EFFECTS AND INTERACTIONS OF ANTIBIOTIC, BUTYRATE, AND TEMPERATURE ON SAILFIN MOLLY (*POECILIA LATIPINNA)* METABOLISM AND GUT MICROBIOTA

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ABSTRACT

Understanding the complex interactions between environmental factors, dietary supplements, and microbiome dynamics is crucial for optimizing fish health and growth, particularly in aquaculture settings. This study examines the effects of antibiotic, butyrate, and temperature treatments on the metabolism and gut microbiota of sailfin molly (*Poecilia latipinna*) alone and in combination. The results indicate that butyrate successfully restored the growth rates due to antibiotic exposure but had little effect when supplied to controls. Interestingly, while elevated temperature has been shown to promote growth through increased metabolic rates growth was not observed, possibly due to stress. Notably, there was a decrease in diversity in the microbiome because of antibiotic treatment. Further, both antibiotics and increased temperature lead to a decrease in the predicted functional capabilities of bacteria related to metabolism and health pathways after using PICRUSt analyses. While butyrate ameliorated the impacts on growth and metabolism caused by antibiotics, the microbiome was mostly unaffected, indicating that butyrate exerts its effects through the host directly. These findings suggest that butyrate could mitigate some negative impacts of antibiotics on fish growth and gut health as well as highlight some of the concerning effects of increasing environmental temperatures and antibiotic use. These insights may help develop strategies in fish population management and aquaculture practices, particularly those involving antibiotics and butyrate supplementation.

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ABBREVIATIONS

ANCOM	Analysis of Composition of Microbiomes
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
DNA	Deoxyribonucleic acid
gDNA	Genomic Deoxyribonucleic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG ORTHOLOGY
MMR	Maximum Metabolic Rate
MS-222	Tricane methanesulfonate
PCA	Principal Component Analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal RNA
SEM	Standard Error of the Mean
SMR	standard metabolic rate
STAMP	Statistical Analysis of Metagenomic Profile
16S	16S ribosomal RNA

CHAPTER 1: GENERAL BACKGROUND

1.1 The importance of fish and aquaculture

The study of fish is essential for several reasons. Primarily, fish exhibit a wide range of biological diversity, thus proving to be an excellent model system for understanding fundamental biological principles. Also, fish share some genetic proximity to other species, such as humans, which makes them valuable for comparative biology. Many medical studies often use zebrafish (*Danio rerio*) as experimental subjects to investigate pathways conserved between zebrafish and humans, such as cancer, neurological, and developmental research (Dudziak et al., 2022; Streisinger et al., 1986; Vargas et al., 2018). Additionally, fish are relatively easy to work with in laboratory settings due to their manageable sizes and life cycles. Furthermore, studying fish can enhance our knowledge of their roles in aquatic ecosystems, which is crucial for maintaining the health and sustainability of these environments (Muller, 2005), as fish help maintain the balance of aquatic environments by contributing to biodiversity and the overall health of the ecosystems (Angermeier, 2011). Finally, fish are vital for billions of people worldwide as fisheries and aquaculture stabilize many communities by providing a consistent food source and nutrients. Indeed, fish significantly contribute to global nutrition as they offer a stable source of food that is important for human health (Thisted et al., 2014).

Specifically, aquaculture has become a critical component of the global food supply. As wild fish stocks face overfishing and climate change pressures, aquaculture provides a sustainable alternative to meet the growing demand for fish (Thisted et al., 2014). The industry has experienced rapid growth over the past few decades, now supplying more than half of the fish consumed globally (Cressey, 2009). This controlled environment allows for better management of fish health and nutrition contributing to the sustainability of the food supply chain (Cressey,

2009). The growth of fish is critical in aquaculture for its overall success as the rate at which fish grow directly impacts the profits and the sustainability of aquaculture (Cressey, 2009). As such, understanding and optimizing factors that influence growth is a primary focus for aquaculture research and industry practices.

Importantly, the gut microbiome, metabolic rate, rearing temperature, and diet are all critical factors involved in fish growth and overall health (Rowland et al, 2018). Firstly, the microbiome, a complex community of microorganisms residing in the digestive tract, plays a significant role in many health functions and is important for the growth performance of fish species (Thibault et al., 2019). Secondly, metabolic rate, the amount of energy expended by an animal, also directly affects growth (Johnston and Dunn, 1987). Indeed, higher metabolic rates can indicate growth under optimal conditions, however, they may also increase the animal's sensitivity to environmental changes and stress (Johnston and Dunn, 1987). Thirdly, rearing temperature is crucial as it influences metabolic processes and enzymatic activities, with optimal temperatures promoting growth, while variations can lead to reduced growth rates and overall, a decrease in health (Clarke and Johnston, 2002). Finally, the diet provides the necessary nutrients and energy for growth, whereas a balanced diet ensures proper development, while nutritional deficiencies can stunt growth and overall health (Singh et al., 2017). Thus, understanding the relationship between the microbiome, metabolic rate, temperature, and diet is essential for improving the growth of fish species.

1.2 Gastrointestinal microbiota and fish growth

The gut microbiome contains diverse communities of microorganisms that reside within the digestive tract of animals (Thursby and Juge, 2017). These microorganisms consist primarily of bacteria (microbiota) that are crucial in maintaining host health and metabolism (Lee and Hase, 2014). Specifically, the gut microbiota are involved in physiological processes which include digestion, absorption, immune response, and signaling pathways (Krishnan et al., 2015) and the composition, function, and dynamics of these microorganisms in the gut can influence the host's overall health and are therefore essential to research (Ha et al., 2014).

The gut microbiota can be categorized based on their various functions as well as how they interact with their host. These bacterial groups can be classified into three categories which include commensal, mutualistic, and pathogenic bacteria (Hornef, 2015). Commensal bacteria are usually harmless and coexist within the digestive system and therefore neutral to the host (Hornef, 2015). They are somewhat beneficial as they can contribute to gut homeostasis as well as help compete for space against pathogenic bacteria (Hornef, 2015). Mutualistic bacteria, on the other hand, provide a beneficial relationship with the host (Hornef, 2015). The function of these bacteria is to aid in digestion and immune response while benefiting from the nutrients from the host (Hornef, 2015). Finally, there are pathogenic bacteria that have a negative relationship with the host and can be harmful as they can cause disease by disrupting the balance of the gut microbiota and can lead to infections and other gastrointestinal disorders (Ivnitski et al., 1999).

The composition of the gut microbiota can be influenced by various factors which include diet, the host's genetics, and environmental conditions (Hasan and Yang., 2019). In fact, temperature is a critical environmental factor that can dramatically influence the composition and

function of gut microbiota (Dehler et al., 2017). Changes in water temperature can lead to shifts in microbial populations, affecting the overall balance within the gut (Dehler et al., 2017). These temperature changes potentially favor the growth of certain bacteria that can have impact on digestion and immune function (Sepulveda and Moeller, 2020). Additionally, these conditions may also promote the proliferation of pathogenic bacteria, increasing the risk of infections and disrupting the microbial equilibrium (Nogales et al., 2011). Therefore, maintaining an optimal temperature is essential for sustaining a healthy and balanced gut microbiota structure.

Each fish species, as well as each individual within a species, possesses a unique microbial makeup (Rossum et al., 2020). The microbiome is under intense selection pressures as the host immune response plays a critical role in selecting and maintaining specific microbial communities (Henry et al., 2021). Overall, the selected composition of the microbiome helps maintain homeostasis by favoring beneficial bacteria that will promote the health and growth of the host while reducing the presence of pathogens (Henry et al., 2021). Additionally, the microbiome is responsible for many complex processes such as breaking down carbohydrates, fibers, and other nutrients that the host normally would not be able to digest (Frame et al., 2020). This allows the production of more nutrients for the host but can also assist in facilitating nutrient absorption which can lead to increased growth of the host (Frame et al., 2020). Understanding the selected composition, as well as the environmental conditions that influence the microbiome, is important for maintaining the health and well-being of fish populations to limit disease and promote growth which can also be a valuable resource for wild and captured fish.

1.2.1 Antibiotics and dysbiosis within the gut microbiome

Antibiotics are widely used in aquaculture, usually as prophylactics, to prevent and treat bacterial infections which are common in highly stocked farms. There are many benefits to their use such as improved survival rates, in some cases increased growth, and overall increased yield (Gaskins et al., 2006). Most importantly, by controlling disease risk within populations of fish this helps maintain the health of the fish and supports the viability of large stocking aquaculture enterprises (Bondad-Reantaso et al, 2023). In aquaculture, the antibiotics can be administered through feed, in the water, or injections.

However, there are many drawbacks to the use of antibiotics in aquaculture. A major concern in its use is the chance to develop antibiotic-resistant bacteria within these fish populations (Hossain et al., 2022). This can lead to a decrease in the effectiveness of treating fish diseases but can also be a huge health risk for humans (Hossain et al., 2022). Antibiotic-resistant bacteria can be transferred to humans after the consumption of a fish that has been infected (Guo et al., 2020). Another concern is for the overall health of the fish with prolonged antibiotic exposures. Inconsistent and long-term antibiotic exposures can lead to dysbiosis, a state of imbalance in the microbial communities (Qian et al., 2021; Figure 1.1). Indeed, when antibiotics are administered, it is impossible to direct the effects between pathogenic and beneficial bacteria which can have undesired impacts on the composition of the microbiome, such as a reduction in microbial diversity (Kriss et al., 2018) or the proliferation of an undesired bacteria species (Vincent et al., 2019). Usually, a diverse microbiome is considered healthy as it can play a wider range of functions that promote the host's health. Therefore, a reduction in this diversity can impair functions such as digestive and absorption pathways and lead to decreased growth rates (Limbu

et al., 2020; Zhou et al., 2018). This dysbiosis can lead to vulnerability in the health and growth of many fish which can offset the initial benefits of antibiotic use in the first place.

The environmental impact of antibiotics is also another major concern in aquaculture. Antibiotic use within fish stocking sites can be released into the surrounding water and contaminate surrounding ecosystems (Han, 2020). This can further lead to broader problems of antibiotic-resistant bacteria and impact the health and fitness of other animals (Han, 2020). Understanding and mitigating the impacts of antibiotics is essential for global health and aquaculture farming practices to ensure the well-being of fish populations. It is also important for us to continue to explore the interactions of antibiotics and temperature to prevent future challenges that might occur in aquaculture with shift global temperatures.

1.3 Metabolic rate and its effect on growth and the gut microbiome

Metabolic rate, or the amount of energy an organism uses over a period of time can be measured in several ways. Firstly, it can be examined at the rate at which an organism performs basic life-sustaining functions (known as standard metabolic rate; SMR). Conversely, metabolic rates can also be examined when animals are expending as much energy as they can sustain (maximum metabolic rate; MMR). The metabolic rate of a fish provides insights into its physiology, behavior, and ecological role. For example, fish with higher metabolic rates tend to be more active and may require more energy to sustain movement and foraging. Predatory or fast-swimming species (like tuna) often have higher metabolic rates compared to more sedentary species (like bottom-dwelling fish). Further, metabolic rate is influenced by the surrounding environment, particularly temperature. Fish in warmer waters generally have higher metabolic

rates than those in colder waters, as metabolic processes speed up with temperature in ectotherms such as fish. Further, fish with higher metabolic rates must consume more food to meet their energy needs. This can affect their feeding behavior and growth rates.

Metabolic rate can directly affect the growth rates of animals by determining how quickly they use energy for bodily functions. A higher standard metabolic rate increases the demand for energy processes such as digestion and cellular activity (Pettersen et al., 2018). Conversely, a lower standard metabolic rate potentially allows more energy to be used for growth (Pettersen et al., 2018). This is because growth involves energy-intensive processes like cell division and protein synthesis which is regulated by metabolic activity. The relationship between SMR and MMR is important for energy allocation and growth. To explain, an organism with a higher MMR can reach higher energy outputs, allowing it to sustain intense activities like foraging or escaping predators. However, if SMR is high relative to MMR, a significant portion of energy is used for stress and maintenance, allocating less energy available for growth. Overall, metabolic rate plays a key role in balancing energy use between maintenance and growth. (Austad, 2009)

Metabolic rate also plays a crucial role in shaping the gut microbiome (Lindsay et al., 2020), which could present a possible mechanism for the relationship between growth and metabolic rate. A higher metabolic rate in fish increases the demand for energy and nutrients, altering the gut environment and in turn the microbiota (Lindsay et al., 2020). As the metabolic rate rises, the consumption of available nutrients can shift the balance of microbial species (Lindsay et al., 2020). Certain microbes that can quickly adapt to the changing availability of nutrients may become more dominant, while others may struggle to survive (Astbury et al., 2015). This shift can affect the overall diversity and stability of the gut microbiome which can have potential

effects on overall function. The relationship between metabolic rate and the gut microbiome is dynamic. A balanced metabolic rate helps maintain a diverse and stable microbiome, which is essential for optimal growth and health in fish (Lindsay et al., 2020). However, fluctuations in metabolic rate, whether due to environmental factors, temperature changes, or stress, can disrupt this balance (Martin et al., 2019). Thus, metabolic rate significantly influences the gut microbiome by affecting nutrient availability and microbial balance (Martin et al., 2019). Maintaining a stable metabolic rate is key to supporting a balanced gut microbiome, which is vital for fish's overall health and growth.

1.4 The effects of temperature on fish growth and the gut microbiome

Temperature is an environmental variable that can influence the physiology and behavior of fish (Reynolds and Casterlin, 1980). Fish are ectotherms, meaning they are more sensitive to changes in the temperature of the water as changes have a major influence over their metabolic rates and bodily functions (Johnston and Dunn, 1987). As climate change causes global increases in water temperatures we need to understand and predict the impact this has on fish populations.

As mentioned, one of the most significant impacts of temperature is on the metabolic rates of fish, which can influence the growth rates (Clarke and Johnston, 2002). With increased water temperatures, there is a direct increase in metabolic rates in fish which leads to increased nutrient demand to maintain homeostasis and grow (Clarke and Johnston, 2002). Fish body temperatures demand a specific range for optimal growth and function (Lindmark et al., 2022). Outside these optimal ranges fish can experience a reduction in growth, increased stress, and even mortality (Lindmark et al., 2022). Additionally, changing water temperatures can affect the availability of oxygen in the water (Diaz and Breitburg, 2009). Increased water temperatures cause less

dissolved oxygen and can create a hypoxic environment which can increase stress (Diaz and Breitburg, 2009). At higher temperatures where fish demand more oxygen consumption to maintain physiological processes, it can lead to poor metabolic rates needed to sustain the growth of the fish (Diaz and Breitburg, 2009).

The gut microbiome composition and activity can also be directly influenced by changes in water temperature (Xavier et al., 2023). Microbiota composition in the gut can be altered by changes in temperature causing possible changes in the function of the microbiome (Xavier et al., 2023). For example, when temperatures shift, it can favor the growth of heat-tolerant bacterial species and suppress the functions of other microbiota (Li et al., 2022). These shifts can alter the composition of beneficial and harmful pathogens which can have major impacts on health and growth. Additionally, these changes in communities of bacteria can potentially lead to dysbiosis and can affect many functions of the microbiome (Xavier et al., 2023).

1.5 Impact of diet and dietary supplements on growth and the microbiome

The specific diet of any species is important for promoting health, growth, and even the sustainability of the microbiome (Voreades et al., 2014). Although each species differs in the nutrients of their diets, they all require a balanced diet containing proteins, lipids, carbohydrates, and vitamins (Singh et al., 2017). In many cases where the host cannot break down certain diet specific bacteria within the gut assist in the digestion of those feeds (Nayak, 2010). The ability to have a diverse and stable gut microbiome is essential in many cases for efficient digestion and nutrient absorption (Nayak, 2010).

Additionally, to promote metabolism, a balanced diet is important for immune response and stress prevention (Voreades et al., 2014). In aquaculture with high population densities of fish they must be fed a sustainable diet that meets the needs to promote growth and health (Hoseinifar et al., 2019). Dietary additives to feeds, such as probiotics, prebiotics, and postbiotics, have been used to increase growth and produce a beneficial microbiome (Hoseinifar et al., 2019). In total, the use of these additives leads to a balanced diet and has been shown to increase growth rates and survival rates which are both key components of sustainable aquaculture production (Hoseinifar et al., 2019).

1.5.1 Role of butyrate

Short-chained fatty acids (SCFA) are crucial metabolites that are produced through anaerobic fermentation of dietary fibers by the gut microbiota. There are three primary SCFAs which consist of acetate, propionate, and butyrate. These SCFAs are involved in many biological pathways and are an important source of energy for the host. Additionally, butyrate has become a common additive to many farming feeds due to its many health benefits (Cook and Sellin, 2001).

Butyrate, though being one of the least produced SCFAs, plays one of the most significant roles in maintaining gut health by being the primary energy source for colonocytes (Robles et al, 2013; Figure 1.2). Additionally, it is involved in many other biological processes that have been shown to promote health in animals including modulating immune responses (Siddiqui and Cresci, 2021). Some studies have shown it to promote health by suppressing inflammatory pathways in the gut that could cause impaired gut health and lead to decreased nutrient absorption (Siddiqui and Cresci, 2021).

Despite butyrate being known to have many positive benefits there is still much to be learned in its impact on the microbiome and host and specifically in aquaculture. Research is needed to look at how butyrate operates either through the microbiome and host as well as in different species, environmental temperatures, with other additives, and overall, how it influences the microbiome of the animal. Butyrate has been known to be important for the development of beneficial feeds as it is effective at increasing animal growth, which is important for aquaculture (Cook and Sellin, 2001).

1.6 Thesis rationale

The use of antibiotics in aquaculture, which is beneficial in many ways for preventing disease, can lead to many adverse effects such as dysbiosis (Qian et al., 2021). The imbalance in the microbiome can lead to reduced growth (Qian et al., 2021) although the exact mechanism is still unclear. Further, butyrate has been shown to promote health in many animal species as well as in fish and has shown to be a possible solution to counter reduced growth (Hany et al., 2020), although again the mechanism of action (either through the host or the microbiome) is unclear. Additionally, although antibiotics and butyrate have been studied independently within some fish species it has not been looked at in conjunction and requires further research. Finally, temperature is another critical environmental factor that is important for study as we progress through climate change due to its effect on metabolism, growth, and immune response within fish (Reynolds and Casterlin, 1980). With increasing temperatures, it is important to understand how shifting energy demands change the physiology of the fish as well as the microbiome that resides within them (Killen, 2014; Xavier et al., 2023). Additionally, at higher temperatures, fish experience higher metabolic rates, but it is unclear how these changes will affect the interactions

with long-term antibiotic use and butyrate supplementation and overall, the growth of the animals. Overall, using possible supplements such as butyrate is critical for developing and sustaining aquaculture practices in a changing world.

The sailfin molly (*Poecilia latipinna*) is the species chosen for this research due to them being eurythermal, meaning they can survive a wider range of temperatures. As such, this species makes an excellent model to explore the change in temperature. Sailfin molly is also herbivorous meaning that most of their diet is fiber-based making them an ideal species as they possibly rely on their microbiome for digestive functions and butyrate production. This information could be applied to majorly farmed fish such as the tilapia that share many similar traits (Alkahem and Ahmad 2007). These similar traits include both living in tropical environments and being herbivorous.

1.7 Study objectives

The main objective of this study was to observe how the herbivorous poikilotherm sailfin molly changes growth and metabolism when given butyrate as an additive as well as antibiotics. We further applied increased temperature as multistressors (e.g. antibiotics and temperature together) are frequently encountered by animals in aquaculture (and nature). Additionally, by using metagenomic sequencing we can identify changes in microbiota compositions and investigate which microbial communities butyrate, antibiotics, or temperature are directly affecting. By identifying bacterial community changes between treatments, we can identify

potential microbial functional changes that the microbiome contributes to and link to host health, growth, metabolism, and other biological processes.

By understanding the interactions between butyrate and antibiotics the current study seeks to identify possible synergistic or antagonistic interactions that may affect the microbiome and health of the fish (resulting in changes in growth). Additionally, by looking at the physiological changes as well as the changes to the microbiota we can investigate how butyrate operates throughout the intestine. This may develop future strategies to mitigate the negative impact of antibiotic use and reduce dysbiosis as well as promote growth. Finally, investigating antibiotic properties at higher environmental temperatures is important to understand the changes in metabolic needs to maintain homeostasis and give us an understanding of how increased water temperature due to climate change can affect these interactions with the host. Ultimately, this research can contribute to improving aquaculture where both treatments can be used in conjunction to develop healthier and more sustainable farming operations in a world with increasing water temperatures.

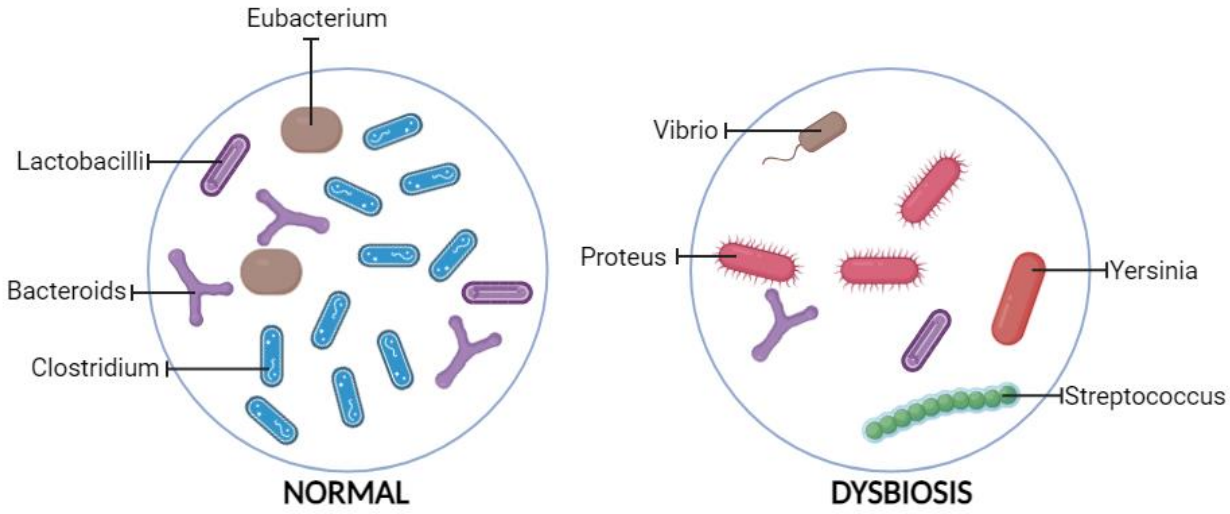


Figure 1.1: Comparison of bacterial communities in normal conditions versus dysbiosis. Dysbiosis is characterized as an imbalance in the gut microbiota composition. Key changes include a reduction in beneficial bacteria and an increase in pathogenic bacteria. (Made with BioRender)

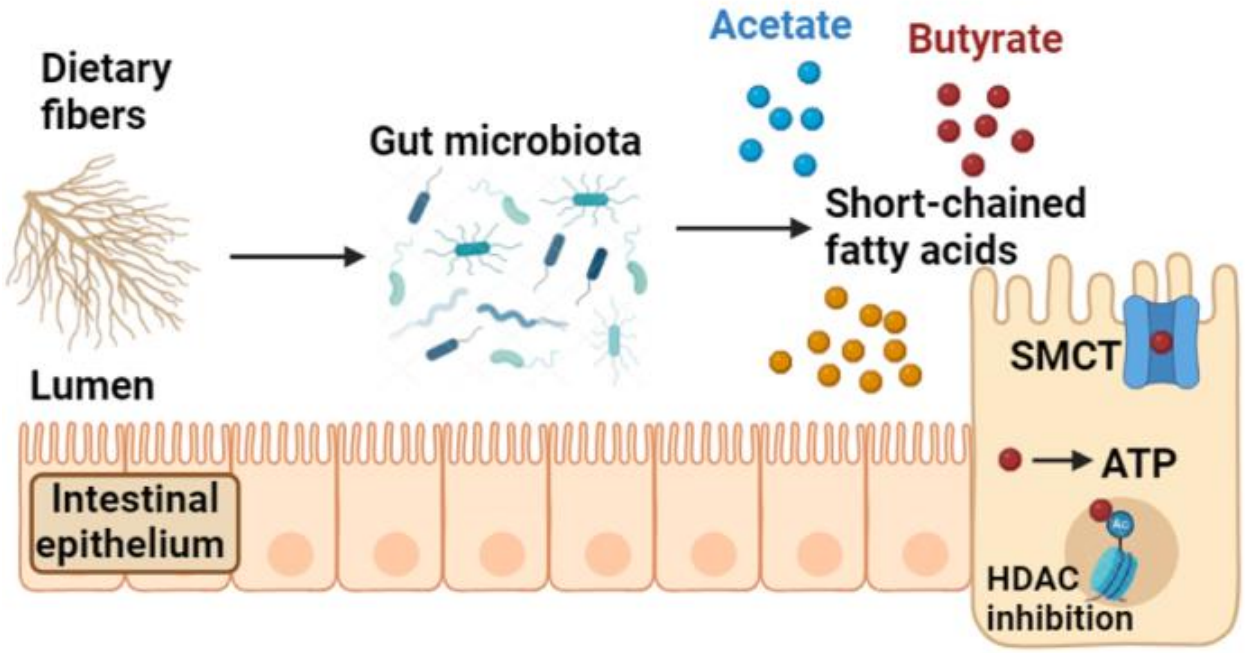


Figure 1.2: Production and function of butyrate and other SCFAs in the gut microbiome. The microbial fermentation of dietary fibers in the gut by microbiota leads to the production of butyrate and other SCFAs. Butyrate is primarily utilized by colonocytes for energy production. In addition to this primary role, butyrate also functions as a histone deacetylase (HDAC) inhibitor, modulating gene expression, and can be transported into cells via sodium-dependent monocarboxylate transporter (SMCT), aiding in its absorption. (Made with BioRender)

CHAPTER 2: PHYSIOLOGICAL RESPONSES OF SAILFIN MOLLY TO BUTYRATE, ANTIBIOTICS, AND TEMPERATURE

2.1 INTRODUCTION

2.1.1 Growth and metabolism

Growth and metabolic rates within fish can be influenced by a variety of factors. This includes their diet, environmental conditions, and importantly their microbiome (Yukgehnaish et al., 2020; Walburn et al., 2018). Understanding these influences is beneficial in predicting the physiological response to these conditions in both natural and aquaculture settings.

Diet is one of the more critical factors that affect growth and metabolism. The quality and composition of the diet are important for sustaining and promoting growth (Kong et al., 2020). Proteins, lipids, and carbohydrates are all important (in an appropriate ratio) to provide the nutrients to sustain regular physiological processes such as tissue growth, tissue repair, hormone production, and overall to provide energy to the host (Ali and Al-Asgah, 2001). Supplementation of nutrients within feeds can also play a role in promoting growth and metabolism (Ali and Al-Asgah, 2001). Many supplements can provide extra nutrients that would not otherwise be received and can be utilized by either the gut microbiota or the host directly (Lyons et al., 2016).

The microbiota is important in aiding the digestion of dietary components, synthesizing essential nutrients, and mediating immune responses (Dawood, 2020). A balanced and rich gut microbiome can increase assimilation efficiency which can in turn promote the growth and metabolism of the host (Thibault et al., 2019). Prebiotics, probiotics, and postbiotics can all help promote the selection of beneficial bacteria while antibiotics can disturb the gut microbiota and can impair many functions within the microbiome (Kim et al., 2019; Panitsidis et al., 2023).

The impacts of diet, environmental factors, and/or gut microbiota on the metabolic rates of fish can present by altering specific metabolic measurements such as Standard Metabolic Rate (SMR), Maximum Metabolic Rate (MMR), and/or Aerobic Scope. SMR represents an animal's basal metabolic rate at rest, reflecting the minimal energy necessary to maintain basic physiological function (Chabot et al., 2016). This measure is crucial for understanding fish's energy requirements under non-stressful conditions and provides insights into how efficiently an animal can sustain itself with minimal energy (Chabot et al., 2016). MMR, on the other hand, shows the peak metabolic rate that a fish can reach during periods of maximum energy output, such as escaping predators or other intense activity (Kasihmuddin et al., 2024). This measurement is important because it shows the upper limit of an animal's metabolic ability and its capacity for intense activity under extreme conditions. Aerobic scope, the difference between SMR and MMR, measures the total energy available for an animal's activities (Eliason and Farrell, 2016). In terms of aerobic scope, the principle of allocation says organisms must balance energy or trade-off across competing functions such as selecting energy for survival and rapid movement or energy for growth, affecting both SMR and MMR (Jutfel et al., 2021). Larger aerobic scopes allow for a larger range of energy allocation towards growth and metabolism while still allowing for rapid shifts in energy expenditure for things such as movement (Jutfel et al., 2021). Conversely, narrow aerobic scopes may decrease energy allocation for either of these rapid energy expenditure pathways, growth, and in some cases both (Eliason and Farrel, 2016). These measurements are vital in aquaculture, as they help predict how fish populations may respond to varying conditions and stressors in their natural habitats or controlled environments.

2.1.2 Effect of butyrate on growth and metabolic rates

Butyrate has been shown to enhance growth in many animals, one of many possible explanations for this is by providing energy to the colonocytes that line the intestinal wall (Ahmad, 2000). This additional energy is used to support the maintenance and health of the intestine to promote gut integrity and function (Ahmad, 2000). This allows for improved nutrient absorption which increases growth and metabolic efficiency (Makowski et al., 2022). Most research conducted on butyrate as a supplement in animals has been mostly on terrestrial animals while some more recent work has shown that butyrate can provide the same health effects in fish (Robles et al., 2013). More recent studies looking at butyrate within the feed of various fish species have significantly increased growth and feed conversion ratio (Robles et al., 2013). For instance, research that looked at the rainbow trout (*Oncorhynchus mykiss*) has shown that supplementation improved the efficiency of feed conversion (Gao et al., 2011). Similar results have been observed in the Atlantic salmon (*Salmo salar*) showing that butyrate has broad applicability as a growth-promoting additive (Bjerkeng et al., 2001).

Other research using butyrate in fish has demonstrated that butyrate supplementation has many secondary effects such as promoting immune response (Fang et al., 2021). Chronic intestinal inflammation in fish is common and leads to energy being used towards fighting off disease and repair of the intestine which impacts growth rates (Dawood, 2020). Butyrate has been shown to reduce intestinal inflammation which promotes digestive functions for optimal growth (Estensoro et al., 2016).

Butyrate may also affect the composition of the gut microbiota which can influence the growth and nutrient metabolism of the host (Laserna-Mendieta et al., 2018). Butyrate and the microbiota create a positive feedback loop that promotes these functions for increased growth and feed conversion (Zhao et al., 2018). However, not much is known about the specific

interactions between butyrate and the microbiota within fish species. We need to understand how alterations in microbiome composition from butyrate alter the function of the microbiome as well as how butyrate is affecting host growth pathways. Further fish research is required for us to understand the connection between butyrate and the microbiota.

2.1.3 Impact of antibiotics on growth and metabolic rates

Antibiotics are commonly used in aquaculture to prevent and treat bacterial infections. This treatment has an impact on the growth and metabolism of fish and has both positive and negative effects. Firstly, the ability to control bacterial diseases is important for the sustainability of aquaculture to keep up with the demand for fish in highly stocked facilities. The reduction in mortality is an overall improvement to a sustainable farm as it has been shown to lead to improved yields and in some cases increased growth. (Chen et al., 2020).

Despite the major upside of antibiotics, there are still many negative factors associated with their long-term use (Limbu et al., 2018). Research has shown that antibiotics can directly influence metabolic processes in fish (Limbu et al., 2020). Directly, antibiotics can interfere with metabolic pathways causing lower feed conversion and decreased growth rates (Limbu et al., 2020). Indirectly, the antibiotics can alter the gut microbiota which can cause dysbiosis and lead to loss of function in many metabolic processes (Kim et al., 2019). Overall, research has shown that antibiotic use in some cases may cause more harm than good (Limbu et al., 2018). Regarding antibiotic residues that are left in the water, they can enter aquatic ecosystems (Limbu et al., 2020). These free-roaming antibiotics can alter the microbial communities in the environment and can lead to broader ecological impacts that can be disruptive (Want et al.,

2020). While the use of antibiotics in aquaculture can be beneficial, we need to understand its effects on growth and metabolism in long-term conditions.

2.1.4 Influence of temperature on growth and metabolic rates

As mentioned, temperature plays a direct role in the growth of fish species as temperature has a direct influence on metabolic rate (Lindmark et al., 2022) and many cellular processes. Indeed, at higher temperatures, more ATP is required to fuel processes which leads to higher cellular kinetic energy and metabolic rates (Sun et al., 2019). Fish species have an optimal temperature range where growth and metabolism are at their most efficient (Lindmark et al., 2022). Variations from their optimal temperature range can lead to stress, reduced feed consumption, and reduced growth (Maderia et al., 2013). At higher temperatures, metabolic rates can increase and lead to faster growth but also cause higher energy demands and thermal stress (Maderia et al., 2013).

Temperature is a fundamental factor for influencing growth in fish species. Maintaining optimal ranges for growth is essential for maximizing growth rates and overall health. With rising global temperatures, we need to understand its influence on the growth and metabolism of farmed fish for aquaculture sustainability.

2.1.5 Hypotheses

The usage of butyrate and antibiotics have been previously researched independently but the interactions between the two are still yet to be studied. Indeed, this prior research has shown that in some cases, butyrate or antibiotics have opposing effects, therefore, it is beneficial for us to

understand at the physiological level how they influence fish species. Additionally, it is beneficial for us to understand how butyrate influences the host either directly from the microbiota or directly through the host machinery. Also, by increasing the temperatures of these treatments, we could potentially see how butyrate and antibiotics operate under different environmental conditions.

It was hypothesized that providing butyrate within the diet of the sailfin molly would increase their growth as this has been seen in other fish species (Robles et al., 2013). Additionally, it was predicted if the growth increased then there would be a corresponding decrease in SMR which would provide more energy for growth within principals of allocation (as basal metabolic activities would consume less energy). For MMR, we would also predict it would decrease due to the predicted decrease in SMR which is the floor for metabolic rate. Aerobic scope is thus predicted to be maintained as SMR and MMR decrease. It was also hypothesized that the long-term use of antibiotics would lead to a decrease in growth rate by negatively impacting functions of the microbiome, such as nutrient acquisition. As some studies have shown long-term exposure to antibiotics can have adverse effects on growth and intestinal health, we predicted that the metabolic rates would be increased in this treatment (Limbu et al., 2020). Specifically, SMR and MMR would both be increased as the energetic demand for basal activities is being increased and taken away from growth pathways and instead allocated for maximum metabolic capacities or possible stress-related responses. Additionally, it was predicted that aerobic scope would remain the same as the MMR would increase the same amount as SMR as the energy allocation for growth shifts to other metabolic pathways needed for survival. Furthermore, it was hypothesized that these effects would be increased at higher temperatures as increased metabolic rates would allow for greater growth from increased metabolic rates and

nutrient breakdown. Finally, it was hypothesized that the combination of butyrate and antibiotics would promote growth and lower metabolic rates which would counter the adverse effects of long-term antibiotic use.

2.2 MATERIALS AND METHODS

All experiments and animal care were carried out under an approved Animal Use Protocol from York University (R2017-14).

2.2.1 Obtaining and housing of experimental animals

Freshwater acclimated sailfin mollies were purchased from Fish and Bird Emporium (Innisfil, Ontario) and transported to York University (Toronto, Ontario). Upon arrival at York University, fish were examined for any illnesses and separated into 37.85 L (10-gallon) aquarium tanks (Aqueon; Franklin, Wisconsin) with 15 fish in each tank, making sure only to select males to avoid reproductive cycles that may occur from the use of females. All fish were acclimated to laboratory conditions for 2 weeks before experimentation. Each tank contained a sponge filter with airflow and a water heater set to 23ºC. Ammonia, nitrite, nitrate, and pH levels were monitored frequently using the Freshwater Aquarium Master Test Kit (API®; Chalfont, Pennsylvania). Water changes and tank cleaning were conducted daily to ensure clean tank conditions. All fish were fed 1mm pellets of Premium Fish food – Veggie Formula (NorthFin™; Toronto, Ontario). These fish were fed once daily at approximately 11:00 AM. Finally, fish were placed in a day-to-night light cycle with 14 hours of light and 10 hours of dark to promote normal circadian rhythm.

2.2.2 Preparation of fish feeds

Store-bought Premium Fish food – Veggie Formula (NorthFin™; Toronto, Ontario) was ground into a fine powder using a pepper grinder. The ground fish food powder was then measured into 10-gram portions using an analytical balance. For the butyrate feed portions, 0.1

grams of sodium butyrate (Thermofisher Scientific, Whitby, Ontario) (equivalent to 1% of the total feed weight) was added to each 10-gram portion of the groundfish food powder. This amount was selected from previous studies that showed 1% sodium butyrate showed increased growth in fish (El-Sharkawy et al., 2023). Nothing was added to the control food powders. Both the control and butyrate-enhanced feed powders were then thoroughly mixed with 20 mL of distilled water to ensure homogeneity. Each feed mixture was then placed in a syringe and squeezed out in lines onto tin foil sheets. The feeds were placed in a preheated oven at 90°C (194°F) and dried for at least 1 hour, or until all moisture was removed. Once dried, the feed was removed from the oven, broken apart into small pellet-like shapes, and stored in marked containers in a refrigerator set at 4°C until used. New feed was produced weekly to ensure freshness.

During diet trials, fish feed was placed into tubes measured to 5% of the total body mass of all fish in each tank. For the antibiotic-treated group, four broad-spectrum antibacterial antibiotics were added to each tube with the dry pellets. First, 0.1 mL of ampicillin (100 mg/mL) (ampicillin solution; Millipore Corp., an affiliate of Merck KGaA, St. Louis, Missouri), 0.1 g of gentamycin (gentamycin sulfate; Fisher BioReagents, Pittsburgh, Pennsylvania), 0.5 mL of penicillin (100 U/mL) (penicillin-streptomycin solution; HyClone, a subsidiary of Cytiva, Marlborough, Massachusetts) and 0.5 mL of kanamycin(50 mg/mL) (kanamycin mono sulfate; TheromoScientific, Whitby, Ontario).

2.2.3 Diet trial

For the diet trial, male fish (n=4) were selected randomly from the main populations and placed in 9 L (2.3 gallon) multistressor tanks (Aquabiotech; Coaticook, Quebec) based on treatment groups (Figure 2.1). Two tanks per treatment (n=8 total) were placed in 57-liter (15-gallon) Salma storage boxes (Ikea, Toronto). The storage boxes were filled with 25 liters of dechlorinated water, and water pumps (Domica mini pump; Amazon, Canada) were used to ensure water flows into the multistressor tanks. Heaters were added to the storage boxes and set to either 23°C or 30°C, depending on the treatment group. Additionally, air lines connected to air stones were placed in each multistressor tank to maintain aeration. All fish were initially fed the re-pelleted control feed for one week before starting the treatments. There were eight treatment groups consisting of control, butyrate, antibiotics, and antibiotic plus butyrate, at either 23°C or 30°C. After acclimation, the fish were fed the appropriate diets at 5% of their body mass once daily around 11:00 AM for a total of six weeks. During the trial, ammonia, nitrite, nitrate, and pH levels in the tank water were monitored, and daily water changes were performed to maintain water quality. The entire experiment was then repeated to ensure adequate sample sizes. Weight measurements were conducted on day 0, and the experiment was to confirm changes in total body mass. To keep track of each fish, pictures of their unique scale colorations were taken and recorded with tank numbers.

2.2.4 Respirometry

After at least four weeks into the diet trials, fish were randomly selected for respirometry measurements. The autoresp v 2.3.0 software (Loligo Systems; Viborg, Denmark) was used for recording data, while the Witrox 4 device (Loligo Systems; Viborg, Denmark), equipped with oxygen and temperature sensors, was utilized for measurements. Fish were immediately weighed

before being placed into the chambers to allow for mass-specific respirometry calculations. The intermittent respirometry was conducted for 24 hours, with mass-specific mO_2 measured as mO_2 kg^{-1} hr^{-1}. The intermittent respirometry cycles were set to 5 minutes of closed measurement, 3 minutes of open flushing phase to reset oxygen levels in the system, and a 1-minute wait phase. The circulating system included a 250 mL glass resting chamber (Core Resting Chamber; Loligo Systems) connected to tubing that contained 100 mL of water with a connected oxygen probe, with a flushing and recirculating pump (Figure 2.2). This system was placed in a 130-liter Salma storage box (Ikea, Canada) which also contained a heater set to either 23°C or 30°C, depending on the treatment group. Continuous airflow was provided to the water via an airline. Before the start of the respirometry cycles, fish were hand-chased for 3 minutes and exposed to oxygen for 1 minute to stimulate maximum oxygen consumption. After each experiment, systems were run for several cycles to calculate the metabolic rate of bacterial build-up over the 24 hours to be deducted from the total metabolic rates. The system was cleaned using 70% ethanol flush through the system to remove bacteria between treatments. Metabolic rate was calculated using equation A. The highest three raw metabolic rate values within the first hour of respirometry were averaged and used to determine the maximum metabolic rate, while the lowest six values within the entire respirometry session were averaged and used to determine the standard metabolic rate. The difference between the maximum metabolic rate subtracted from the standard metabolic rate was used to calculate the aerobic scope.

(A):

$$\boldsymbol{Metabolic\ Rate} = \frac{\boldsymbol{\Delta O_2 \times V_w}}{\boldsymbol{M \times t}}$$

Where $\boldsymbol{\Delta O_2}$ the change in oxygen concentration in the water in the system from the start to the end of the cycle (mO_2/L), $\mathbf{V_w}$ is the volume of water in the respirometer chambers and tubes (L), **M** is the mass of the fish (kg), and **t** is the duration of time which the oxygen consumption is measured (hour).

2.2.5 Statistical analysis

All raw data was transferred into GraphPad Prism (version 10, San Diego, California). All statistical analyses were conducted using GraphPad Prism Software, which is appropriately described within their respective table and figure captions. All data was through analyzed with a two-way ANOVA, based on treatment and temperature, to determine interaction effects. Only the main effects were found to be significant with no interaction from all data sets so Uncorrected Fisher's LSD multiple comparisons were chosen as post-hoc tests. Statistically significant differences were only assumed if $p<0.05$. All graphs were created using GraphPad Prism and the bars represent column means ± standard error of the mean (SEM).

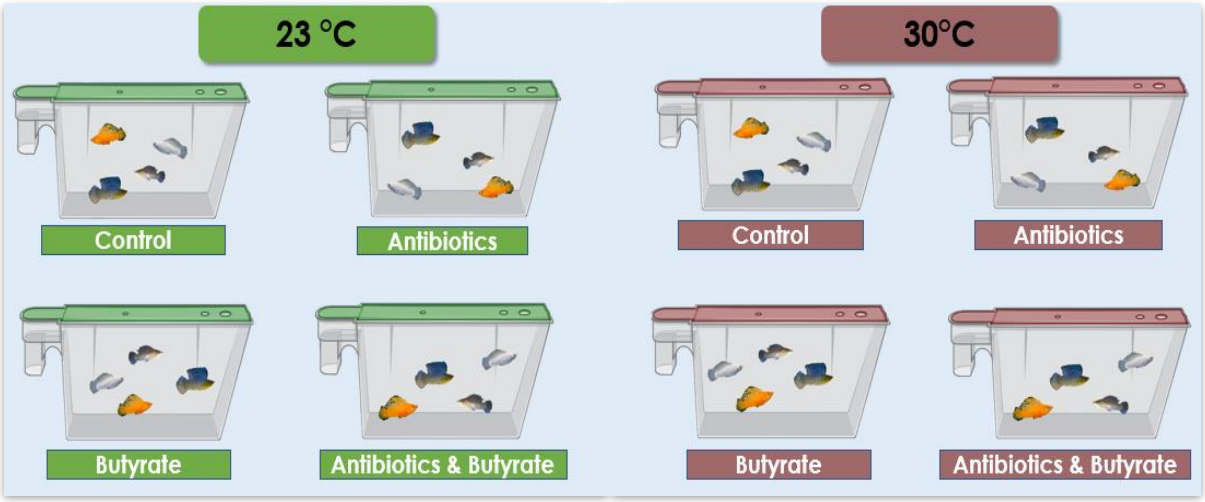


Figure 2.1: Diagram of Diet trial tank setup with sailfin molly split between control, butyrate, antibiotics, and butyrate plus antibiotics groups at 23°C or 30°C (*n*=8). (Made with BioRender)

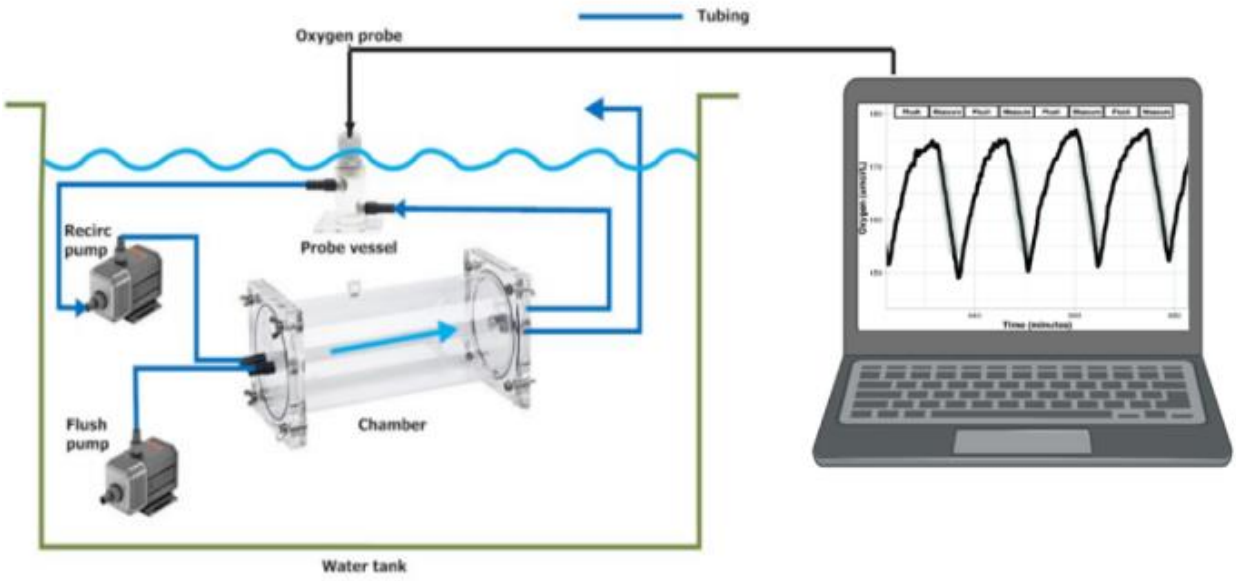


Figure 2.2: Diagram of intermittent respirometry setup using Loligo Systems chambers and software. Intermittent respirometry chamber with primary components and connections. Respirometry chambers are connected to tubing for circulating water which are connected to pumps for flushing and recirculating. The oxygen probe records the reading and transfers it to the computer. (Made with BioRender)

2.3 RESULTS

2.3.1 Growth

At 23°C, the control group gained an average mass of 0.6 ± 0.2 g ($p < 0.05$; Figure 2.3) throughout the experiment. In contrast, the butyrate group exhibited the highest mass gain, averaging 1.2 ± 0.15 g, which is approximately a 100% increase in growth compared to the control group ($p < 0.05$; Figure 2.3). Interestingly, the antibiotics group had a significantly lower mass weight gain of 0.23 ± 0.11 g, only gaining about one-third of the mass compared to the control group ($p < 0.05$; Figure 2.3). Finally, the antibiotic plus butyrate group showed a moderate mass gain of 0.58 ± 0.2 g, which was significantly less than the butyrate group but higher than the antibiotics group ($p < 0.05$; Figure 2.3).

At 30°C, the pattern of mass change across the treatments was slightly different than observed at 23°C. Similar to 23°C, the antibiotics group gain of 0.06 ± 0.13 g ($p > 0.05$; Figure 2.3) throughout the experiment. Additionally, the antibiotic plus butyrate group had a mass gain of 0.6 ± 0.17 g, significantly higher than the antibiotics group ($p < 0.05$; Figure 2.3) as seen in the 23°C groups. In contrast to the 23°C groups, the 30°C control group had similar mass gain compared to the 23°C treatment (0.26 ± 0.07 g; $p > 0.05$; Figure 2.3). Additionally, the butyrate group showed similar growth compared to both the control and antibiotic groups at 0.39 ± 0.07 g ($p > 0.05$; Figure 2.3). Further, some fish showed a decrease in total body mass in this treatment. Comparing treatments across temperature the butyrate group was shown to be the only group to be significantly decreased ($p < 0.05$; Figure 2.3).

2.3.2 Maximum metabolic rate

At 23°C, the control group had an MMR of approximately 1508 ± 70 mO_2 kg^{-1} hr^{-1} (Figure 2.4). Interestingly, the butyrate group showed a significantly lower MMR, averaging around 1031 ± 100 mO_2 kg^{-1} hr^{-1} compared to the controls ($p < 0.05$; Figure 2.4). Similar to the control group, the antibiotics group had an MMR of about 1276 ± 121 mO_2 kg^{-1} hr^{-1}($p < 0.05$; Figure 2.4). Finally, the antibiotic plus butyrate group showed an MMR like the butyrate group, with a significant decrease to 1297 ± 55 mO_2 kg^{-1} hr^{-1} when compared to the control group and antibiotic group ($p < 0.05$; Figure 2.4). These results suggest that butyrate supplementation alone, and when applied with antibiotics, significantly alter MMR at 23°C.

At 30°C, the MMR values increased across all groups compared to 23°C ($p < 0.05$), except when comparing the butyrate plus antibiotic groups ($p > 0.05$; Figure 2.4). Firstly, the control group had an MMR of around 2181 ± 186 mO_2 kg^{-1} hr^{-1}, significantly higher than its 23°C counterpart ($p < 0.05$). Similar to the 23°C treatment, the antibiotic group showed no significant difference in MMR compared to controls ($p > 0.05$; Figure 2.4). As well, the antibiotic plus butyrate group had an MMR of about 1297 ± 55 mO_2 kg^{-1} hr^{-1}, which was significantly lower when compared to all the other treatments at this temperature ($p < 0.05$; Figure 2.4). In contrast to what was seen at the lower temperature however, the butyrate group showed no significant difference in MMR compared to the control group ($p < 0.05$; Figure 2.4), reaching about 1876 ± 227 mO_2 kg^{-1} hr^{-1}. These results suggest that butyrate supplementation alone does not significantly alter MMR at 30°C, but butyrate and antibiotics together do.

2.3.3 Standard metabolic rate

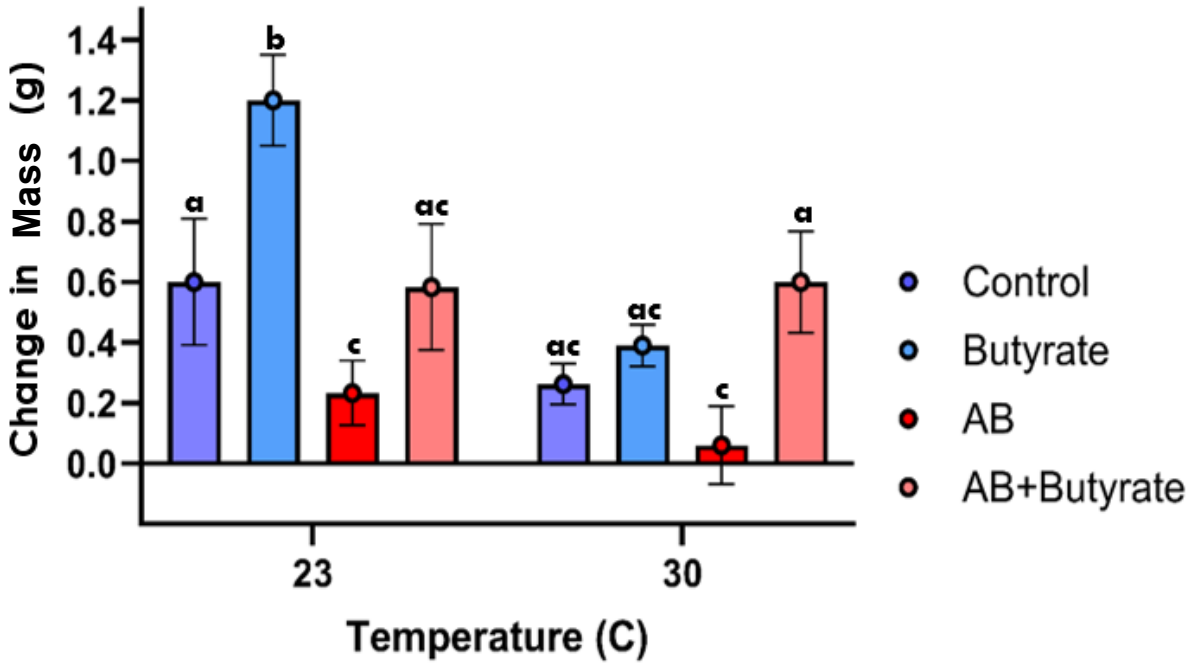
At 23°C, the control group had a SMR of approximately 748 ± 52 mO_2 kg^{-1} hr^{-1}. As well there appeared to be no impact of antibiotics, as this group had a similar SMR to the control group (Figure 2.5); just as was seen with the MMR. Further, like with the MMR, the antibiotic plus butyrate group showed an SMR significantly less than the control group, around 530 ± 54 mO_2 kg^{-1} hr^{-1} ($p < 0.05$; Figure 2.5). However, unlike with the MMR, the butyrate group exhibited a similar SMR to controls, averaging around 562 ± 39 mO_2 kg^{-1} hr^{-1} ($p > 0.05$; Figure 2.5). These results suggest that butyrate supplementation does not significantly alter SMR, while antibiotics plus butyrate does.

At 30°C, the SMR values increased across all groups compared to 23°C as was seen with MMR. Specifically, the control group had an SMR of around 1237 ± 132 mO_2 kg^{-1} hr^{-1}, significantly higher than its 23°C counterpart ($p < 0.05$; Figure 2.5). Just as with the 23°C SMR (but unlike 23 MMR) with the butyrate group showed an SMR of approximately 1086 ± 97 mO_2 kg^{-1} hr^{-1}, which was not significantly different compared to the control group ($p < 0.05$; Figure 2.4). Similarly, the antibiotics group exhibited an SMR of about 1155 ± 87 mO_2 kg^{-1} hr^{-1}, also not significantly lower than the control group ($p > 0.05$; Figure 2.5). Interestingly, the antibiotic plus butyrate group had an SMR of around 880 ± 47 mO_2 kg^{-1} hr^{-1}, which was significantly lower compared to all other treatments at this temperature ($p < 0.05$; Figure 2.5). Once again, we find a decrease in metabolic rate in the antibiotic plus butyrate group showing an interesting interaction when used in conjunction.

2.3.4 Aerobic scope

At 23°C, the control group exhibited a mean aerobic scope of approximately 760 ± 39 $mO_2\ kg^{-1}\ hr^{-1}$ (Figure 2.6). Interestingly, the butyrate group showed a mean aerobic scope of around 468 ± 80 $mO_2\ kg^{-1}\ hr^{-1}$, which was lower than the control group ($p < 0.05$; Figure 2.6) which was driven solely by the reduction in MMR (Figure 2.4) as SMR did not change (Figure 2.5). Next, the antibiotic group had an aerobic scope at approximately 681 ± 77 $mO_2\ kg^{-1}\ hr^{-1}$, indicating no significant difference in aerobic scope compared to the control group ($p < 0.05$; Figure 2.6) as MMR (Figure 2.4) and SMR (Figure 2.5) were not altered by this treatment. Finally, the antibiotic plus butyrate group had a mean aerobic scope of about 430 ± 49 $mO_2\ kg^{-1}\ hr^{-1}$, which was also significantly lower than the control group but did not differ significantly from the butyrate group ($p < 0.05$; Figure 2.6). This was driven by changes in both MMR (Figure 2.4) and SMR (Figure 2.5).

At 30°C, the control group demonstrated an increase in aerobic scope, compared to 23°C, reaching about 945 ± 136 $mO_2\ kg^{-1}\ hr^{-1}$ (Figure 2.6). As with 23°C, the butyrate group showed a decrease in aerobic scope compared to the control group at approximately 790 ± 166 $mO_2\ kg^{-1}\ hr^{-1}$ ($p > 0.05$; Figure 2.5). This occurred despite both MMR (Figure 2.4) and SMR (Figure 2.5) being statistically unaffected by treatment. Conversely, the antibiotic group also showed no significant difference compared to the control at 802 ± 82 $mO_2\ kg^{-1}\ hr^{-1}$ ($p > 0.05$; Figure 2.6), as MMR (Figure 2.4) and SMR (Figure 2.5) were not altered compared to controls. Finally, as MMR decreased (Figure 2.4) more than SMR (Figure 2.5), the antibiotic plus butyrate group reached a mean aerobic scope of about 417 ± 36 $mO_2\ kg^{-1}\ hr^{-1}$, which was significantly lower than all other groups at 30°C ($p < 0.05$; Figure 2.6).



<u>Figure 2.3:</u> Butyrate, antibiotics, and temperature impact the mass (g) of the sailfin molly over a 6-week diet trial. Butyrate increases the growth while antibiotics show to decrease in growth. Butyrate is shown to restore growth rates to control amounts when used in conjunction with antibiotics. Increased temperature seems to follow a similar trend but with no significant impact between treatments. Bars represent treatment groups separated based on temperature on the x-axis. The bars represent column means ± SEM. Bars that share letters are not statistically different. Comparisons were conducted across treatments within each temperature and across each temperature within the same treatment. A two-way ANOVA with an Uncorrected Fisher LSD multiple comparisons test was performed ($p < 0.05$; n = 8-12).

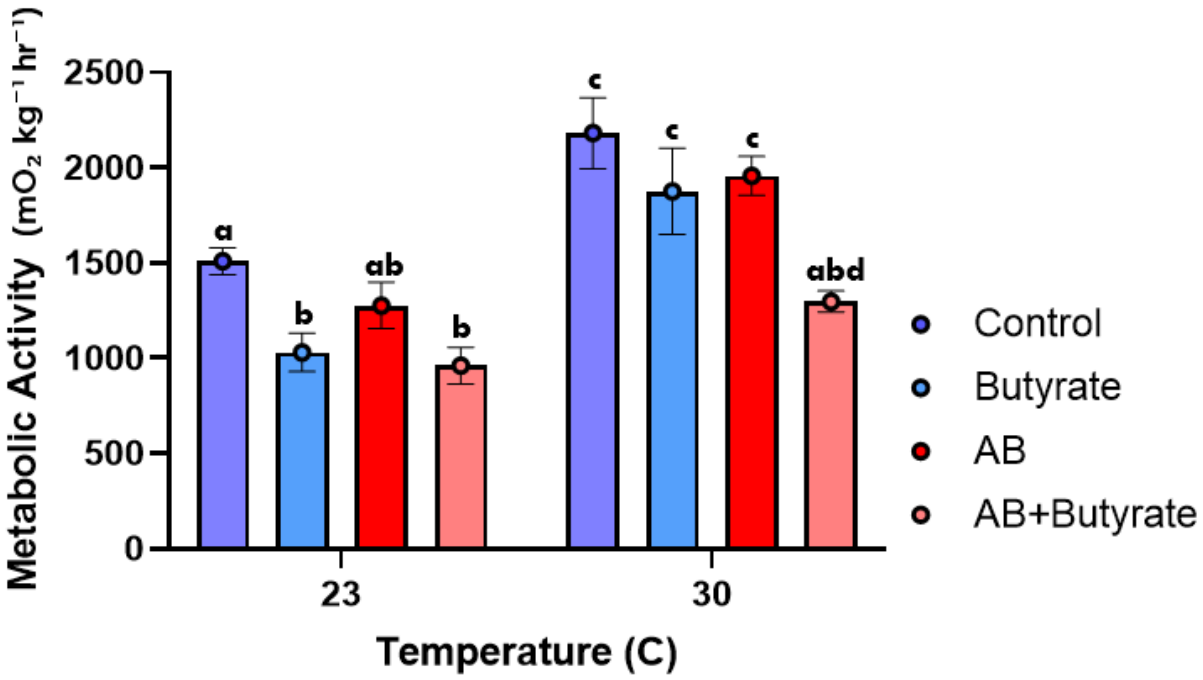


Figure 2.4: Butyrate and temperature altered the maximum metabolic rate (mO_2 kg^{-1} hr^{-1}; MMR) of the sailfin molly. Butyrate is shown to decrease the maximum metabolic rates in both the butyrate and the butyrate plus antibiotics group while this is not seen in fish just treated with antibiotics. Increased temperature also shows to increase in metabolic rates in all treatments except the butyrate plus antibiotic group. Bars represent treatment groups separated based on temperature on the x-axis. The data bar represents column means ± SEM. Bars that share letters are not statistically different. Comparisons were conducted across treatments within each temperature and across each temperature within the same treatment. A two-way ANOVA with an Uncorrected Fisher LSD multiple comparisons test was performed ($p < 0.05$; n = 8).

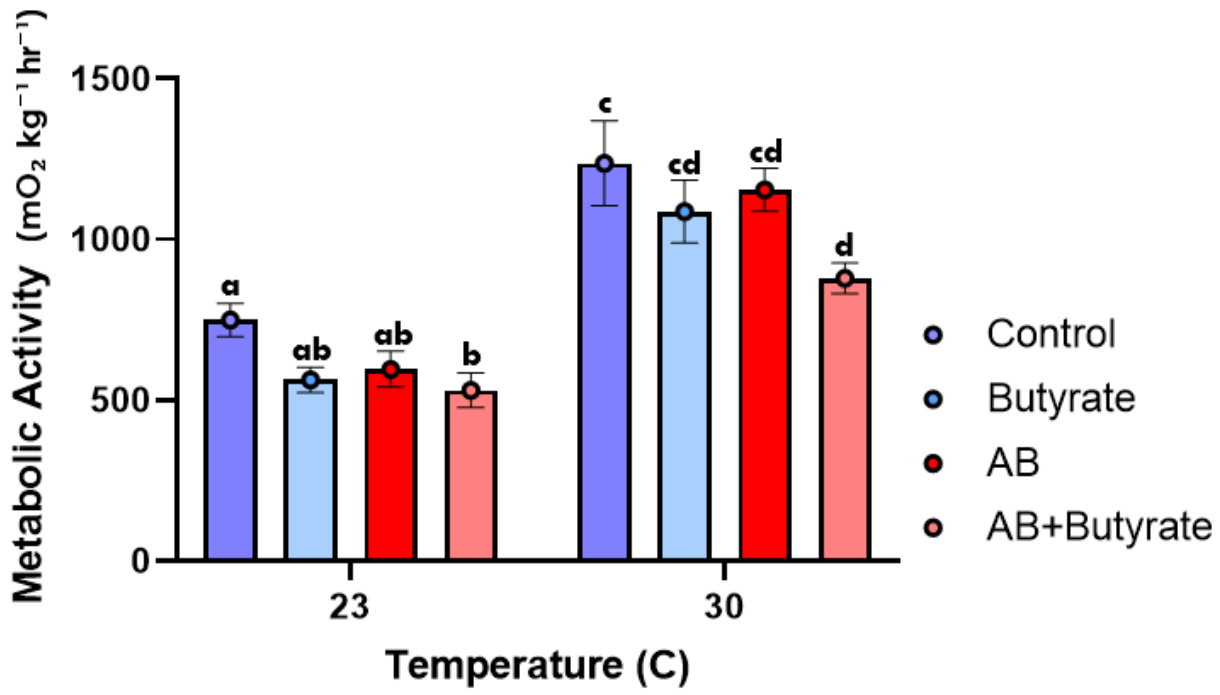


Figure 2.5: Butyrate plus antibiotics and temperature impact the standard metabolic rate (mO_2 kg^{-1} hr^{-1}; SMR) of the sailfin molly. Butyrate plus antibiotic-treated fish showed a decrease in SMR. Increased temperature also increased the SMR but once again at 30°C butyrate plus antibiotics treated fish showed a decrease in SMR. Bars represent treatment groups separated based on temperature on the x-axis. The data bar represents column means ± SEM. Bars that share letters are not statistically different. Comparisons were conducted across treatments within each temperature and across each temperature within the same treatment. A two-way ANOVA with an Uncorrected Fisher LSD multiple comparisons test was performed ($p < 0.05$; n = 8).

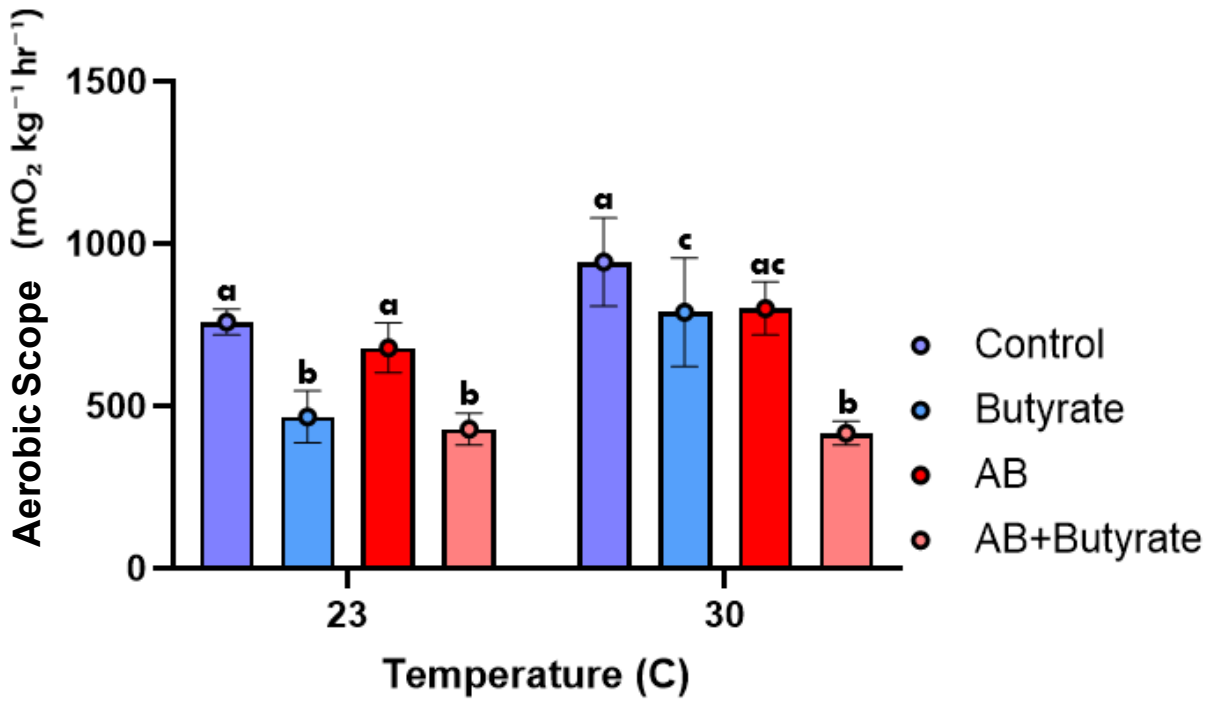


Figure 2.6: Butyrate and butyrate plus antibiotic treatment decreased the aerobic scope (mO_2 kg^{-1} hr^{-1}) of the sailfin molly. The butyrate treatment showed a decrease in their aerobic scope similar to the butyrate plus antibiotics. Interestingly there was no effect on the antibiotic-treated fish. Temperature increased aerobic scope in all treatments except antibiotics plus butyrate. Bars represent treatment groups separated based on temperature on the x-axis. The data bar represents column means ± SEM. Bars that share letters are not statistically different. Comparisons were conducted across treatments within each temperature and across each temperature within the same treatment. A two-way ANOVA with an Uncorrected Fisher LSD multiple comparisons test was performed ($p < 0.05$; n = 8).

2.4 DISCUSSION

2.4.1 Growth

The effects of butyrate, antibiotics, and their combination on the growth of sailfin molly at higher temperature revealed notable patterns. As we hypothesized, the 23°C butyrate-fed fish exhibited a remarkable 100% increase in growth compared to the control group (Figure 2.3). This significant growth enhancement could potentially be attributed to the known benefits of butyrate in promoting gut health and nutrient absorption, which likely led to improved overall metabolism and growth efficiency (Ahmad et al., 2000). Indeed, sodium butyrate is the preferred energy source of colonocytes (Ahmad et al., 2000). By providing the intestine with a raw energy source they are likely utilizing this towards growth-promoting pathways. These findings have been seen in other fish species but is important to confirm how it affects the sailfin molly specifically and confirm that 1% sodium butyrate supplementation is sufficient to increase growth. In a study using yellow catfish (*Pelteobagrus fulvidraco*), they investigated varying amounts of sodium butyrate and its effects on growth. They found that too high of a dosage of 2% sodium butyrate can lead to no improvement in growth and a decrease in nutrient retention-related gene expression (Zhao et al., 2021).

Conversely, antibiotics alone resulted in a decrease in growth, which supported our hypothesis (Figure 2.3). This reduction may be due to the detrimental impact of antibiotics on the gut microbiota, which can disrupt normal digestive processes and nutrient absorption, leading to stunted growth. The disruption of the microbiome in fish from antibiotics has been well documented and, in some cases, leads to a similar decreased growth rate (Limbu et al., 2020). Dysbiosis of the gut microbiome can lead to the loss of bacteria communities that are important

for many functions regarding growth and metabolism (Kakakhel et al., 2023). In terms of the use of antibiotics in aquaculture, it is important to monitor the physiological effects.

Most interestingly, antibiotic-treated fish supplemented with dietary butyrate had growth rates returned to normal, supporting our hypothesis that butyrate can counter adverse side effects of antibiotics (Figure 2.3). This suggests that butyrate supplementation may mitigate the negative growth effects associated with antibiotics. One possible explanation for this is a similar one for why butyrate alone increased growth - butyrate serves as an energy source for colonocytes. Another possibility is that butyrate promotes the integrity of the gut barrier. This interaction with butyrate and the integrity of the intestine has been documented in other marine teleost (Estensoro et al., 2016). Enhancing gut health may counter such secondary effects of antibiotics such as "leaky gut" which increases intestinal permeability and can lead to impaired nutrient absorption, and inflammation, or disrupt the gut microbiota (Limbu et al., 2018). Another possible explanation for this finding could be influenced by the butyrate effect on other microbiota. In largemouth bass (*Micropterus salmoides*) that were fed sodium butyrate researchers found that butyrate significantly elevated the relative abundance of *Bacteroidetes* and *Firmicutes* which are thought to generally produce butyrate and thus promote growth (Chen et al., 2021). By promoting beneficial microbiota in the gut this could lead to possibly secondary functions that can promote the health and growth that was observed.

In contrast to what we hypothesized, at 30°C there was not a significant increase in growth compared to 23°C (Figure 2.3). Further, increases in growth associated with butyrate treatments were eliminated at this temperature. This lack of response might be due to the higher temperature potentially masking the effects of these treatments on growth, as metabolic rates and physiological processes are generally elevated at higher temperatures. High temperatures can

accelerate metabolic processes, potentially diminishing the distinct impact of dietary supplements like butyrate (Rowe et al., 2018). Additionally, with the increased metabolic processes nutrient demand is also needed to sustain growth and maintain bodily function even with the extra energy provided from butyrate. Also, higher temperatures can cause significant stress in fish and can impact growth. It has been documented that teleost exposed to higher temperatures can experience increased levels of stress which can depress growth (Balasch and Tort, 2019). Although sailfin molly are tropical fish and experience higher temperatures daily, being constantly at 30°C over long periods could be the reason for decreased growth. Similar to how antibiotics reduced growth indicating that antibiotics may have a detrimental impact on growth. However, when both butyrate and antibiotics were combined, there was a noticeable recovery increase in growth compared to the antibiotic treatment alone – just as in 23°C. The complimentary effect of these treatments at higher temperatures could be from butyrate providing an energy source that can support the growth and repair from high temperatures, and the antibiotics are working to stop pathogens. The combined treatment might have helped restore gut microbiota balance by targeting pathogens and promoting beneficial bacteria or allowing the host to utilize the raw energy of butyrate. It suggests that optimizing growth in aquaculture requires careful consideration of these factors, as their effects can vary significantly with temperature changes.

2.4.2 Maximum metabolic rate

In the 23°C group, we observed a decrease in MMR in the butyrate group (Figure 2.4). This finding supports our hypothesis and the observed increase in growth (Figure 2.3). Many possibilities could contribute to this increased growth such as changes in energy allocation,

being a source of energy for the host, or possibly promoting beneficial energy for microbiota to have improved functions. Additionally, we find improved growth rates in fish species with increasing temperatures due to increased metabolic and physiological processes (Kasihmuddin et al., 2024). A potential explanation for this phenomenon could be found in the dual role of butyrate as both an energy source and a signaling molecule. According to Donohoe et al. (2011), butyrate can reduce oxidative stress and inflammation, which could contribute to a lower MMR by decreasing energy demands for these costly processes. Interestingly, increased growth and decreased MMR were observed which suggests that energy is being reallocated within this treatment. The energy that usually is put into maintaining high metabolic rates for maintenance or stress is being allocated towards growth. Consequently, fish that do not have to put energy into fighting stress and inflammation may have lower MMRs but be allocating energy more effectively for rapid movement as well as growth. In the antibiotic group, no significant difference in MMR was observed. Antibiotics primarily target bacterial infections and may not directly affect the metabolic processes of the host organism. However, some studies suggest that antibiotics can alter metabolic activity indirectly by affecting the gut microbiota. These results suggest that antibiotics do not affect energy allocation of the host but changes in growth imply these effects might be caused by dysbiosis and the function of the gut microbiota. For instance, Ianiro et al. (2016) indicated that antibiotics can disrupt gut microbiota, potentially leading to metabolic disorders indirectly, but this effect may not be significant enough to observe from just MMR measurements.

Conversely to the hypothesis at 30°C, no difference in MMR was observed across the groups treated with antibiotics or butyrate alone (Figure 2.4). One possible explanation for this is that the organisms are already operating at their maximum metabolic output due to the higher

temperature. According to a study by Kumar et al. (2020), metabolic rates of Iridescent sharks (*Pangasianodon hypopthalmus*) increase with temperature until an optimum level is reached, beyond which further increases in temperature do not result in higher metabolic rates. Therefore, at 30°C, the organisms might have already reached this optimum level. Interestingly at both temperatures, when both butyrate and antibiotics were administered together, MMR decreased. This indicates that butyrate is a common factor influencing metabolic rates across these groups. When looking at the combination of antibiotics and butyrate we do see a decrease in MMR, and this may be due to complimentary effects. Antibiotics can reduce harmful bacteria that may be competing for butyrate utilization (Olymon et al., 2024).

2.4.3 Standard metabolic rate

In the 23°C group, we observed no significant changes in SMR with either butyrate or antibiotics alone, which did not support our hypothesis (Figure 2.5). This could be because the organisms are already at their baseline metabolic rates, and these treatments such as butyrate can't further reduce SMR. Baseline metabolic rates are often tightly regulated. According to Konarzewski and Książek (2013), standard metabolic rates are typically stable and are determined by fundamental physiological processes and pathways that are not easily altered by simple changes in diet. Although butyrate increased growth, no changes in SMR could indicate that butyrate is being used for the basal metabolic process and allowing for extra energy to be allocated toward growth. However, we observed a significant difference in SMR when both butyrate and antibiotics were combined, like the effect we saw with MMR. This reinforces the idea of the additive effect where the presence of both treatments together exerts a more pronounced impact on the metabolic processes. A possible explanation is that butyrate and

antibiotics together could be altering the gut microbiota in a way that influences host metabolism more significantly than either treatment alone.

Once again in the 30°C group, the same trend was observed with no significant changes in SMR with either butyrate or antibiotics alone, but a noticeable difference with the combination of the two. At higher temperatures, organisms have higher metabolic output required to sustain life. As a result, like the 23°C group, butyrate and antibiotics alone might not exert a significant influence on SMR. However, the combined treatment could facilitate a unique interaction that affects metabolic regulation. A possibility is that the higher temperature interactions between butyrate and antibiotics are where energy is allocated to survival mechanisms to reduce overheating and optimize energy use (Chabot et al., 2016).

2.4.4 Aerobic scope

Aerobic scope is a measure of the difference between the maximum metabolic rate (MMR) and the standard metabolic rate (SMR). It represents the capacity of an organism to increase its metabolic rate above the baseline level to support activities such as growth, reproduction, and movement (Eliason and Farrel, 2016). In both the 23°C and 30°C groups, we observed a consistent trend where the use of butyrate, either alone or with antibiotics, resulted in a decrease in aerobic scope. Butyrate might have this effect because it could be shifting the energy allocation in the fish from basal metabolic processes and the ability for rapid movement towards growth. Additionally, at 30°C we saw no significant difference in SMR or MMR but a significant decrease in aerobic scope. This trend shows that subtle changes in energy allocation though not significant in basal or maximal metabolic rate can significantly impact energy

allocation for growth. According to Louis and Flint (2009), butyrate can improve gut health and increase the efficiency of nutrient utilization, which might lead to more energy being allocated toward growth rather than being available for aerobic activities. Additionally, since we did not observe significant changes in SMR with butyrate alone, the changes in aerobic scope are driven by the decreased MMR. The reduction in MMR when butyrate is used indicates a reduction in maximum metabolic output. This might be because the energy usually needed for these rapid activities is being redirected towards growth and maintenance, which might make sense for fish within aquarium conditions and do not require rapid changes in metabolic output. In terms of the novel nature of this finding, while the exact interaction between butyrate, antibiotics, and aerobic scope might not have been extensively documented, the general principles of energy allocation and nutrient absorption are well-supported. This could represent a new insight into how these treatments interact specifically in the context of temperature and metabolic rates in fish. However, the specific impact of butyrate and antibiotics, individually and together, on metabolic rates in fish remains unknown. The results of this study suggest that further investigation is necessary to understand where fish species are allocating energy resources.

2.4.5 Conclusions

This research revealed that butyrate and antibiotics have distinct and interactive impacts on fish growth and metabolism. Butyrate significantly increased growth, which supported our hypothesis, possibly due to its role in improving gut health and enhancing nutrient absorption, as supported by Louis and Flint (2009). It also led to a decrease in MMR and aerobic scope at 23°C, which partially supported our hypothesis, and suggested that butyrate promotes growth by allowing the fish to utilize the excess energy toward growth pathways. Additionally, antibiotics

showed decreased growth with no changes in metabolic rate, suggesting that they do not influence metabolic rates directly. However, this decrease in growth could indicate a shift in energy allocation, where energy normally used for growth is being diverted to address the consequences of antibiotic treatment, such as managing gut microbiota disruptions or other stress responses. As a result, while the overall metabolic rate remains unchanged, less energy is available for growth. Interestingly, butyrate can counteract the negative effects of antibiotics on growth, making it a beneficial additive for aquaculture to optimize fish growth and this supported our hypothesis. These results for butyrate indicate that the energy it provides is being used by the host to support growth directly through the host as previously discussed, however as we know butyrate may work on other areas such as the gut microbiome further investigation is required to determine the mechanism of action. At higher temperature, no additional growth was seen. This is consistent with the findings of Clarke and Fraser (2004), who noted that organisms might reach their maximum metabolic output at elevated temperatures. Overall, our results emphasize the need for further research on how environmental factors such as temperature interact with diet to influence fish metabolism and growth.

CHAPTER 3: METAGENOMIC RESPONSE OF THE SAILFIN MOLLY MICROBIOME TO BUTYRATE, ANTIBIOTICS, AND TEMPERATURE

3.1 INTRODUCTION

3.1.1 Role of the gut microbiome

The microbiome is a complex and dynamic community of microorganisms that plays an essential role in maintaining host health and facilitating growth (Rajilic-Stojanovic and de Vos, 2014). The gut microbiome has been extensively studied for its role in various physiological processes. Microbiota interactions within the gut can be both mutualistic and competitive (Coyte and Rakoff-Nahoum, 2019). Cooperative interactions often involve the production and exchange of metabolic byproducts, which benefit multiple microbial species (Cavaliere et al., 2017). For example, certain bacteria can break down complex sugars into simpler molecules that other microbes can utilize (Flint et al., 2012). This bacterial interaction is critical for the efficient energy production from the diet. Additionally, some microbes produce essential vitamins and other nutrients that the host and other microbiota require (Flint et al., 2012). Alternatively, microorganisms can compete for limited resources, such as nutrients and space, using various strategies (Ghoul and Mitri, 2016). Some bacteria produce antimicrobial compounds, such as bacteriocins, to inhibit the growth of competitors (Ghoul and Mitri, 2016).

Understanding how different microbial species interact, compete, and cooperate, and how these interactions affect the host is important for manipulation and understanding the consequences of different treatments of fish. Additionally, investigating the effects of various interventions on the microbiome will provide insights into optimizing health and growth through the microbiome.

3.1.2 Impact of butyrate on gut microbiota

The gut microbiota in fish plays a crucial role in maintaining host health, influencing various physiological processes including digestion, immune function, and metabolic regulation (Yukgehnaish et al., 2020). One of the most important metabolic byproducts produced by gut bacteria is butyrate. Butyrate is primarily produced through the fermentation of dietary fibers by certain beneficial gut bacteria, primarily from the phylum *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (Vargas et al., 2023).

Butyrate exerts several beneficial effects on the gut microbiota and the host. Generally, it promotes the growth of beneficial bacteria while inhibiting the proliferation of pathogenic bacteria (Canani et al., 2011). This helps maintain a balanced gut microbiota composition, which is crucial for overall gut health. The mechanism may relate to the ability of butyrate to influence the gut environment by lowering the pH through its acidic properties; which creates an unfavorable environment for pathogenic bacteria while promoting the growth of beneficial bacteria (Fu et al., 2018). Moreover, butyrate's influence on the gut environment and microbiota composition has implications for broader host health. Butyrate's impact on the microbiome composition has not been deeply explored and requires more research into the secondary effects it may have. Butyrate has shown that it is multifaceted but is unclear what specific impacts are happening to gut microbiota composition and host. A balanced and healthy gut microbiota can enhance nutrient absorption, improve metabolic efficiency, and enhance the immune system (Kaur and Ali, 2022). This interconnected relationship between butyrate, gut microbiota, and host health implies the importance of dietary fibers and butyrate-producing bacteria in fish diets, especially in aquaculture settings where maintaining fish health and growth is critical.

3.1.3 Mechanisms of antibiotics on gut microbiota

Antibiotics are essential for combating bacterial infections and impact the gut microbiota by disrupting its complex and dynamic ecosystem (Fassarella et al., 2021). In particular, broad-spectrum antibiotics, such as ampicillin, gentamicin, kanamycin, and penicillin all target both gram-positive and gram-negative (Kakoullis et al., 2021). As such, broad-spectrum antibiotics are commonly used to treat a wide range of diseases within aquaculture facilities (Ibrahim et al., 2020). The removal of bacterial species by antibiotics can have effects on the gut microbiota. In particular, when antibiotics eliminate rare species with beneficial properties this leads to reduced microbial diversity and altered metabolic activities (Ramirez et al., 2020). The gut microbiome functions similarly to an ecosystem, where each bacterial species occupies a specific niche and contributes to the overall stability and function of the ecosystem. Removing or significantly reducing populations of certain species through antibiotic treatment can have major effects on the microbiome. This disturbance can lead to reduced microbial diversity, loss of beneficial functions, and an increased risk of infections. Understanding the dynamics of the gut microbiome and the impact of antibiotics within bacterial taxa is crucial for developing strategies to reduce these adverse effects and promote gut health during and after antibiotic treatment.

3.1.4 Temperature effects on gut microbiota

Temperature is a critical environmental factor that influences the composition and function of gut microbiota in aquatic organisms (Dehler et al., 2017). Firstly, the microbial communities in water are sensitive to temperature changes (Sieburth, 1967). Higher temperatures can influence the metabolic demands of aquatic microbes, leading to shifts in community

composition and potentially favoring opportunistic or pathogenic species (Nogales et al., 2011). These changes in the aquatic environment can directly affect the gut microbiota of marine organisms, as they are responsible for colonizing the gastrointestinal tract of fish (Sepulveda and Moeller, 2020). Furthermore, high temperatures can increase the host's metabolic rate, altering nutrient availability and immune function, and ultimately impact gut microbial composition (Sepulveda and Moeller, 2020). For example, higher temperatures may promote the growth of certain bacterial species while inhibiting others, leading to a less diverse but more specialized gut microbiota (Minich et al., 2018; Sepulveda and Moeller, 2020). Additionally, temperature fluctuations can influence the gut environment, such as pH and oxygen levels which further affects microbial communities (Nguyen et al., 2021). This imbalance in the microbiota can reduce the beneficial functions provided by the microbiota, such as nutrient absorption and immune regulation, and increase the host's susceptibility to infections and diseases (Minich et al., 2018).

Understanding the impact of temperature on gut microbiota is important, especially in the context of climate change and its effects on aquatic ecosystems. It is important to consider how long-term exposure to higher temperatures might alter the gut microbiota and overall health of organisms. This information can be useful for managing aquaculture practices and fish populations in the long run.

3.1.5 Current metagenomic analysis

Current research has taken advantage of the many uses of metagenomic analysis. Metagenomics is a key tool that analyzes genetic material extracted from samples which

provides insights into the microbial communities present in the sample (Langille et al., 2013; Douglas et al., 2018). Metagenomics allows for the study of entire microbial populations in their natural environment. This technique is important for studying bacterial differences because it provides a more accurate view of microbial communities. When examining variations the bacterial genomes, this approach can find bacterial species and their potential functions (Langille et al., 2013; Douglas et al., 2018). For example, metagenomics can be used to predict how shifts in bacterial populations might affect metabolic processes, immune responses, or other physiological traits in fish. By analyzing the bacterial communities in different environmental conditions, researchers can learn how microbes contribute to the growth, health, and metabolism of species.

To get an understanding of microbial communities both alpha and beta diversity tests are used (Chao and Ricotta, 2019). Alpha diversity refers to the diversity within a single sample or environment, with common indices such as Chao1 and Shannon providing insights into species richness and evenness (Chao and Ricotta, 2019). Chao1 estimates the total number of species in a community, including rare species, while the Shannon index accounts for both the abundance and evenness of species. Richness refers to the number of different bacterial taxa present in a community while evenness measures how evenly taxa are distributed between each sample (Chao and Ricotta, 2019). These indexes are chosen to compare richness and evenness across samples by comparing significant findings overlapping. Overall, the measurements help to understand the complexity and health of microbial communities within each treatment.

Beta diversity compares the differences between microbial communities across different samples or environments (Myers et al., 2012). Metrics like Weighted and Unweighted UniFrac, and Bray-Curtis dissimilarity, are commonly used. Weighted UniFrac considers both the

presence/absence of species and their relative abundance, making it sensitive to differences in dominant species (Myers et al., 2012). Unweighted UniFrac, however, only considers the presence or absence of species, emphasizing differences in community composition. Bray-Curtis dissimilarity, on the other hand, measures the compositional dissimilarity between two samples based on species abundance, providing a view of how microbial communities differ in abundance (Myers et al., 2012). Each of these metrics shows pieces of the community compositions between treatments and together can illustrate how bacterial communities differ across treatments in terms of taxa present and their relative abundances.

Analysis of Composition of Microbiomes (ANCOM) is another crucial tool, which allows researchers to identify differentially abundant species between groups (Mandal et al., 2015). ANCOM tests are particularly useful because they account for the bacterial composition of the microbiome, making it a robust choice for detecting changes in microbial populations at different taxonomic levels and across different environmental conditions or treatments (Mandal et al., 2015). In addition to diversity analyses, predictive functional profiling using tools are commonly used in conjunction, like PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). PICRUSt allows for the prediction of the functional capabilities of microbial communities based on their phylogenetic composition (Mandal et al., 2015). By inferring bacterial gene sequences, researchers can predict the functional potential of a community, shedding light on how microbial functions might shift in response to environmental changes.

These methods and analyses are chosen as they provide a comprehensive overview of microbial communities, illustrating diversity and functional potential. These tools are useful to detect differences in community composition, identify key microbial communities, and predict

potential functional changes, offering an overview approach to understanding microbiomes in various treatments or environments.

3.1.6 Hypotheses

This study aims to investigate the individual and combined effects of butyrate, antibiotics, and temperature on the gut microbiome of the sailfin molly. Understanding these influences is crucial for revealing the interactions within the microbiota and how these interactions affect the host's metabolism and growth, especially in light of the observations from Chapter 2.

We hypothesize fish given dietary butyrate will increase the abundance of beneficial bacteria such as *Firmicutes*, *Proteobacteria*, and *Actinobacteria* which are associated with improved gut health and metabolic efficiency as well as butyrate producers themselves. This increase in beneficial bacteria is expected to increase functions associated with growth and nutrient assimilation and benefit the microbiota. Conversely, antibiotics are known to disrupt the gut microbiome by reducing microbial diversity by eliminating both pathogenic and beneficial bacteria. Hence, we hypothesized that the administration of antibiotics would create dysbiosis in the gut microbiome and overall lead to a reduction in the diversity and richness of the microbiota present in the gut which can help explain the impacts seen in Chapter 2. This dysbiosis was expected to cause a significant reduction in beneficial bacteria, leading to a loss of metabolic and health-promoting functions. By then investigating butyrate treatments in animals with reduced microbiomes we hope to determine if butyrate is directly working through the host's metabolic pathways by supplementing butyrate from microbiota or possibly interacting with the bacterial

communities or even a combination and could help explain the observed impact of butyrate on growth observed in Chapter 2. Finally, we hypothesize that elevated temperatures will impair the microbiome's metabolic functions and health benefits by reducing microbial diversity and increasing the presence of pathogenic bacteria. We predict that butyrate supplementation will have a reduced impact on the microbiome based on the little impact from Chapter 2 following temperature treatments.

Overall, this study will allow us to observe changes in the composition of the microbiota and understand how butyrate, antibiotics, and temperature individually and in combination affect the overall health and metabolism of the sailfin molly. We aim to explore how changes in microbial communities affect fish health and growth, which could have broader implications for aquaculture and environmental management practices.

3.2 MATERIALS AND METHODS

Please refer to Chapter 2.2 Materials and Methods for experimental setup, diet trials, and animal housing information.

3.2.1 Dissection

Fish from all treatments (*n*=8) were sampled in the same manner, 24 hours after their last feeding session. During euthanasia, each fish was placed in anesthetic water containing buffered tricane methanesulfonate (2.0g/L) (MS-222; Syndel Canada, Nanaimo, British Columbia). The pH of the solution was checked using a pH meter and adjusted to 7.5 using NaOH. Following an anesthetic procedure transactional cut of the spinal cord was performed. Sterile dissection techniques were used for each dissection. Scissors and forceps were sterilized in 70% ethanol as well as left under UV light for 10 minutes. Dissections were conducted in a fume hood that was sprayed and wiped down with 70% ethanol. Scissors, forceps, and fume hoods were sterilized between each fish. During the dissection, the intestine was cut in half at the midpoint. Since any distinct characteristics did not separate both sections, a halfway point was chosen to ensure consistency across the samples. The samples were placed in marked tin foil, flash frozen using dry ice, and then stored at -80ºC until further analysis.

3.2.2 DNA extractions

gDNA and bacterial DNA from the intestinal tissue from each treatment condition were extracted using Qiagen's PowerSoil Pro Max Kit (Qiagen; Germantown, Maryland; *n*=8). Following the manufacturer's protocol, DNA extraction was performed under sterile conditions in a UV fume hood. Metal tools such as forceps and scissors were sterilized by soaking in 70%

ethanol for 15 minutes and UV exposure for 10 minutes. The intestinal tissue was cut into small pieces using sterile scissors and placed into a PowerBead Pro Tube with solution CD1. The sample was vortexed to disperse it in the buffer, dissolve humic acids, and protect nucleic acids. It was then homogenized horizontally at maximum speed for 40 minutes using a Fisher Vortex 12-812 Genie 2 (Fisher Scientific). After, the tube was centrifuged, solution CD2 was added to the supernatant and vortexed to mix, precipitating non-DNA organic and inorganic materials. After another centrifugation step, solution CD3 was added to the supernatant, vortexed, and the mixture was loaded onto the MB Spin Column, allowing DNA to bind to the membrane. Wash buffers EA and C5 were sequentially added and centrifuged to remove contaminants. Finally, solution C6 was added to elute the DNA, which was then stored at -20ºC until further use. To ensure sterility, a blank extraction was performed with each set of DNA extractions, following all steps without adding tissue samples.

3.2.3 Amplicon Sequencing

The extracted DNA samples were sent to Genome Québec (Montreal, Quebec), where they used 16S rRNA amplicon sequencing. Genome Quebec's protocol handled all library preparation at the facility. The amplification targeted 16S rRNA V3-V4 hypervariable regions (Table 3.1). After samples were processed using the Illumina NextSeq platform with a paired-end 300 bp (PE300) read configuration. Genome Québec performed the library preparation, sequencing, and initial data quality assessment.

3.2.4 Importing and trimming data

The raw sequence data in FASTQ format were initially quality-checked using FastQC to ensure the integrity of each sample. Each sample was assigned a Q score indicating the confidence of each base in the sequence with the cut-off being Q30 (99.9% accuracy) up to a maximum score of Q40 (99.99% accuracy); all samples passed the quality check. The sequences were then imported into QIIME2 (Version 2024.5) for further analysis using a Python environment. Sequence data were demultiplexed using the demux plugin in QIIME2, and quality filtering was performed with the DADA2 plugin, which includes steps for denoising, dereplicating, and chimera removal and removing all additional sequences above the lowest amount of sample (~100,000 reads per sample). During this quality filtering step, additional parameters were applied to remove non-bacterial species, including chloroplast DNA, mitochondrial DNA, and any sequences classified as unknown. The feature table and representative sequences were used for downstream analysis. Taxonomic classification of the features was carried out using the SILVA 138 database.

3.2.5 Alpha diversity

To assess the alpha diversity of the microbial communities, we calculated the Chao1 and Shannon diversity index using the QIIME2 alpha diversity plugin, which measures species richness and evenness. These tests were chosen to provide insights into the complexity of the microbial community within each sample. The QIIME2 diversity alpha plugin was also used to compute the Shannon index for each sample. The resulting diversity metrics were then analyzed

to compare the microbial diversity across different experimental groups. The index's values were visualized using boxplots.

3.2.6 Beta diversity

To assess the beta diversity and compare the microbial community composition between samples, we calculated Bray-Curtis, Unweighted, and Weighted UniFrac distances using the QIIME2 beta diversity plugin. Quality-filtered, denoised sequences were used to generate an ASV table, ensuring high-quality input data. Simply, the Bray-Curtis index measures abundance without phylogeny, the Unweighted UniFrac looks at presence with phylogeny, and the Weighted UniFrac considers both abundance and phylogeny. Together these indices provide a full overview of changes in present, abundance, and phylogenetic differences. The resulting distance metrics were then analyzed to compare the microbial community composition across different treatment groups. The distance values were visualized using PCA plots to illustrate the differences in beta diversity between the groups, offering a visualization of the similarities and differences between microbial communities across the samples.

3.2.7 ANCOM

Microbial community composition was analyzed using QIIME2. After quality filtering, denoising, and taxonomic classification of the data, the resulting feature table was used for downstream analysis. To identify differentially abundant bacterial taxa between sample groups, we performed an Analysis of the Composition of Microbiomes (ANCOM). ANCOM was chosen for its ability to handle microbial sequencing compositions and identify differences in microbial

taxa across treatments. The feature table was filtered to remove unidentified taxa and bacterial groups which was done by removing groups containing fewer than 500 reads (0.05% of total reads), which reduced noise and enhanced the statistical power of the analysis to ensure that only relevant taxa were considered. The QIIME2 composition ANCOM plugin in QIIME2 was used to compare the relative abundance of bacterial taxa across groups. The data was displayed as a stacked bar plot which showed the bacteria phylum. The plot depicted the relative abundances of the top 10 bacterial communities, which make up ~99% of the total relative abundance, in each sample to highlight the most relevant bacterial communities.

3.2.8 PICRUSt analysis

Functional predictions were performed using the PICRUSt plugin in the QIIME2 software. The ASV table was first normalized for copy number to account for varying gene copy numbers across different taxa. Metagenome predictions were then generated and annotated using the KEGG KO database (2011 version), which was freely available to produce an in-house mapping file. This mapping file linked KO values to bacterial taxa, providing a functional profile of the microbial communities.

The KO mapping file values generated were then imported into R 4.4.1, where they were assigned to each bacterial taxon. These values were aggregated to obtain absolute totals for each predicted function, allowing for an overview of the functional potential of the microbial communities. Additionally, KO values were organized into levels according to a functional level. For instance, L1 analysis provided a broad overview of the dominant functional categories such

as metabolism and disease, while L2 and L3 analyses allowed for more detailed functions of specific metabolic pathways and proteins associated.

The output from the statistical analysis, which included comparisons of functional group abundances across different samples or conditions, was visualized using STAMP. This visualization helped in identifying significant differences in predicted functional capabilities and provided a clear representation of the functional diversity and potential roles of the microbial communities under study.

3.2.9 Statistics

All statistical analyses were conducted using QIIME2, R 4.4.1, and STAMP software as indicated above. Specifically, QIIME2 data from Chao1 and Shannon indexes were analyzed using the Kruskal-Wallis test to determine the significance between groups in R. For the PICRUSt data containing comparisons across all treatments, STAMP was used for analysis using a Tukey-Kramer test followed by the Benjamini-Hochberg post hoc correction. The Tukey-Kramer test was selected because it allows for multiple comparisons while controlling for the family-wise error which is essential when comparing multiple groups in large sample sizes. The QIIME2 composition ANCOM plugin in QIIME2 was used to compare the relative abundance of bacterial taxa across groups. The Benjamini-Hochberg correction was applied to control the false discovery rate, making it a robust choice for reducing the likelihood of type I errors. For the main effects, comparisons between temperature and treatment were conducted using Welch's t-tests in STAMP. Statistically significant differences were only assumed if $p < 0.05$. All figures

were produced using R 4.4.1 and STAMP software, where the bars represent column means ± standard error of the mean (SEM).

Table 2.1: Primers and Sequences Used for 16S rRNA Gene (V3-V4 Region) Amplicon Sequencing. Primers and their corresponding nucleotide sequences were utilized for the amplification of the 16S rRNA gene V3-V4 region in amplicon sequencing using the Illumina NextSeq platform.

Gene	Region	Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence
16S	V3-V4	341F	CCTACGGGNGGCWGCAG	805R	GACTACHVGGGTATCTAATCC

3.3 RESULTS

3.3.1 Alpha diversity

The Chao1 index (Figure 3.1A), indicates species richness, while the Shannon index (Figure 3.1B), reflects species evenness and richness. Notably, the Chao1 index reveals significant differences in species richness between treatment groups at both temperatures ($p < 0.05$; Figure 3.1A). Specifically, the control group showed the highest species richness, while the antibiotics and antibiotics plus butyrate group had significantly lower richness in both temperatures ($p < 0.05$; Figure 3.1A). Meanwhile, the butyrate group showed intermediate richness levels as it was not significantly different from either the control or antibiotic groups ($p > 0.05$; Figure 3.1A), again in both temperatures. Conversely, the Shannon index did not show significant differences across any of the groups, with the average Shannon index being 2.9 ± 0.1 across all treatments ($p > 0.05$; Figure 3.1B).

3.3.2 Beta diversity

The PCA plot of Bray-Curtis dissimilarity (Figure 3.2A) shows a clear separation of samples along both Axis 1 (20.3%) and Axis 2 (15%). Specifically, axis 1 primarily separates the groups based on the presence or absence of antibiotics, with antibiotic-treated groups clustering on the left separately from the non-antibiotic groups (control and butyrate). Additionally, Axis 2 appears to separate the samples based on temperature, particularly the 23°C (triangles) and 30°C (squares) groups. The grouping clearly shows in the control and butyrate being separated by Axis 2. Also, this trend is seen in the antibiotic groups which are shown inversely but separate the 23°C (triangles) and 30°C (squares) groups in the top left corner (Figure 3.2A). These patterns

suggest that temperature plays a crucial role in shaping the presence and absence of microbial communities.

In contrast, the PCA plot for Weighted UniFrac (Figure 3.2B), which considers both species abundance and phylogenetic relatedness of the bacteria, indicates that the separation of samples is based on antibiotics along Axis 1 (34%). This suggests that the phylogenetic composition of the communities is affected by the presence of antibiotics regardless of butyrate. Unlike the Bray-Curtis plot (Figure 3.2A), which only considers the abundance of bacteria, the 23°C (triangles) and 30°C (squares) groups do not form distinct clusters, demonstrating that temperature influences the abundance of bacteria but affect a broad set of bacterial taxa evenly.

Finally, the PCA plot using Unweighted UniFrac (Figure 3.2C), which measures phylogenetic differences without taking in relative abundances of bacteria, mirrors the findings of the Bray-Curtis plot, showing separation based on both treatment and temperature. Axis 1 (16.6%) differentiates the samples based on the presence of antibiotics, with control and butyrate groups (without antibiotics) clustering separately from the antibiotic-treated groups. This indicates that antibiotic treatment significantly alters the presence/absence of microbial taxa. Moreover, axis 2 (13.6%) further separates the samples based on temperature. There is a clear grouping of 23°C (triangles) and 30°C (squares) separately on the Axis 2 (13.6%). This separation is more pronounced in the control and butyrate groups. This suggests that temperature impacts the composition of microbial communities more in the control and butyrate group than in the antibiotic groups but overall, there is a significant impact of the presence of antibiotics on microbial species. Additionally, this means that temperature directly impacts specific species' presence and absence but is not affect phylogenetically related groups.

3.3.3 Taxonomical variations

The relative abundance of bacterial phyla across different treatments and temperatures was analyzed, as depicted in the stacked bar plot (Figure 3.3). The plot represents the top 10 bacterial phyla in each treatment. The phylum level was chosen to focus on the broader taxonomic differences that are more pronounced at this level, which reflect major shifts in the microbial community structures.

Actinobacteriota showed a significant difference between the treatment and temperatures (Figure 3.3). At both temperatures, *Actinobacteriota* comprises a significant portion of the bacterial community in the controls. In contrast, within the antibiotic treatments (antibiotic and antibiotic plus butyrate groups), *Actinobacteriota* makes up much less of the overall abundance. When butyrate is applied alone, there are temperature-specific trends, where at 23°C *Actinobacteriota* makes up a similar proportion of the whole community compared to controls, while at 30°C *Actinobacteriota* reduced similarly to antibiotic treatments (Figure 3.3). Additionally, when looking across temperature the abundance of *Actinobacteria* is much less in the control and butyrate groups at high temperatures. Temperature and the presence of antibiotics both play a significant role in the presence or absence of Actinobacteria.

Desulfobacterota is another phylum that changes with temperature and antibiotic exposure. At 23°C, in the control group, *Desulfobacterota* is present but only in a small abundance, while this increases at 30°C (Figure 3.3). In the butyrate group, *Desulfobacterota* makes up a larger relative abundance at both temperatures (Figure 3.3). Interestingly, in the antibiotic-treated groups, *Desulfobacterota* is completely absent at both temperatures, showing that antibiotics completely removed the group. As with *Actinobacteriota* , temperature appears to increase the abundance of *Desulfobacterota* as long as antibiotics are not applied (Figure 3.3).

Firmicutes, which make up a small proportion of the community, show distinct patterns across the treatments and temperatures. Notably, at 23°C, *Firmicutes* are present in both the control and butyrate groups but make up less than 1% of the relative abundance of bacteria. Additionally, at 30°C, *Firmicutes* are highly present in both the control and butyrate groups but have increased in abundance indicating a temperature-dependent increase in bacterial presence as also seen with *Actinobacteriota* and *Desulfobacterota*. Additionally, *Firmicutes* are completely absent in both antibiotic-treated groups at both temperatures, like the pattern observed for *Desulfobacterota* (Figure 3.3). This pattern shows that temperature and the presence of antibiotics play a significant role in the abundance of *Firmicutes*, but butyrate had no effect.

3.3.4 Predicted function

3.3.4.1 Overall functional groupings

The PCA of the total predicted functions of the microbiota revealed patterns regarding the functional differences among the various treatment groups and temperatures (Figure 3.4). When examining the control groups, there was a clear separation between samples at 23°C and those at 30°C. This distinct clustering indicates that temperature affects the functional composition of the control group. Similarly, the butyrate samples also exhibited clear separation between 23°C and 30°C. This separation of temperature in these groups could be attributed to the PC1 axis separation (49.3%) Additionally, when looking at antibiotic groups at 23°C and 30°C we do not get distinct clustering but instead overlapping between those groups which can be explained by PC2 (21.6%). These observations suggest that temperature has a pronounced effect on the functional profiles of both the control and butyrate-treated groups but no effect on groups

treated with antibiotics. It appears that the antibiotics exert a dominant influence on community function that overrides the temperature, maintaining similar functional characteristics across different temperatures.

Notably, there was no significant difference between the control and butyrate groups at either temperature. This suggests that butyrate alone does not substantially alter the predicted functional composition compared to the control despite changes in bacteria phyla abundance (Figure 3.3). There was no significant difference between the antibiotic and antibiotic plus butyrate groups, indicating that the addition of butyrate to antibiotic treatments does not further alter the functional profile imposed by the antibiotics alone.

3.3.4.2 Predicted function of bacteria

The predicted functional profiles of microbiota involved in metabolism exhibited significant differences among the treatment groups (Figure 3.5A). Notably, at both temperatures, the control group had the highest proportion of sequences related to metabolism. This was significantly higher than the antibiotics and antibiotics plus butyrate groups. The only temperature-related difference is that the butyrate treatment was similar to controls at 23C but lower than controls at 30C (Figure 3.5A).

These differences suggest that antibiotic treatment may negatively affect the metabolic functions of the microbiota. Additionally, the combination of temperature and butyrate appears to contribute to a greater loss of predicted metabolic function in the microbiota. The predicted functions associated with human diseases varied markedly across the treatments (Figure 3.5B).

The control and antibiotic groups exhibited the lowest proportion of predicted functional disease pathways while antibiotics showed increased proportions. This suggests that antibiotic treatment may enhance microbial functions related to disease pathways. When comparing across temperatures, the control and butyrate groups are significantly reduced in the 30°C temperature. This indicates that higher temperature impacts can lead to a decrease in the predicted function of preventing diseases but are restored by antibiotics. Overall, the predicted function of bacteria involved in disease pathways makes up less than 2% of the function.

There were notable differences in the proportion of sequences involved in cellular processes among the treatments shown in Figure 3.5C. At both temperatures, 23°C and 30°C, the same trend is seen across both treatments. There is no significant difference between the control and butyrate groups which make up ~7% and 8% respectively. The antibiotic treatments do show a significant increase in predicted function regarding cellular processes making up about 9%. Overall, there is no impact of temperature seen across the treatments and all significant differences in the predicted function of cellular processes are due to the involvement of antibiotics.

The predicted functional profiles related to organismal systems also showed significant variation among the treatment groups shown in Figure 3.5D. Specifically, at 23°C, the control group had the highest proportion of sequences, about 1.3%, suggesting a high amount of predicted microbial function related to organismal systems. Furthermore, the butyrate group had a slightly lower proportion at around 1.2%, while the antibiotics and antibiotics plus butyrate groups had the lowest proportions, approximately 0.9% each. Interestingly, at 30°C both control and butyrate are significantly less. Additionally, we find no significant differences across

treatments at 30°C. This decrease indicates that temperature plays a significant role in predicted functional pathways related to organismal systems while at lower temperatures antibiotics cause significant changes as well.

Overall, these results highlight the varying impacts of different treatments on the predicted functional profiles of microbial communities, with the majority of the predicted function of the bacteria being responsible for metabolism and significantly less for cellular processes, disease pathways, and organismal systems.

3.3.4.3 Functional differences regarding temperature

The predicted functional profiles related to metabolism exhibited significant differences between the two temperature conditions (Figure 3.6). Functions such as Amino Acid Metabolism, Lipid Metabolism, and Xenobiotics Biodegradation and Metabolism showed higher proportions at 23°C compared to 30°C. For diseases, the functions related to Cancers and Neurodegenerative Diseases showed notable differences between the temperatures. The proportion of sequences associated with Cancers was lower at 30°C. Additionally, Neurodegenerative Diseases had a higher mean proportion at 23°C. The cellular processes category included functions such as Cellular Processes and Signaling, Membrane Transport, and Transport and Catabolism. The results showed that Cellular Processes and Signaling had a higher proportion at 30°C. Membrane Transport also exhibited a higher mean proportion at 30°C. Conversely, Transport and Catabolism showed a significant decrease at 30°C between the temperatures. The functions related to organismal systems, including Glycan Biosynthesis and

Metabolism, Nervous System, and Sensory System, also exhibited significant variations between the temperatures. Glycan Biosynthesis and Metabolism had a higher proportion at 30°C. The Nervous System function was more predominant at 23°C. Sensory System functions followed a similar trend, with a higher mean proportion at 23°C.

3.3.4.4 Functional differences regarding treatment

The predicted functional profiles related to metabolism showed significant differences between the antibiotic and no antibiotic treatments (Figure 3.7). Functions such as Amino Acid Metabolism, Carbohydrate Metabolism, and Xenobiotics Biodegradation and Metabolism were all significantly decreased in groups with antibiotics. Interestingly, the predicted functions associated with diseases, such as Cancers and Infectious Diseases, varied significantly between the treatment groups. The proportion of sequences related to Cancers was higher in the antibiotic group. Infectious Diseases also showed a higher mean proportion in the antibiotic group. Within the cellular processes category, functions such as Cellular Processes and Signaling, Membrane Transport, and Signal Transduction showed notable differences. Cellular Processes and Signaling had a higher mean proportion in the antibiotic group. Membrane Transport also exhibited a higher mean proportion in the antibiotic group. Similarly, Signal Transduction showed a significant increase in the antibiotic group. Also, the functions related to organismal systems, including Glycan Biosynthesis and Metabolism, Endocrine System, and Nervous System, also exhibited significant variations between the treatments. Glycan Biosynthesis and Metabolism showed a higher mean proportion in the antibiotic group. The Endocrine System function was more predominant in the no antibiotic group.

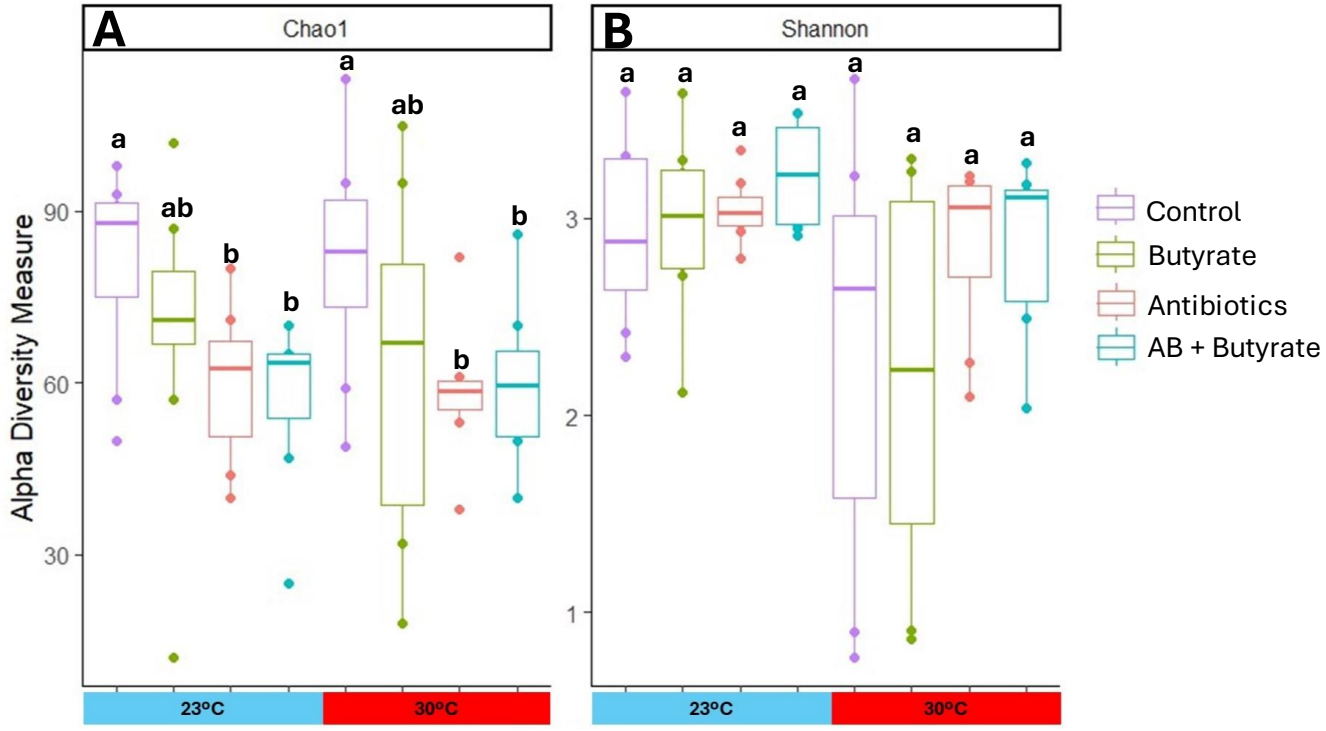
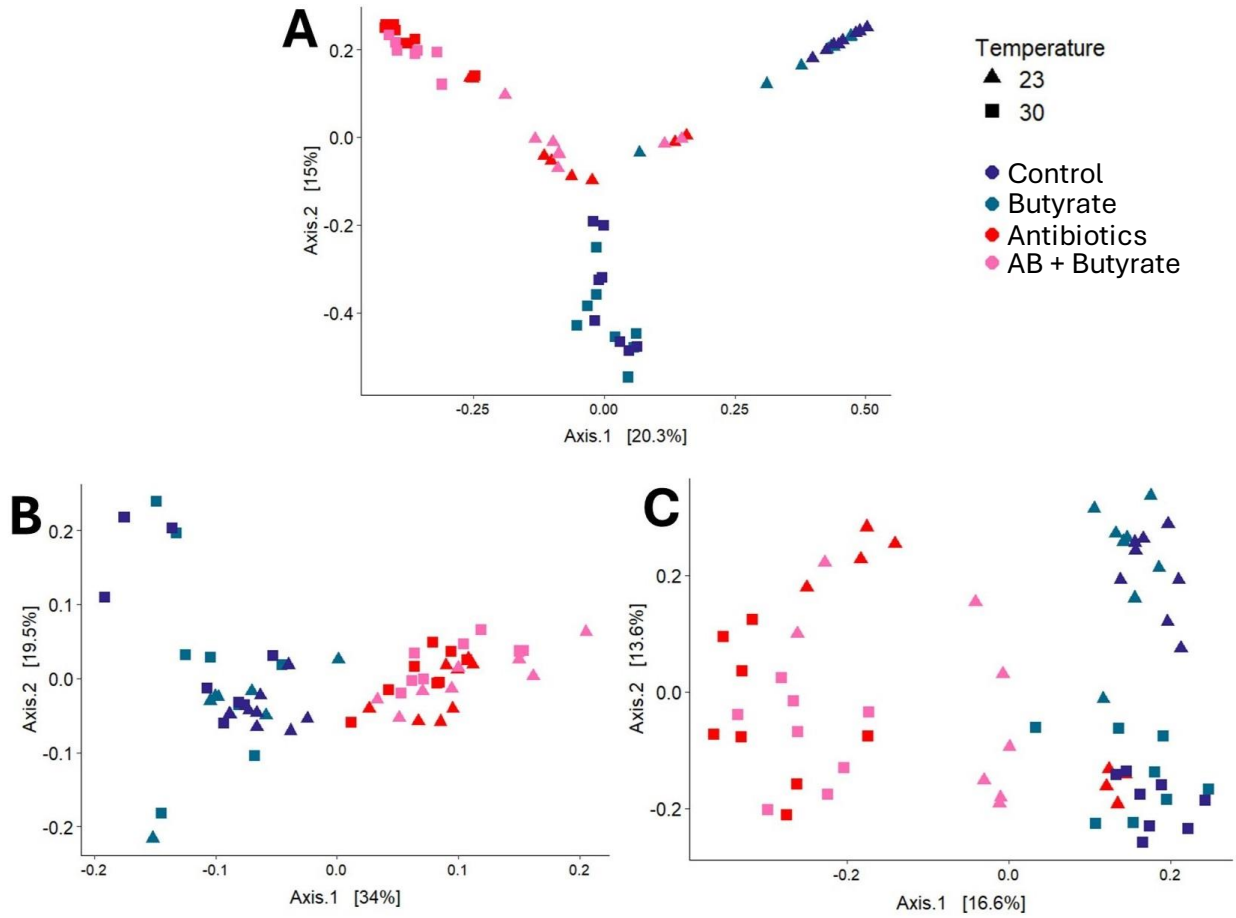


Figure 3.1: Antibiotics impact species richness when comparing Chao1 and Shannon indexes. Boxplots display the Chao1 (A) and Shannon (B) diversity indices for different treatment groups. Boxes that share the same letter are not significantly different from each other (Kruskal-Wallis test, $p < 0.05$).



<u>Figure 3.2(A to C):</u> Beta diversity analysis reveals changes in microbial structure when exposed to antibiotics and temperature using Bray-Curtis (A), Weighted UniFrac (B), and Unweighted UniFrac (C). PCA plot emphasizes phylogenetic relationships between samples regarding treatment and temperature.

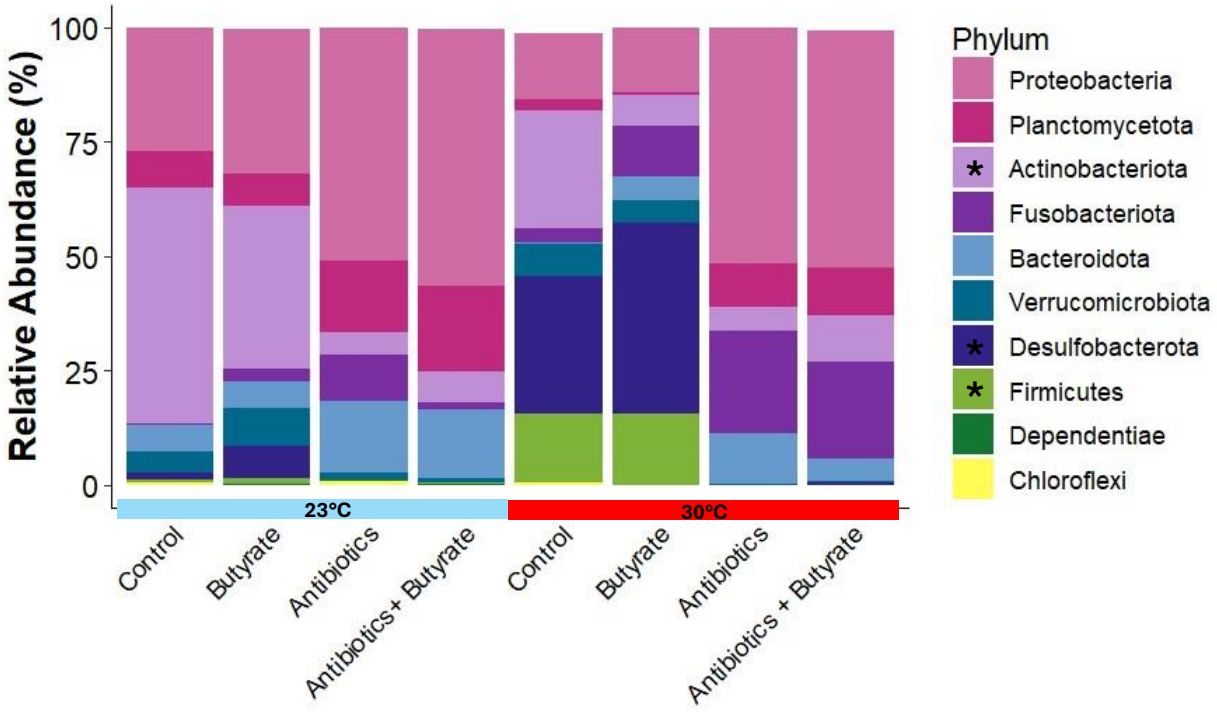


Figure 3.3: Antibiotics reduce bacterial taxa abundances in *Actinobacteriota, Desulfobacterota, and Firmicutes*. The stacked bar plot represents bacteria phyla with each color representing a different phylum. The top 10 phyla are represented. Statistical significance was determined using an ANCOM (p <0.05, n=8). Asterisks indicate significant differences in the specific phylum between treatment groups.

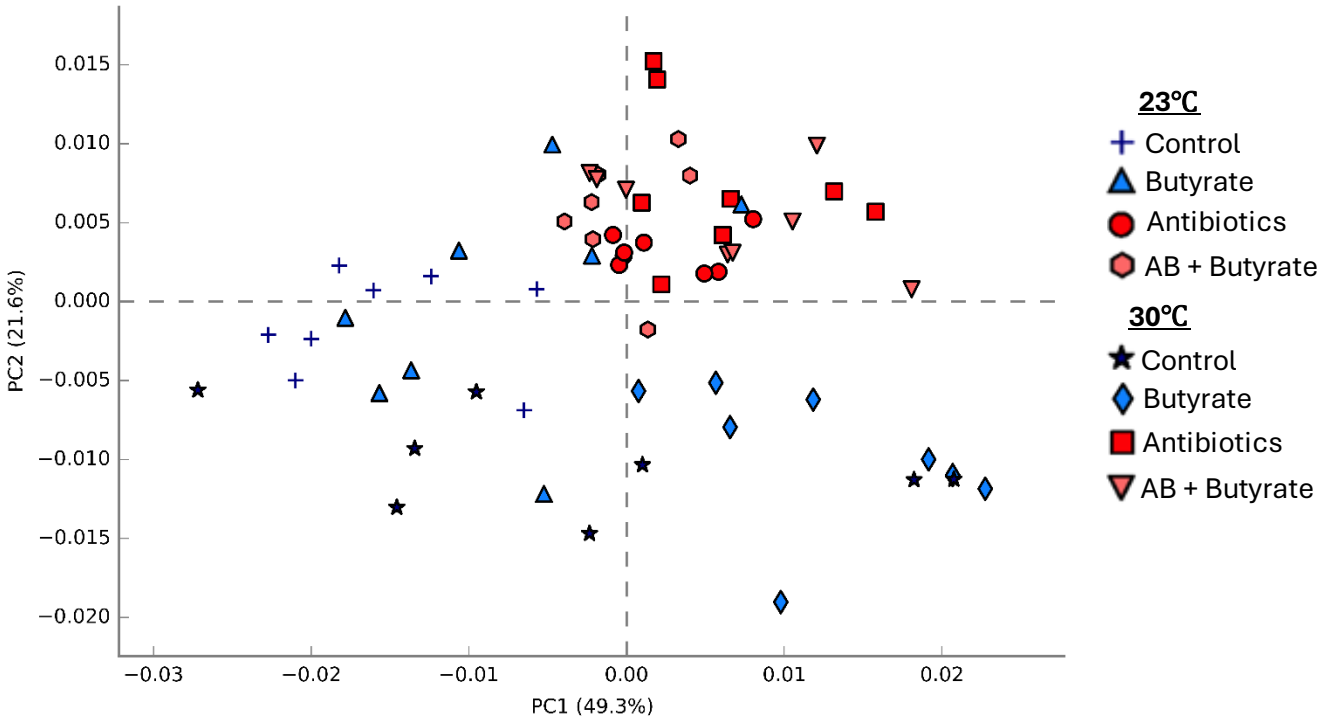
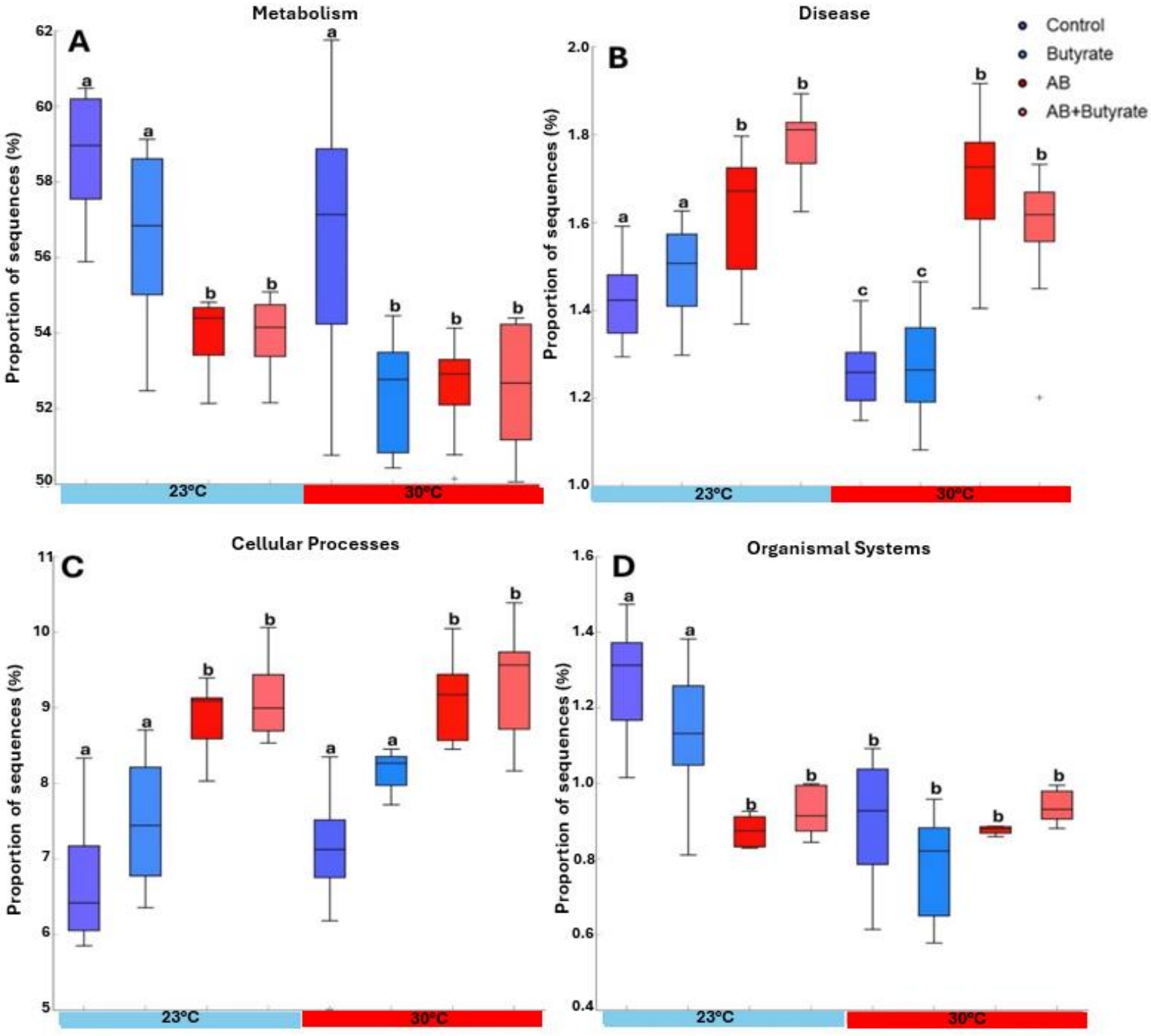
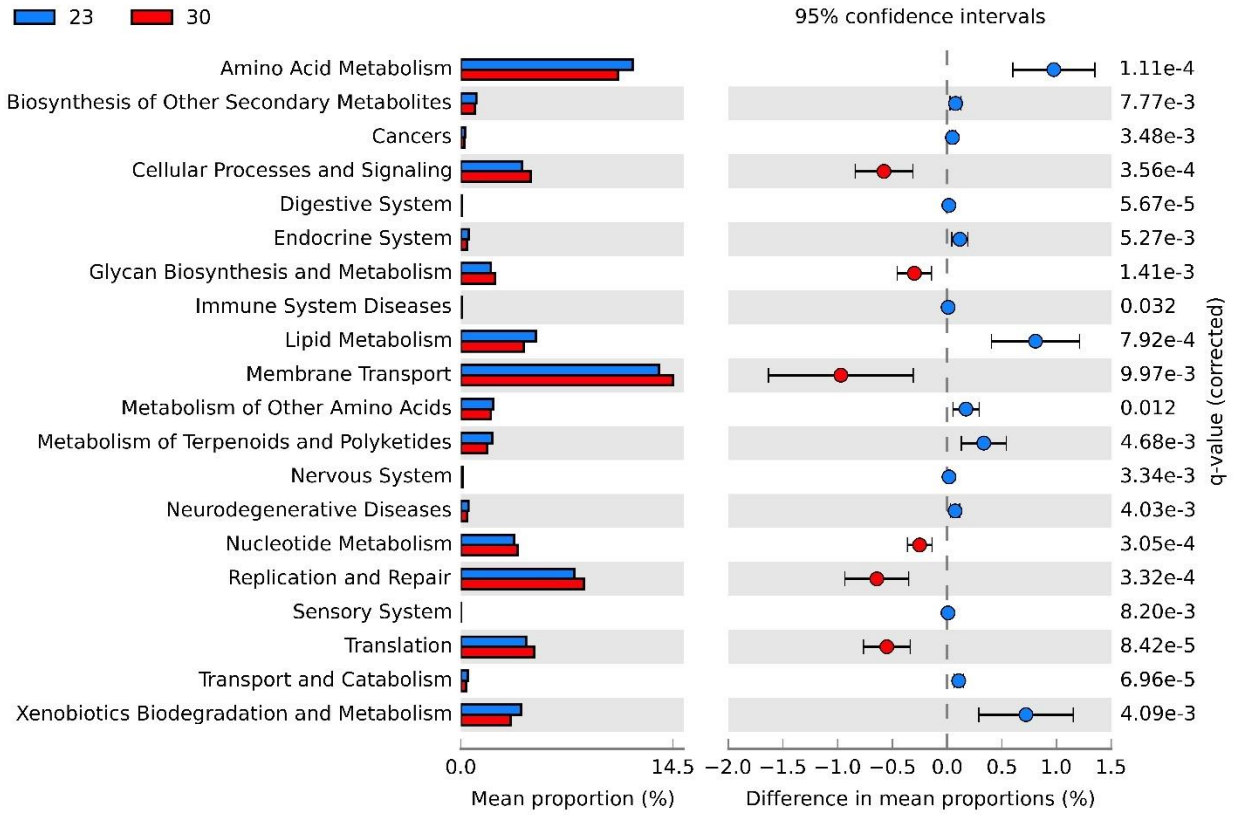


Figure 3.4: Antibiotics and temperature impact predicted bacterial functions in the gut microbiome. The PCA plot emphasizes the predicted functional differences between samples regarding treatment and temperature.



<u>Figure 3.5(A to D):</u> Antibiotics and temperature impact predicted functional profiles of microbiota of metabolism (A), disease (B), cellular processes (C), and organismal systems (D). Data boxes represent means ± SEM. Bars that share letters are not statistically different. Statistical differences were measured using the Tukey-Kramer test followed by the Benjamini-Hochberg post hoc test ($p < 0.05$; n = 8).



<u>Figure 3.6:</u> Temperature reduces predicted functions in health and metabolism pathways in microbial communities. Extended error bar plot for two-group analysis comparison based on temperature using Welch's t-test for two groups. Only predicted functions with $p < 0.05$ are shown. Bar plots on the left side display the mean proportion of each KEGG pathway while the dot plots on the right show the differences in mean proportions.

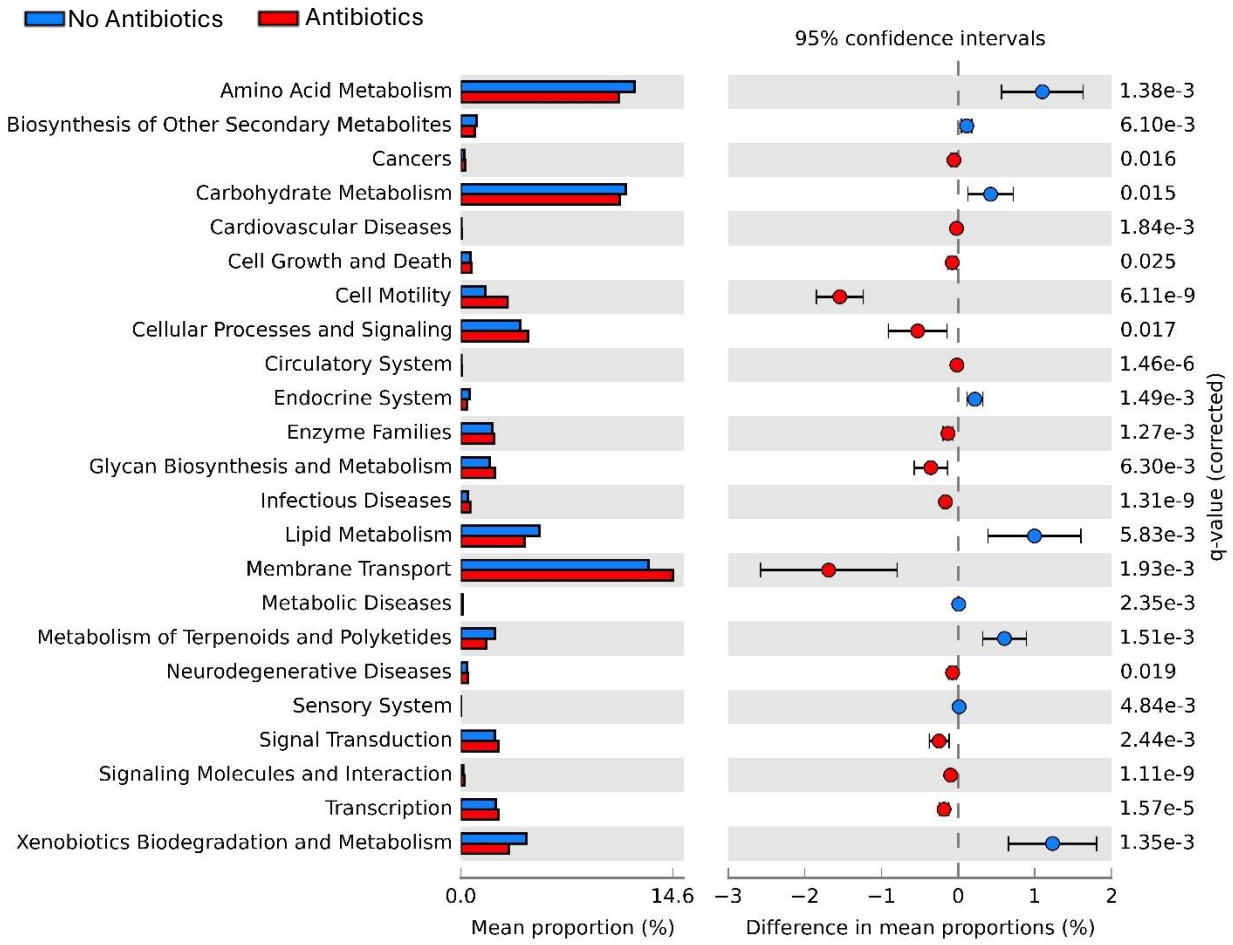


Figure 3.7: Antibiotics reduce gut microbiotas predicted metabolic and health functions. Extended error bar plot for two-group analysis comparison based on treatment using Welch's t-test for two groups. Only predicted functions with $p < 0.05$ are shown. Bar plots on the left side display the mean proportion of each KEGG pathway while the dot plots on the right show the differences in mean proportions.

3.4 DISCUSSION

3.4.1 Impact of butyrate

To understand how butyrate affects microbial communities, we first examined its impact on richness, diversity, and predicted function. These results in conjunction with the growth observed in Chapter 2 will allow us to determine if butyrate's effect influences host and/or microbial functions to influence the physiological changes. Firstly, the Chao1 index, which measures species richness, and the Shannon index, which accounts for species richness and evenness, showed intermediate richness levels in the butyrate group (Figure 3.1 A and B) suggesting that butyrate did not significantly impact microbial richness. Furthermore, our beta diversity analyses (Figure 3.2) indicate that butyrate does not seem to affect the gut microbiome in a large capacity. In contrast to our results, previous studies suggest that butyrate can promote the growth of beneficial bacteria (Knudsen et al., 2018). One explanation for this could be butyrate dosage. Butyrate effects are dose-dependent (Abd El-Naby et al., 2019). Though the effects of butyrate on growth shown in Chapter 2 are significant, the effects on microbial composition may be subtle with this dosage.

Butyrate did not change the alpha or beta diversity (Figures 3.1A and B), which is supported by our lack of change in our taxonomic analysis using ANCOM where we found that butyrate did not have any significant effects on the microbial taxa (Figure 3.3). However, butyrate may still impact microbial communities on a scale not detectable by this statistical method. In broilers and mice, butyrate is a known modulator of microbial communities, influencing bacterial metabolism and interactions (Wu et al., 2018; Yu et al., 2019). It could be an energy source for certain gut bacteria, promoting the growth of beneficial taxa and potentially suppressing pathogenic ones (Wu et al., 2018; Yu et al., 2019). These results suggest butyrate

may exert subtle effects on microbial community structure, which could become more apparent with more sensitive analysis or possibly in the functional profile of the microbiomes.

Now looking at the predicted functional capabilities of the microbiota with butyrate we saw a slightly lower proportion of sequences related to metabolic functions compared to the control, though not significantly different at 23°C (Figure 3.5A). In the context of disease pathways, the control and butyrate groups exhibited lower proportions of sequences related to disease pathways than the antibiotic-treated groups, suggesting a protective effect of butyrate (Figure 3.5B). Thus, butyrate may significantly affect microbial functions to allow for immune-specific signaling pathways. Many other studies have reported in fish that butyrate has increased the immune function and in turn allows for improved intestinal health for improved growth (Tian et al., 2017). Regarding cellular processes, the butyrate group showed no significant differences compared to the control (Figure 3.5C). Additionally, butyrate did not significantly alter the functional profiles related to organismal systems compared to the control group, though it did exhibit a slight decrease (Figure 3.5D). These results for cellular processes and organismal systems suggest that butyrate alone does not substantially impact these functions (Kircher et al., 2022). In total, we see very minor differences in predicted microbial functions except in the immune pathways which show that butyrate effect in the microbiome is minimal or subtle to detection and the most prominent impact appears to be immunological.

Our hypothesis that butyrate would alter the gut microbiome to benefit specific microbial taxa and in turn promote metabolic functions to influence growth was not supported (Wu et al., 2018; Yu et al., 2019). These results suggest that the increased growth from butyrate-fed fish (Chapter 2) must be directly working through the host mainly, although we cannot discount that

some may be attributed to the beneficial immune pathways that may allow for energy to be used for growth over repair (Kircher et al., 2022).

3.4.2 Impact of antibiotics

The results observed in Chapter 2 showed that long-term antibiotic exposure leads to decreased growth although the mechanism was not clear. Here we investigated how antibiotics may lead to gut dysbiosis and to understand how these affect the functional profile of the microbiota. The Chao1 index revealed a significant reduction in the antibiotic-treated groups (Figure 3.1A) indicating that the antibiotics exerted an influence on microbial diversity, reducing rare taxa, which are crucial for community stability and resilience (Shade et al., 2012). Reduced species richness can make microbial communities more vulnerable to environmental stressors, as less diversity limits the range of functions the community can perform (Philippot et al., 2021). While temperature is known to influence microbial community structure (Wang et al., 2018), our results indicate that the effect of antibiotics on microbial richness remained consistent across different temperatures, highlighting the robust impact of antibiotics.

In contrast, the Shannon index showed no significant differences across treatment groups at either temperature (Figure 3.1B). This evenness suggests that, although antibiotics reduced the total number of species, the remaining bacterial community maintained a balanced distribution of relative abundance. This could mean that dominant bacterial species were unaffected by the treatments, while rare species—often crucial for specific functional roles within the community—were disproportionately impacted (Maier et al., 2021). As antibiotics tend to target

a broad spectrum of bacteria, rare taxa may be more susceptible, leading to a reduction in specialized functions, as observed in previous studies (Rosen et al., 2017).

In addition to differences in alpha diversity, beta diversity was also impacted by antibiotics (Figure 3.2A; Figure 3.2B; Figure 3.2C). Specifically, antibiotic-treated groups clustered distinctly from the non-antibiotic groups in the Bray-Curtis PCA, and the weighted and unweighted UNIFRAC PCAs, which indicates that antibiotics are a significant factor in shaping microbial community composition and revealed that antibiotics significantly affect the phylogenetic composition of microbial communities. This suggests that antibiotics exert a broad-spectrum impact, influencing both the abundance and the phylogenetic relatedness of the taxa. This supports the evidence we see in the alpha diversity where antibiotics have a larger effect on rare taxa (Maier et al., 2021) suggesting that we have induced dysbiosis.

In terms of taxonomic differences in the antibiotic-treated fish *Actinobacteriota, Desulfobacterota*, and *Firmicutes* all showed a similar trend of reduced abundance in response to antibiotic treatments (Figure 3.3). *Actinobacteriota*, mainly known for nutrient cycling, were significantly reduced in antibiotic-treated groups at both 23°C and 30°C, which aligns with findings that antibiotics disrupt *Actinobacteriota* populations (Horvathova et al., 2019). *Desulfobacterota* is crucial for sulfur cycling and sulfate reduction and was eliminated in all antibiotic treatments. *Firmicutes*, which are important for fermentation and short-chain fatty acid production, followed a similar pattern, being present in control and butyrate groups but absent in antibiotic-treated groups across temperatures (Sun et al., 2022).

The reduction and elimination of microbial taxa from antibiotic exposure was reflected in the decrease in many predicted functions. Firstly, the control group had the highest proportion of sequences related to metabolic functions, which was significantly lower than in the antibiotics

and antibiotics plus butyrate groups (Figure 3.5A). Antibiotic treatment, as supported by Perez-Cobas et al. (2013), can reduce the metabolic capabilities of microbial communities by disrupting their composition, which impairs the microbiota's ability to perform essential metabolic processes, impacting the host's metabolic health and growth performance. Specifically for metabolic functions, antibiotic treatments resulted in a notable decrease in functions related to amino acid metabolism, carbohydrate metabolism, and xenobiotics biodegradation and metabolism (Figure 3.7). This reduction in metabolic functions can have huge effects on the host, as these pathways are essential for nutrient assimilation. The decrease in these metabolic functions suggests that antibiotics disrupt the microbial community's ability to perform critical metabolic activities, which could lead to impaired growth and overall health of the sailfin molly. This finding is supported by Qian et al., (2021), who showed that antibiotics can significantly reduce growth rates in fish. This impact on the microbiome may be a significant cause of lower feed conversion and absorption rates leading to the decreased growth that we have seen in this treatment (Chapter 2).

In the context of disease related function, antibiotics led to a higher proportion of sequences related to disease pathways compared to the control and butyrate groups (Figure 3.5B). This suggests that antibiotic treatment may enhance microbial functions related to diseases. The increase in disease-related functions in antibiotic-treated groups aligns with findings by Duan et al. (2017), which showed that low concentrations of antibiotics led to an abundance of antibiotic-resistant bacteria that contribute to disease-related pathways. When looking at specific predicted functions associated with diseases, they showed significant variations between the antibiotic treatments as well (Figure 3.7). The higher proportion of sequences related to cancers and infectious diseases in the antibiotic group indicates that

antibiotics might promote microbial functions associated with disease pathways. Bonomo, (2000) shows that there is a trend between antibiotic resistance and infectious bacteria. This overlap of bacteria that can survive antibiotics and have the functions of infectious disease can explain why these functions are more predominant in antibiotic treatments. This increased disease-related functionality is important as antibiotic use may make fish more susceptible to infections and other health issues after treatment.

Antibiotic treatment also resulted in a significant increase in predicted cellular processes at both temperatures (Figure 3.5C), likely due to the upregulation of stress response and cellular maintenance genes in response to antibiotic pressure, as found by Harms et al. (2016). These responses may promote resilience mechanisms but could also lead to the proliferation of antibiotic-resistant genes. Within the cellular processes category, functions such as cellular processes and signaling, membrane transport, and signal transduction were significantly higher in the antibiotic-treated groups (Figure 3.7). Dawan and Ahn (2022) reported that antibiotics could lead to an upregulation of stress response genes and immune responses, which might be also selected for specific bacteria that promote these functions as well. While the initial immune response may be targeting and reducing stress, it may also be permitting bacterial communities that benefit the host's cellular and signaling repair pathways (Limbu et al., 2018).

Lastly, antibiotic-treated groups had the lowest proportions of sequences related to organismal systems pathways, highlighting the disruptive impact of antibiotics on these functions, likely due to the broad-spectrum effect that reduces the functional capacity of microbial communities (Figure 3.5D). The functions related to organismal systems, including glycan biosynthesis and metabolism, endocrine system, and nervous system, also exhibited significant variations (Figure 3.7). The higher mean proportion of glycan biosynthesis and

metabolism in the antibiotic group suggests an increased need for cell wall modifications possibly as a response to antibiotic stress. Additionally, the endocrine system functions were increased in the non-antibiotic groups, indicating that antibiotics might disrupt the contributions to endocrine processes. These disruptions could have implications for the host's hormonal balance and stress responses, as microbial communities play a role in modulating host endocrine functions (Rastelli et al., 2019).

The observed changes in functional profiles as well as bacterial communities due to antibiotic treatments have significant implications for the health and metabolism of the sailfin molly, explaining the results we found in Chapter 2. The reduction in metabolic functions could lead to compromised nutrient absorption and metabolic pathways, negatively impacting growth and overall health. The increase in disease functional pathways suggests a risk of infectious diseases possibly after antibiotic treatment is done, which could further stress the host. Additionally, combining antibiotics and butyrate failed to alter any diversity (Figure 3.1; 3.2) or functional metrics (Figure 3.5). This means that by using butyrate with antibiotics we saw a restoration of growth (Chapter 2) but no improvement in bacterial function. This further supports that butyrate is operating on the host and not the bacteria to provide energy for growth in replacement of microbial functions. Overall, these results indicate disruption to the gut microbiota communities leads to shifts in predicted functions that have significant measurable changes in growth and metabolism shown in Chapter 2. Understanding these functional shifts is crucial for developing strategies to mitigate the negative effects of antibiotics in aquatic organisms.

3.4.3 Impact of temperature

The interaction between temperature and treatment has shown to play a pivotal role in shaping microbial community structure, with this study demonstrating that temperature can have significant impact on physiological changes. While temperature is known to influence microbial community structure (Wang et al., 2018), our results indicate that the effect of antibiotics on microbial richness remained consistent across different temperatures similar to the observed physiological changes. Additionally, temperature played a crucial role in butyrate and control treatments, highlighting that both antibiotics and temperature influence the microbial community structure. Previous research supports these findings, showing that environmental factors, such as temperature, significantly impact microbial community composition (Zhou et al., 2016).

In our study, the effect of temperature was more pronounced within specific treatments, while antibiotics have a dominant effect on community composition that removes the noticeable effects of temperature. Specifically, the Bray-Curtis plot shows dissimilarity between communities based on the abundance of taxa. The distinct clustering observed in the Bray-Curtis plot (Figure 3.2A) suggests that antibiotic treatments significantly alter the microbial community, likely by reducing sensitive species as shown in the alpha diversity tests.

For the predicted functional roles of microbiota at 23°C, the control group had the highest proportion of sequences related to metabolic functions, which was higher than in groups treated with antibiotics or butyrate (Figure 3.5A). At 30°C, the same trend was observed, with the control maintaining the highest metabolic function levels, but the butyrate group experienced a significant decrease in metabolic function compared to its 23°C counterpart. Higher temperatures negatively affected microbial metabolic functions in the presence of butyrate, likely due to the increased volatility of butyrate and higher stress on microbial communities. For specific

predictive metabolic functions, at 23°C, the higher proportions of functions related to Amino Acid Metabolism, Lipid Metabolism, and Xenobiotics Biodegradation and Metabolism suggest that increased temperatures hurt metabolic pathways in the microbiome (Figure 3.6). The decrease in these metabolic functions at 30°C indicates a potential metabolic inefficiency, which is why we might see a compromise in growth and metabolism. The temperature-related metabolic shifts align with findings by Volkoff and Ronnestad, (2020), who showed that temperature significantly affects metabolic rates and nutrient absorption in aquatic organisms. This decrease could be heavily impacted by the removal of important bacterial taxa and the critical functions they provide.

Regarding disease pathways, higher temperature significantly reduced predicted disease-related functions in the control and butyrate groups (Figure 3.5B). Although increased temperatures are generally associated with a higher rate of disease spread (Karvonen et al., 2010), this was not observed here, possibly due to the microbiome's efficiency at higher temperatures in preventing pathogens. Specifically, the differences in disease-related functions between the temperatures further illustrate the impact of temperature on the microbiome. The lower proportion of predictive function in the 30°C treatments involved in cancer and neurodegenerative disease may indicate a lower risk to these pathways (Figure 3.6). Indicating that fish at this higher temperature may be less susceptible to these diseases through these pathways.

For organismal systems, control temperature supported higher functional levels, likely due to optimal growth conditions for microbial taxa involved in organismal systems, such as cell communication and membrane transport (Figure 3.5D). Increased temperature disrupted these functions, which may lead to lower intestinal integrity and nutrient absorption, as found in

studies by Volkoff and Ronnestad (2020) and Wang et al. (2018). The variations in functions related to organismal systems between the temperatures further illustrate the temperature's role in shaping microbial functions. At 30°C, the higher proportion of glycan biosynthesis and metabolism that gut bacteria may shift their metabolic processes to adapt to lower temperatures (Figure 3.6). This could be a selective response to the potential weakening of gut integrity at these temperatures. Other research has shown that temperature has a profound impact on shaping microbial composition to combat environmental stress (Huus and Ley, 2021). As we see here, growth may be limited at higher temperatures due to the microbiome's stress.

The effects of temperature were independent of cellular processes, with the control and butyrate groups displaying no significant changes across temperatures (Figure 3.5C). In cellular processes, the higher proportion of functions related to cellular processes and signaling and membrane transport at 30°C suggests an increased cellular activity and communication (Figure 3.6). Additionally, the significant decrease in transport and catabolism functions at 30°C indicates potential a disruption in nutrient transport and degradation processes, which could impair energy balance and metabolic efficiency further impacting the growth and metabolism we have seen (Suzuki and Mittler, 2005). As we know temperature can put stress on cellular processes that may not be able to cope with increased temperatures (Suzuki and Mittler, 2005).

The results highlight the significant impact of temperature on the predicted functional profiles of microbial communities. Overall, we find different expressions of disease functions suggesting that temperature changes could alter disease susceptibility and stress responses in the sailfin molly. These functional shifts have implications for the growth, metabolism, and overall health of the host and support our hypothesis that temperature would disrupt the microbial communities. By connecting the results from Chapter 2, the impacts of temperature on the

microbiome predictive functions may be reducing the overall growth rates within the sailfin molly that we observed. These complex interactions between temperature, microbial functions, and host health show the importance of maintaining optimal environmental temperature to support the health and growth of aquatic organisms.

3.4.4 Conclusions

This study investigated the individual and combined effects of butyrate, antibiotics, and temperature on the gut microbiome of the sailfin molly. The goal was to elucidate the interactions within the microbiota and how these translate to changes in microbial functions. Overall, these results allow us to link predicted functional changes in the microbiome to the physiological changes that were observed in Chapter 2. Additionally, by employing PICRUSt for predictive functional profiling, I wanted to understand any putative variations in metabolic functions, disease-related pathways, cellular processes, and organismal systems across various treatments and temperatures. These findings reveal that antibiotics have an influence on microbial diversity and lead to a reduction in the richness of bacterial taxa. Additionally, this leads to microbial functional diversity often reducing metabolic activities and enhancing disease-related functions possibly explaining the decrease growth seen in Chapter 2. Additionally, temperature changes significantly affect microbial functions, particularly those related to metabolism and organismal systems. These results illustrate the complexity between environmental factors and chemical interventions in shaping microbial communities and functions.

Our hypothesis that butyrate supplementation would increase the abundance of beneficial bacteria such as *Firmicutes, Proteobacteria, and Actinobacteria*, was not supported and showed no significant impact. These results indicate that butyrate increased growth (Chapter 2) most likely directly working through the host regardless of bacterial presence, instead of altering the microbiome to allow for more nutrient uptake. However, butyrate's role may be more subtle in terms of shaping microbial communities and functions that cannot be detected by broad testing methods. Additionally, we hypothesized that antibiotics would create dysbiosis in the gut microbiome, leading to a reduction in microbial diversity and richness. This hypothesis was supported as diversity and richness decreased. The impact of elevated temperature was complex, partially supporting our hypothesis. While higher temperature did not reduce microbial diversity, it did affect metabolic functions.

The study provided insights into how butyrate, antibiotics, and temperature individually and in combination affect the overall microbiome and functionality. Antibiotics had the most profound impact, causing significant dysbiosis and reducing beneficial microbial functions. While elevated temperatures also negatively impacted microbial diversity and metabolic functions. Butyrate's effects were less clear, suggesting a need for further research to determine its role under different conditions. Understanding these interactions can help develop strategies for optimal aquaculture practices. The results contribute to a broader understanding of how environmental and chemical factors influence the gut microbiome and, the health of aquatic organisms.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

4.1 Concluding remarks

This study investigated the individual and combined effects of butyrate, antibiotics, and temperature on the gut microbiome and physiological functions of the sailfin molly. The findings provide informative information on how these factors influence microbial communities on a compositional and functional level and how those translate to physiological changes in the sailfin molly. Finally, these discoveries could be applied to optimize growth and microbiome compositions in aquaculture to promote healthier and more sustainable fish production.

The results demonstrated many trends for us to understand the interactions and use of butyrate supplementation. Butyrate, opposite to our hypothesis, did not significantly increase the abundance of beneficial bacteria such as *Firmicutes, Proteobacteria,* and *Actinobacteria*. Overall, we found no significant differences in microbial diversity and predicted functional changes. Therefore, we conclude that butyrate's impact is through its energy production directly to the host. Butyrate significantly increased growth, supporting our hypothesis.. Interestingly, butyrate also led to a decrease in maximum metabolic rate and aerobic scope, suggesting that it promotes growth by redirecting energy towards maintenance and growth rather than supporting high metabolic activity needed from survival responses. This unexpected result highlights the trade-offs in metabolic allocation induced by butyrate. Next, antibiotic treatment created significant dysbiosis in the gut microbiome, leading to a reduction in microbial diversity and richness, primarily due to the disappearance of rare taxa. This finding aligns with our hypothesis, resulting in a loss of beneficial microbial functions and an overall decline in community resilience. Additionally, this correlates with the decreased growth rates and concludes that the reduction in beneficial bacteria in the microbiome is having a direct impact on the growth of the

sailfin molly. Antibioticsdecrease growth without altering metabolic rates. This suggests that antibiotics disrupt growth through mechanisms other than metabolic rate modulation, most likely by inducing dysbiosis and impairing nutrient utilization. Interestingly, butyrate was able to counteract the negative effects of antibiotics on growth, making it a promising additive for aquaculture to optimize fish growth despite antibiotic use. This finding is consistent with our hypothesis and demonstrates butyrate's potential to mitigate some adverse effects of antibiotics. Higher temperatures did not promote additional growth but did show significant increases in metabolic rates. Elevated temperatures were the other main effect that showed a significant interaction with the microbiome. While higher temperatures did not reduce microbial diversity, they did negatively impact metabolic functions. This partial support of our hypothesis suggests that temperature influences microbial communities. When compared to the growth results, we can conclude that the increase in temperature is impacting microbial functions that are overall reducing the growth rates that we predicted to see. Additionally, we found data that supports that increased temperatures are causing other prolonged health problems which may be attributed to the stress of the animal. These results highlight the ways environmental factors can modulate microbial communities and their functions.

Overall, the results emphasize the need for further research on how environmental factors such as temperature interact with diet to influence fish microbiota, metabolism, and health. Understanding these interactions can help develop strategies for managing microbial communities to maintain fish health and optimize growth in aquaculture. This study contributes insights into the functional changes in the microbiota and their implications for the growth and metabolism of fish species, offering potential applications for aquaculture management.

4.2 Limitations and future directions

While this study provides valuable insights into the interactions between butyrate, antibiotics, temperature, and the gut microbiome, several limitations should be acknowledged. The scope of functional analysis was restricted to predictions rather than direct measurements, highlighting the need for future studies to incorporate more advanced meta-analysis. Focusing exclusively on the sailfin molly limits the generalization of the results, so more comparative studies across multiple fish species are important to determine whether the effects are consistent or species-specific. Also, measuring oxygen consumption using intermittent respirometry assumes that the metabolic rate remains constant during the measuring period. However, during regular activities metabolic rates can fluctuate due to factors such as stress or environmental conditions that should also be considered in more applicable situations. Furthermore, measuring the total metabolic rate only gives a general sense of energy expenditure. Oxygen consumption only reflects aerobic metabolism but does not consider anaerobic generated energy. This total activity could be underestimated when not considering all energy allocation pathways provided by the microbiota or anaerobic pathways. A possible solution may be using oxygen-to-nitrogen ratios. As oxygen, becomes depleted, anaerobic pathways such as glycolysis will kick in and cause a release of nitrogen. Measuring oxygen to nitrogen ratios may be a way to identify and quantify anaerobic energy consumption to get a full profile of the energy use. Next, conducting these experiments under controlled laboratory conditions may not fully replicate the variability of natural environments. Future directions should include long-term studies to assess prolonged effects after each treatment to understand the severity of each condition to a fuller effect. Understanding microbial interactions and host physiological responses will enhance our knowledge of the mechanisms of microbial communities and their impact on host health,

ultimately contributing to the development of effective strategies for managing microbial communities and optimizing fish health and growth in aquaculture.

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