			*Veillonella_dispar*	0.02				
Bacteroides_nordii	0.02	*Balneimonas_*	0.07			*Asarum_canadense*	0.01				
*Facklamia_*	0.02	*Lactobacillus_reuteri*	0.07								
Sphingomonas_asaccharolytica	0.02	*Anaerovibrio_*	0.07								
Blautia_producta	0.02	*Polyangium_fumosum*	0.07								
Staphylococcus_aureus	0.02	*Phascolarctobacterium_*	0.06								
*p_*	0.02	*Microbacterium_lacticum*	0.06								

*Gardnerella_*	0.01	*Anaeromyxobacter_*	0.06								
*Thermus_*	0.01	*Nonomuraea_*	0.06								
Lactococcus_garvieae	0.01	*Brevundimonas_diminuta*	0.06								
Propionibacterium_acnes	0.01	*Labrys_*	0.06								
Pantoea_agglomerans	0.01	*Allobaculum_*	0.05								
*Oxobacter_*	0.01	*Deinococcus_*	0.05								
		*Cupriavidus_*	0.05								
		*Nocardioides_*	0.05								
		Streptococcus_alactolyticus	0.05								
		*Novosphingobium_*	0.05								
		Butyricicoccus_pullicaecorum	0.05								

		*Gemmata_*	0.04								
		*Sporosarcina_*	0.04								
		*vadinCA02_*	0.04								
		*Dechloromonas_*	0.04								
		*Ancylobacter_*	0.04								
		Corynebacterium_kroppenstedtii	0.04								
		Victivallis_vadensis	0.04								
		*Paenibacillus_*	0.04								
		*A17_*	0.04								
		Acidopila_rosea	0.04								
		Ruminococcus_bromii	0.04								

		Bifidobacterium_pseudolongum	0.04								
		*Comamonas_*	0.03								
		*PD_*	0.03								
		*Mycobacterium_*	0.03								
		*Syntrophomonas_*	0.03								
		*Arthrobacter_*	0.03								
		*Thauera_*	0.03								
		Methylobacterium_komagatae	0.03								
		*Dietzia_*	0.02								
		*Cryocola_*	0.02								
		Bifidobacterium_animalis	0.02								

		*Sutterella_*	0.02								
		*Rhodoferax_*	0.02								
		*Fusobacterium_*	0.01								
		Thiobaca_trueperi	0.01								
		*Oxobacter_*	0.01								
		*rc4_*	0.01								
		*Phycicoccus_*	0.01								
		*Oleibacter_*	0.01								
		Clostridium_botulinum	0.01								
		*Paracoccus_*	0.01								
		*Megasphaera_*	0.01								

TOTAL	**99.98**		**99.96**		**100**		**100.1**		**100**		**99.99**