The Phage Behind the Curtain: Determining the Role of Bacteriophage in the Restructuring of Microbiomes During Disease

by

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Abstract

The gut microbiome is a dynamic microbial ecosystem inhabited by all domains of life and is essential for the homeostatic function of almost all organ systems within the host. Additionally, the mammalian intestine is home to the largest immune network within the body and is tasked with the challenge of maintaining tolerance to commensal microorganisms while also retaining the ability to respond to invading pathogens. In steady state, the gut microbiome is dominated with Bacteria and Bacteriophage, with smaller populations of Archaea and Eukaryotes. In times of disease, such as Obesity, there is a deviation from the steady-state composition of the microbiome that potentially worsens the severity of disease. Though many studies have explored the link between the microbiome and disease state, many questions remain unanswered. For example, much less is known about how other constituents, such as Bacteriophage, contribute to disease. Further, many of these studies focused on the connection between the microbiome and the fully developed disease. Therefore, the purpose of this dissertation was to determine how bacteriophage might change and contribute to the development of disease and disentangle a possible mechanism by which this occurs. Using a novel model of obesity, the Mangalica pig, I found that bacteriophage populations rapidly change in response to the development of obesity, while bacterial populations were much more resilient over the course of 18 weeks. Obesity is associated with low-grade, chronic inflammation. Therefore, I also aimed to determine how immune products might lead to changes in bacteriophage populations seen during obesity. To do this, I characterized bacteriophage reproduction in the presence or absence of immune stressors in two different bacteriophage species: the virulent bacteriophage PF2 and the temperate

bacteriophage Lambda. I found that immune stressors inhibited adsorption of PF2, but not Lambda, to its host in a dose-dependent manner. Additionally, hydrogen peroxide, but not hypochlorous acid, decreased progeny production in PF2. Finally, hydrogen peroxide, but not hypochlorous acid, led to an increase in prophage activation in bacteriophage Lambda. Taken together, my dissertation highlights the intricacy and interplay between bacteria and bacteriophage during the development of disease.

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Chapter 1

Literature Review

Introduction to the Gut Microbiome

Microorganisms can be found in almost every environment on earth. Traditionally, these microbes have been viewed as a single species interacting with its environment – such as a pathogenic bacterium invading a host. With recent advances in culture-independent technologies, a new appreciation for the microbiome, a complex community of microorganisms interacting with its environment, has begun to emerge. Almost all environments have an associated microbiome, such as soils, oceans, and most organisms [1]. Within Metazoans, microbiomes can be found in and on various body sites, such as the skin, lung, and the intestinal tract[2].

The intestinal microbiota is a complex community containing trillions of microbial cells, including bacteria, viruses, eukaryotes (such as fungi) and archaea. A vast majority of these microorganisms are considered commensals or mutualistic[2]. Though all domains of life are represented in the intestinal microbiota, Bacteria are by far the best characterized and contribute most to the overall biomass[3]. Collectively, the gut harbors over 100 trillion bacteria, representative of over 11 phyla and roughly 5000 species [3–5]. Within Bacteria, the gut is dominated by 4 main Phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia, with Bacteroidetes and Firmicutes being the most abundant [6]. The collective genome, known as the metagenome, of the microbiome is comprised of roughly 3 million genes - 150 times larger than the human genome[5]. One often overlooked component of the microbiome are viruses that predate on bacteria, known as bacteriophage, which represent a significant selective force on a microbial community. Until recently, phage have evaded commonly used sequencing

technology, such as 16S sequencing. Given the advancements in shotgun metagenomic technology, the impact of bacteriophage on their community and environment have only begun to be appreciated. Though Bacteria have been thought to make up much of the microbial community in the gut, bacteriophage have recently been found in equitable numbers to Bacteria[7]. However, much less is known about bacteriophage function and composition in the gut.

The specific microbial composition of an individual is influenced by a variety of extrinsic factors, which include birth route, diet, antibiotic usage, and geographical location[8–11]. It's important to keep in mind that even though certain factors can be linked to certain microbes, causal mechanisms for these connections have just begun to be explored. In addition to this, interindividual variability exists, making it hard to truly determine what represents a 'healthy' and 'diseased' microbiome. Recently, a 'healthy' microbiome has been described as one that is temporally stable, and can handle transient stressor such as travelling, diet change, and antibiotic usage.

Environmental Factors that Influence the Form and Function of the Gut Microbiome

Environmental factors shape the microbiome starting immediately at birth. It's been observed that infants born via Caesarian section have a much lower abundance of microbial taxa that are considered commensal microbes, such as multiple *Bacteroides* strains, and a higher abundance of various opportunistic pathogens, including *Enterococcus*, *Enterobacter*, and *Klebsiella* spp., as compared to infants born via natural birth[12]. Antibiotics, even given for a few days, can disrupt a stable microbiota. When healthy male patients were treated with an antibiotic cocktail over a 4-day period, it took an average of 1.5 months for their gut microbiota to return to baseline. Researchers also noted that 9 beneficial species, found in the gut before antibiotic

treatment had not returned after 6 months[9]. These included members of the *Bifidobacterium* genera, and butyrate-producing bacteria including members of the *Coprococcus* and *Eubacterium* genera.

The eons of coevolution have led to an interdependent relationship between a microbe and its host, and this stands true in the relationship between the gut microbiota and host[13]. The gut microbiota has been implicated in multiple functions that benefit the host; its effects are far reaching and can influence every organ in the human body[13]. It has been shown to be so important to normal function of the host, it has been dubbed 'the newest organ'. The host relies on the microbiome for functions such as protection against pathogens[14], nutrient metabolism[15], education of the immune system and correct intestinal development[16,17], and energy biogenesis[18] (5-10% of the host's daily requirements).

The gut microbiome is required for the correct development of the overall gut structure and education of the mucosal immune system. The role for the gut microbiota in these processes became quite clear in many studies performed in germ-free (GF) mice. In terms of intestinal structure and biology, GF mice have many irregularities when compared to conventional counterparts. Overall, the gastrointestinal tract of GF mice has a reduced mass and surface area, with thinner villi and shallower crypts, with the small intestine being most affected[19]. In addition, GF mice have a weakened intestinal barrier due to a decrease in tight junction expression, which leaves them more susceptible to intestinal injury[20].

Germ-free mice also harbor an underdeveloped immune system, with both the innate and adaptive immune system being affected[19,20]. Antigens and pathogen-associated molecular patterns (PAMPs) are extremely important to the development and continual education of the host's mucosal immune system. In the absence of the gut microbiota, the gastrointestinal tract is

characterized by an overall dampened presence of the immune system in the lamina propria[5,20]. It's important to note that it is not just the mere presence or absence of the gut microbiota that can have profound effects on the host, the composition of the gut microbiota can also greatly influence the health and function of the host.

As mentioned above, the gut metagenome is comprised of roughly 3 million genes, and many of these genes encode for metabolic enzymes not present in the host genome. Microbes in the gut gain access to nutrients in the food the host ingests, and in turn produces a large variety of metabolites that have can profound effects on host physiology[21]. Claus *et al* highlighted this in their study, in which they saw significant effects on the metabolites present in not just the gastrointestinal tract, but also in organs such as the liver and kidneys of germ-free mice[22]. Though the gut microbiota metabolizes many substrates present in our diet, they most notably metabolize microbe-accessible carbohydrates (MACs) for the host by way of fermentation. A variety of bacteria, such as *Faecalibacterium prauznitzii*, and *Clostridium spp.* contain numerous glycoside hydrolases that aid in the breakdown of molecules such as cellulose and starch that the host is unable to metabolize themselves [23,24]. From this process, several important byproducts for the host are produced, increasing the energy harvested from their diet. One of the primary end products from the fermentation of MACs are short chain fatty acids (acetate, propionate, butyrate), which contribute 5-15% of the total caloric requirement in humans[25,26].

There is a major intersection between the effects of the microbiome on host physiology, and the extrinsic and intrinsic factors that influence the composition of the microbiome. The microbes present or absent within the microbiome dictate how it will influence host physiology. This is exemplified most obviously in terms of diet. Diet is one of the most influential factors that determines the composition of the microbiome, and the link between diet and host

health/physiological status lies within the function of the gut microbiome[27]. This intersection between diet, the gut microbiome, and host health has been a point of discussion for the past two decades. The macromolecules that make up a specific diet, namely carbohydrates, protein, and fats, all have specific effects on the form and function of the microbiome. Of these, carbohydrates and fats have been best characterized[27].

Dietary carbohydrates represent a dichotomy; as a diet high in complex or simple carbohydrates have opposing effects on the microbiome, and ultimately host health status. Overall, diets high in simple sugars seem to negatively impact the microbiome and host health. Mice fed a high-fructose or high-glucose diet for 12 weeks displayed a decrease in overall diversity of the microbiome, with a reduction in organisms belonging to the Bacteroidetes phylum, and an increase in *Desulfovibrio* spp. Along with this, mice fed these high sugar diets became insulin resistant, and had a higher fat mass, but not an increase in body weight, as compared with the control[28]. The host effects of a high-fructose diet have been confirmed in a more recent study, but the impact of this diet on the microbiome remains unclear, as this most recent study produced conflicting results[29].

In contrast, diets high in complex carbohydrates, such as the MACs discussed above, seem to have positive influences on the microbiome and host health status. David et al. found that when participants were fed a plant-based diet for 5 days, populations of carbohydrate-fermenting microbes, such as *Roseburia* spp. and *Faecalibacterium prauznitzii*, expanded and short-chain fatty acid (SCFA) production increased as compared to those fed an animal-based diet[30]. This pattern was confirmed in a more recent study, in which participants were fed a diet high in resistant starches for 2 weeks. Again, this drove the expansion of SCFA-producing bacteria, such as *Faecalibacterium, Roseburia,* and *Ruminococcus* spp[31]. Using metabolomics

and metaproteomics, Maier et al. were able to explore the changes in protein expression and metabolite production of the microbiome. They found that a diet high in resistant starch significantly increased several proteins involved in carbohydrate metabolism and transport, specifically ones involved in butyrate metabolism, as well as a general increase in butyrate production[31].

Like dietary carbohydrates, dietary fats have been highly studied. A diet high in fats has a significantly negative effect on both the host and gut microbiome. Bisanz et al. conducted a robust meta-analysis examining the response of the microbiome to a high fat diet[32]. Across multiple alpha diversity measurements, there was no correlation between a decrease in diversity and a high fat diet, a proposed metric of an 'unhealthy' microbiome. Using a machine learning approach, they identified 228 OTUs that were associated with a high fat diet, with a majority belonging to the families Lachnospiraceae, Ruminococcaceae, and S2407 Murilbaculaceae[32]. The high fat diet is tightly linked to poor health status; in particular diet-induced obesity[27].

Gut Microbiome and Obesity

Recently, obesity has become an epidemic in some highly developed countries. In 2018, 42.4% of adults in the United States were considered obese[33]. Obesity can generally be described as an over-accumulation of adipose tissue, that is most often accompanied by chronic, low-grade inflammation [34]. Additionally, obesity often facilitates the development of many comorbidities, such as cardiovascular disease, type 2 diabetes mellitus, and certain types of cancers- these together represent a larger collection of features belonging to Metabolic Syndrome [35]. The etiology of obesity is complex and not fully understood but is thought to involve interactions between genetic, behavioral, and environmental factors acting through multiple physiological

mechanisms to increase adipose tissue deposition. Genome-wide association studies have identified more than 300 genes that could contribute to obesity[35]. It's important to note that we cannot rely on sequencing studies alone to truly determine whether there is a causal link between a given gene and obesity; mechanistic studies need to be performed and have been done in a few cases[36–38]. Though these causal links exist, it's important to put them into context along with strong environmental factors, such as the gut microbiome.

The connection between obesity and the gut microbiome was realized almost 2 decades ago. Bäckhed et al. observed that germ-free (GF) mice seeded with a conventional rodent gut microbiome had a 60% increase in fat mass within 14 days[39]. This supported the gut microbiome playing a role in energy harvest. Since then, this idea has been corroborated by additionally transferring the gut microbiomes of diet-induced obese mice or obese humans, both of which induced an obese phenotype in GF mice [40,41]. More recently, many studies have highlighted that it isn't only the mere presence of the microbiome that contributes to the development of obesity, the microbial composition, which influences the function of the microbiome, is crucial to its role in metabolic homeostasis. Several observational and mechanistic studies have been performed exploring the role of the gut microbiome and the development of obesity, largely executed in humans as well as mouse models. However, it has been difficult to determine specific microbial signatures of obesity. Initially, it was thought that an increased Firmicutes:Bacteroidetes ratio was a hallmark signature of obesity. This was observed in both obese animal models as well as obese human subjects[39,40,42,43]. It was proposed that the mechanistic underpinnings of this phenomena lie within the ability of microbes belonging to the phyla Firmicutes to harvest energy from a diet more efficiently that microbes belonging to the phyla Bacteroidetes[40,44]. However, other studies have not been able to confirm this trend[45–47]. This includes 3 meta-analyses, in

which very few associations between distinct microbial signatures and an obese phenotype could be found [48–50]. However, the most recent study investigating this link, amongst other studies, did find significant associations between an increase in *Clostridium XIVa* and *Roseburia* spp. and an obesogenic phenotype[45]. In addition to tracking compositional shifts in the microbiome, researchers have gained insight on how the functionality of the microbiome changes in response to obesity, and how that might affect the development of this disease. In a study by Zierer et al., they found a significant correlation to a dysregulation in the fecal metabolome among obese patients[51]. Specifically, they found an enrichment of 48 metabolites, including amino acids and fatty acids[51]. Based on the information above, it's clear that additional studies need to be performed to determine the true association between the gut microbiome and obesity. Further, most studies look at the end point of obesity. <u>This creates a need to determine how the microbiome restructures in response to the development of obesity</u>. In addition to this, much less is known about how other members of the microbiome, namely bacteriophage, contribute to the development and maintenance of obesity.

Intestinal Bacteriophage and their Influence on the Gut Microbiome

In recent years, it has become clear that bacteriophage, bacteria-targeting viruses, contribute greatly to the form and function of the gut microbiome. Intestinal bacteriophage, termed the phageome, make up a significant portion of the virome, and has only begun to be appreciated, thanks to technological advancements in metagenomic sequencing. It was originally thought that bacteriophage were less numerous as compared to their bacterial hosts in the gut microbiome, but recent estimates argue that they are nearly in a 1:1 ratio with their host.

Depending on their lifestyle and host range, the impact of bacteriophage species on the gut microbiome can vary.

Bacteriophage replicate through 2 life cycles, and, depending on whether they utilize one or both strategies, can be categorized into 2 different lifestyles. Virulent bacteriophages replicate strictly through the lytic pathway, while temperate phages can utilize both the lytic and lysogenic pathway. Phages undergoing the lytic life cycle begin by infecting their host and exploiting the host machinery to produce new phage particles (20-200 new virions/host cell) within 30-60 minutes. These new virions will then lyse the host cell and go on to infect sensitive hosts in the vicinity. In stressful conditions, such as nutrient stress, lytic replication of virulent phages can pause for an extended time. This process, termed pseudolysogeny, is poorly understood, but is hypothesized to occur in the intestinal environment[52]. Temperate phages use a mix of the lytic and lysogenic lifestyle, most often referred to as the 'lytic-lysogenic decision'. Initial infection with temperate phage is followed by the lytic cycle, like virulent phages, or they utilize the lysogenic cycle, in which the phage becomes dormant, and its genome is either integrated into the host genome or resides as an extrachromosomal element within the cytoplasm. At this point, many of the phage genes are repressed and the prophage resides in its host cell until an activation cue is detected. The rate and fate of the lysogenic decision depends on numerous variables, such as host-cell density, phage-phage interactions, and other environmental cues[53,54].

Once integrated as a prophage, most are highly stable. However, external cues and stressors can trigger their induction, leading to the completion of the lytic cycle. For example, the extensively studied bacteriophage Lambda uses an intricate system of repressor proteins to maintain lysogeny. The maintenance of lysogeny in Lambda relies on the repressor protein cI,

which represses the expression of lytic phage genes while activating its own transcription through the regulation of other proteins, namely cII[55]. When environmental stressors or DNA damage is sensed through the SOS response, the Cro protein decreases expression of the cII protein, which in turn lowers cI expression, allowing the lytic cycle to occur.

In vitro, rates of spontaneous prophage induction tend to generally be low. However, studies have shown that prophage activation tends to by higher in the gut than in classical *in vitro* cultures, most likely due to increased activation of the DNA damage SOS response[56]. For example, Quinolone usage has been the most described prophage inducer in the intestinal tract, due to its ability to cause DNA double-stranded breaks[57]. More recently, other groups have highlighted the ability of microbiome-generated metabolites as inducers of prophages[58]. However, the literature still lacks clarity on how other components of the gut ecosystem, such as the intestinal immune system and its products, influence prophage activation.

Much like the bacterial community within the gut, the virome is a highly dynamic and complex community. However, until recently, the composition and function of the virome remained elusive. This has been due to the experimental limitation in isolating phages from stool samples and genome annotation. Because phage lack a universal gene marker, like the bacterial 16S sequence, a whole genome sequencing approach must be taken. In addition to this, gut phage genomes are small, averaging around 30kb, and have a highly variable structure[59]. Some groups have found that up to 90% of gut-derived viral sequences share little to no homolog to current reference databases; but in recent years, efforts have been made to curate databases specifically for gut-derived bacteriophages[7,60–62]. Though the contribution of bacteriophages in the gut has started to be explored, their overall effect on the form and function of the gut microbiome remains poorly understood.

In the context of community structure dynamics, there have been multiple attempts to utilize ecological models to describe host-phage interactions in the gut. Among them, 2 have been cited most frequently: the "kill-the-winner" model and the "piggy-back-the winner: model. The "kill-the-winner" model describes an environment in which the most abundant bacterial hosts are killed by their phages, allowing another bacterial population to take over the empty niche and subsequently be killed by their phages[63]. However, this dynamic model disregards lysogeny and extrinsic cues that could influence the lytic-lysogenic decision, and it's been reported that temperate phage dominate gut phageome[64]. Considering this, the "kill-the-winner" model does not seem to fully describe the relationship of phage and host within the gut. On the other hand, the "piggyback-the-winner" dynamics model considers the lytic-lysogenic decision in the context of host cell density. Traditionally, it was thought that temperate phage enter into lysogeny when the ratio of bacteriophage:host was high. However, it has been recently proposed that phage lysogeny is favored at both low and high densities of their bacterial host[65]. Because of this, phage can "piggyback" from the success of their host in the gut by maintaining lysogeny. It's important to keep in mind that these models have limitations, as it does not reflect major extrinsic factors affecting bacterial composition, and further bacterial physiology, such as diet and disease state.

Unlike the bacterial portion of the gut microbiome, there is still not a consensus of whether there is a stable, core collection of phages shared across populations. In a landmark study, Shkoporov et al. performed a longitudinal metagenomic analysis on 10 healthy human subjects for a year to attempt to determine whether there is a shared, stable core of phages residing in the gut[7]. They found that the phageome of each individual was specific to the host, but temporally stable. Amongst this study and others, bacteriophage belonging to the families

Myoviridae, Siphoviridae, and Podoviridae (order Caudovirales) are most abundant, followed by CrAss-like phages and Microviridae[7,62,66]. However, it seems that the small core of CrAss-like phages and Microviridae are most common among individuals. These above-mentioned groups of phages target well known gut symbionts belonging to the 4 most prominent bacterial phyla within the microbiome, most notably the genera *Bacteroides* (CrAss-like phages and Microviridae), *Parabacteroides*, *Prevotella*, *Faecalibacterium*, and *Clostridium*[7,62,66]. In addition, Shkoporov et al. observed "transient" communities of phage that targeted genera such as *Streptococcus* and *Escherichia.* At first glance, one might assume that the presence of a long-term, stable phageome would support the "piggyback-the-winner" dynamic model being utilized in the gut. However, Shkoporov et al. found no evidence to support the idea that the temperate lifestyle is dominant within the gut microbiome and suggests that mechanisms other than lysogeny might be used to maintain stable populations of virulent phages at high levels[7]. Further work to elucidate this mechanism is warranted.

Intestinal Bacteriophage and Disease

Like the bacterial populations in the gut, alterations in gut phage populations have been linked to certain diseases, namely irritable bowel disease (IBD) and, more recently, obesity. Initial reports linked an expansion of bacteriophages in patient with Crohn's Disease and Ulcerative Colitis, inflammatory diseases that are collectively known as IBD[67]. Interestingly, this expansion and diversification of phages did not seem secondary to changes in bacterial populations, highlighting intestinal phage's ability to contribute to disease-specific alterations in the microbiome. More recently, this dataset was re-analyzed and expanded upon by including unannotated viral clusters. It was found that a stable core of virulent phages, much like the one

described above, was replaced with a highly expansive temperate phage populations in disease patients. In contrast, much less is known about how gut bacteriophage populations respond to a high fat diet or the development of obesity. Two robust studies have been published looking at the link between obesity and alterations in the gut virome, and both provide conflicting results[68,69]. An initial study performed in 2016[68] found an enrichment of temperate free phage in diet-induced obese mice as compared to lean counterparts, while a more recent study found a significant decrease in the integrase gene, one necessary for lysogeny to occur, in high-fat fed mice[69]. Though both studies saw significant alterations within the phageome, there were no consistent taxa associated with an obese status. The sparse and conflicting nature of this literature warrants additional studies exploring the link between the phageome and obesity. Moreover, much like the bacterial portion of the microbiome, it is unclear how the phageome contributes to the development of obesity and warrants longitudinal metagenomic studies to be performed.

Hypothesis and Goals

In light of the literature, the hypothesis for this dissertation states that during times of intestinal inflammation phage host dynamics are affected within the gut microbiome. This hypothesis was tested by addressing two goals. First, I determined how bacteriophage populations change in response to the development of obesity. Second, I characterized a mechanism by which intestinal phage populations might be changed in this model – products generated by the immune system during aberrant inflammation seen in obesity.

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Chapter 2

Longitudinal Analysis of the Intestinal Microbiome in the Obese Mangalica Pig Reveals Alterations in Bacteria and Bacteriophage Populations Associated with Changes in Body Composition and Diet

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Abstract

Due to its immunomodulatory potential, the intestinal microbiota has been implicated as a contributing factor in the development of the meta-inflammatory state that drives obesity-associated insulin resistance and type 2 diabetes. A better understanding of this link would facilitate the development of targeted treatments and therapies to treat the metabolic complications of obesity. To this end, we validated and utilized a novel swine model of obesity, the Mangalica pig, to characterize changes in the gut microbiota during the development of an obese phenotype, and in response to dietary differences. In the first study, we characterized the metabolic phenotype and gut microbiota in lean and obese adult Mangalica pigs. Obese or lean groups were created by allowing either *ad libitum* (obese) or restricted (lean) access to a standard diet for 54 weeks.

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Mature obese pigs were significantly heavier and exhibited 170% greater subcutaneous adipose tissue mass, with no differences in muscle mass compared to their lean counterparts.
Obese pigs displayed impaired glucose tolerance and hyperinsulinemia following oral glucose challenge, indicating that a metabolic phenotype also manifested with changes in body composition. Consistent with observations in human obesity, the gut microbiota of obese pigs displayed altered bacterial composition. In the second study, we characterized the longitudinal changes in the gut microbiota in response to diet and aging in growing Mangalica pigs that were either limit fed a standard diet, allowed *ad libitum* access to a standard diet, or allowed *ad libitum* access to a high fat-supplemented diet over an 18-week period. As expected, weight gain was highest in pigs fed the high fat diet compared to *ad libitum* and limit fed groups. Furthermore, the *ad libitum* and high fat groups displayed significantly greater adiposity consistent with the development of obesity relative to the limit fed pigs. The intestinal microbiota was generally resilient to differences in dietary intake (limit fed vs *ad libitum*), though changes in the microbiota of pigs fed the high fat diet mirrored changes observed in mature obese pigs during the first study. This is consistent with the link observed between the microbiota and adiposity. In contrast to intestinal bacterial populations, bacteriophage populations within the gut microbiota responded rapidly to differences in diet, with significant compositional changes in bacteriophage genera observed between the dietary treatment groups as pigs aged. These studies are the first to describe the development of the intestinal microbiota in the Mangalica pig and are the first to provide evidence that changes in body composition and dietary conditions are associated with changes in the microbiome of this novel porcine model of obesity.

Introduction

The prevalence of obesity in adult populations is approaching pandemic levels. For instance, currently more than 650 million adults worldwide and greater than 40% of adults in the United States are considered obese[1] This poses a serious public health crisis as obesity is associated with multiple comorbidities including metabolic disease, cardiovascular disease, and gastrointestinal diseases[1]. While the etiology of obesity is not fully understood, it is clear that a chronic dysregulation of energy balance drives expansion of adipose tissue, and this associates with the development of meta-inflammation that in turn promotes impaired insulin sensitivity and ultimately the myriad of downstream comorbidities[2]. Due to the immunomodulatory and energy-harvesting potential of the intestinal microbiota, it has been implicated as a contributing factor in the development of metainflammation. Further, obesity-associated changes in the intestinal microbiota have also been linked to the promotion of the overnutrition underlying the expansion of adipose tissue[3–9]. A better understanding of these links could allow the development of targeted therapies to either prevent the onset of obesity or to uncouple obesity from downstream disease states.

The mammalian gastrointestinal tract is home to a large collection of microorganisms collectively known as the gut microbiota. All domains of life are represented within this diverse and dynamic microbial ecosystem, with a majority of constituents being bacteria and viruses, namely bacteriophage. [6,7,10]. This bacterial component is dominated by 4 major bacterial phyla (in order of dominance): Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria[4,6,7,10]. Investigations of the intestinal bacteriome have revealed a metabolically active microbial community that has many bidirectional interactions with the mammalian host[8,10,11]. For example, diets high in plant polysaccharides have been directly linked to an increase of bacterial species belonging to the phylum Bacteroidetes. More specifically, *Prevotella* spp. have a strong

connection to diet high in fiber due to their ability to metabolize complex carbohydrates [12–14]. Interestingly, *Bacteroides* spp. tend to decrease in response to the same diet and be more tightly linked to a diet rich in animal products [12,13,15,16]. However, bacteria do not exist in isolation within the microbiome; their predators, bacteriophage, often exist in similar abundance as their bacterial host [17]. The impact of bacteriophage on the form and function of the gut microbial ecosystem has only just begun to be appreciated.

Other features of the mammalian host can contribute to the composition and proportion of microbial symbionts within the gut microbiota, such as the host's environment, disease state, antibiotic usage, and diet[18]. Among these, the impact of diet on the microbiome has been the most thoroughly examined. There is now a large body of literature examining the interplay between diet, the microbiome, and host health, yet studies often report conflicting results. For example, a recent meta-analysis found no correlation between specific bacterial taxa and bacterial richness with diet-induced obesity [19]. Further, even less is known about how bacteriophage populations are impacted by an obesogenic state [20]. Establishment of novel models that facilitate the study of these dynamics should promote an enhanced understanding of mechanisms linking the microbiota to the health status of the host.

To date, most published studies defining the composition and functionality of the gut microbiota in human health and obesity have utilized mouse models. However, pigs have been proposed as a more accurate model for human obesity, as more similarities are seen between pigs and humans in terms of anatomy, physiology, and nutrient digestion[21,22]. Both pigs and humans are omnivores and lack anatomically discreet depots of brown fat within the vasculature. Additionally, the proportion of skeletal muscle and adipose tissue to total body mass and circulating levels of glucose are very similar between pigs and humans[21,22]. Furthermore, given

the well-developed literature concerning the use of swine as a biomedical model to study atherosclerosis, cardiovascular disease, and diabetes, pigs are well-positioned as an experimental solution to overcome limitations inherent in using rodent models to study metabolic syndrome and obesity[23,24].

While multiple porcine models for obesity currently exist, each have some limitations. To date, no swine model spontaneously displays the full spectrum of metabolic dysregulation associated with human obesity. Also, few swine models of hyperphagic obesity currently exist[25,26]. In this regard, the fatty Mangalica pig, capable of achieving a body composition comprised of 70% adipose tissue by mass, represents a novel alternative[27]. A voluntary chronic overnutrition drives this extreme, early onset, morbidly obese phenotype that associates with a spontaneous proinflammatory, insulin resistant metabolic phenotype in these pigs as they age (Roberts, et al, under review). Despite the Mangalica displaying great potential to serve as a relevant animal model of obesity and its complications, neither the normal development of nor diet-induced effects upon its gut microbiota have been characterized.

Because the gut microbiota of this novel obese porcine model has never been explored, we characterized the metabolic phenotype and gut microbiota in adult lean and obese Mangalica pigs. Further, we then characterized the longitudinal changes in the gut microbiota over an 18-week period in response to diet and aging utilizing juvenile Mangalica pigs that were either given restricted access to a standard diet (lean), allowed *ad libitum* access to a standard diet (obese), or allowed *ad libitum* access to a high fat-supplemented diet (diet-induced obese). These studies are the first to describe the overall development of the intestinal microbiota in the Mangalica pig and represent a key first step toward the development of this breed as a useful

model to study mechanisms linking the intestinal microbiota to the development of obesity and downstream metabolic states.

Materials and Methods

Animals, Diets, and Fecal Sample Collection

Purebred Mangalica pigs were obtained from the Auburn University research herd housed at the Auburn University Swine Research and Education Center. In the first study, ten weaned pigs were individually housed in pens and provided *ad libitum* access to water. Voluntary feed intake was determined daily by weighing back orts, and body weights were determined weekly. To establish our limit fed (lean) and *ad libitum* (obese) groups, five pigs were allowed *ad libitum* access to the balanced basal diet (Table 2.1) while the remaining five pigs were fed the basal diet at levels that were 40% of the voluntary intake of their *ad libitum* fed counterparts for the previous day. Despite feed restriction, daily rations for all pigs exceeded the nutrient recommendations for healthy growth of the breed[28]. Body composition was assessed via ultrasound at 26 and 52 weeks on trial. Pigs underwent oral glucose tolerance tests to assess glycaemia and insulinemia. Fecal samples were collected aseptically from post-pubertal pigs once clinical parameters of obesity were observed within the *ad libitum* group. For the second (longitudinal) study, twelve post-weaned pigs were housed two per pen. Pigs penned together received the same dietary treatment. Limit fed and *ad libitum* groups were established by utilizing the same dietary strategy as described above. The *ad libitum* + high fat (HF) group was established by allowing *ad libitum* access to the basal diet supplemented with 28% dietary fat (Table 2.1). All pigs were provided *ad libitum* access to water.

Table 2.1 Formulation and composition of experimental diets (as-fed basis)

Item	Diet	
	Control	High Fat
Ingredient, g/kg	--	
Corn	727.00	577.00
Soybean meal, 47.5% CP	107.00	86.00
Dried Distillers Grains[1]	100.00	80.00
Dicalcium Phosphate	0.16	0.13
Limestone	11.51	9.78
Salt	4.00	3.25
Vitamin-trace mineral premix	0.45	0.45
Soybean oil	46.00	250.00
Calculated composition	--	
ME[1], mcal/kg	3.47	4.75
Crude protein, %	13.80	10.90
Fat, %	4.60	25.00
Ca, %	.68	.54
Available P, %	.45	.36

[1] Metabolizable energy

Fecal samples were collected aseptically one week prior to the assignment of pigs to their respective dietary treatments and then one-, ten-, fourteen-, and eighteen weeks following their transition to the respective diets. Body weights were recorded weekly and body composition was assessed via ultrasound bi-weekly while pigs were on trial.

Ultrasound

Real-time ultrasound was performed on all pigs to assess body composition in growing animals by determining on test ultrasound 10th rib subcutaneous fat depth and Longissimus muscle depth according to Perkins et al[29]. All ultrasound data was collected by the same Ultrasound Guidelines Council certified technician using an Aloka 500 (Aloka America, Wallingford, CT) with a 17 cm transducer using CUP Lab image capture software.

Oral Glucose Tolerance Test (OGTT) and insulin measurements

Lean and obese pigs from the first study were subjected to an OGTT when obese pigs reached an average body weight of 160 kg. Pigs were fitted with jugular catheters and allowed to recover for 7 days. Catheters were flushed with heparinized saline twice daily to maintain patency. During the OGTT, pigs were fasted for 24 h and then offered a control diet equal to 1% of their body weight that had been supplemented with glucose equivalent to 2 g per kg BW. Blood was obtained 15 minutes before and 15, 30, 60, 120, and 180 min after consumption of the glucose dose. Blood was directly analyzed for glucose using a clinical glucose analyzer (YSI 2300 STAT Plus, YSI Inc., Yellow Springs, OH). To facilitate insulin measurement, blood samples were centrifuged (3000 x g, 10 min, 4ºC) and resulting plasma was collected and stored at -80ºC until analysis. Plasma insulin (porcine insulin ELISA kit, ALPCO, Salem, NH) was determined using commercially available kits according to manufacturer instructions. Glucose (mg/dl) and insulin (µU/ml) data are presented as area under the curve (AUC).

Shotgun Metagenomic Sequencing of the Gut Microbiota

Immediately after collection, DNA was extracted from fecal samples using the E.Z.N.A kit (Omega). DNA samples were sent to Hudson Alpha Genome Sequencing Center (Huntsville, Al.) for shotgun metagenomic sequencing. Sequencing was carried out using an Illumina HiSeq 2500 v4 with a 2 x 125 paired-end sequencing 200 million reads. For the preliminary study, each sample was sequenced individually, while in the longitudinal study, DNA samples were pooled by pen (2 pigs per sample). For annotation of samples, an in-house annotation pipeline was used. The metagenomic pipeline can be found at: https://github.com/haleyhallowell/metagenome-annotation-pipeline/blob/main/annotation.sh. Briefly, quality was assessed using FastQC[30]. Using Trimmomatic, sequencing adapters and low-quality sequences (Q-score < 30) were removed[31]. Host sequences were then removed using BWA by mapping reads to the host genome (*Sus scrofa* NCBI v10.2) [32]. Reads were then assembled using the Iterative De Brujin Assembler (IDBA-UD), and reads were mapped back to the assembly using Bowtie2 [33,34]. Mapped reads were then annotated using MetaPhlAn3 [35].

Statistics

For statistical analysis in the first study, growth and clinical characteristics were analyzed as a completely randomized block design using a mixed linear model of SAS v9.2 with individual animal serving as the experimental unit, i.e., individual block (SAS Institute, Inc., Cary, NC). In the second (longitudinal) study, weights and back fat measurements were analyzed using a one-way ANOVA with a Tukey's *post-hoc* test. This was performed in the R-studio platform. Microbiome analysis was performed using outputs generated through MetaPhlAn3; this included both relative abundance and raw hit counts. To determine the alpha diversity between treatment

groups in both sets of samples, raw hit counts were used to generate a Bray-Curtis matrix and plotted using non-metric multidimensional scaling (*nMDS*) using the vegan package in R [36]. Significant differences between different treatment groups, as well as interactions between covariates, was determined using a PERMANOVA, by employing the adonis function in the vegan package [36]. Stacked bar plot displaying relative abundance of the datasets were generated in the phyloseq [37]. To detect differentially abundant taxa between our treatment groups, a differential abundance analysis was performed using DeSeq2 [38]. Briefly, raw hit counts were rlog transformed, and a Wald's test was used to determine significantly different taxa. Adjusted p-values (q-values) were then generated using the Benajmini-Hochberg (FDR) correction to account for false positives [39]. Pearsons' correlation coefficients were calculated using the package *psych* [40]. Pearsons' correlation plots were generated in R studio using the package *ggcorplot* [41], only including relationships with a correlation coeffect greater than |0.6|.

Results

Mature lean (restricted) and obese (ad libitum) Mangalica pigs exhibit divergent body composition and metabolic phenotypes.

Our first study was conducted to characterize the intestinal microbiota of mature lean and obese Mangalica pigs. Initial body weights of the juvenile pigs were not different between the limit fed and *ad libitum* groups ($p > 0.92$). As expected, the final body weights of mature *ad libitum* pigs weighed 53% more than their limit fed counterparts ($p < 0.001$; Table 2.2). Subcutaneous fat depth was 70% greater in *ad libitum* vs. limit fed pigs ($p < 0.001$) while muscle depth between the two groups was not significantly different ($p > 0.73$) suggesting differences in body weight were reflective of differences in adiposity rather than skeletal muscle mass (Table 2.2).

To determine the impact of adiposity on blood glucose and insulin levels in the mature limit fed and *ad libitum* pigs, an oral glucose tolerance test (OGTT) was performed. Fasting glucose levels were significantly higher in *ad libitum* versus limit fed pigs at time -15 min ($p < 0.05$; Figure 1A). In response to glucose administration, a significant increase in blood glucose levels was observed above baseline by 15 minutes in limit fed and *ad libitum* pigs, with values being significantly higher in *ad libitum* versus limit fed pigs at the peak of the curves. Furthermore, blood glucose values returned to baseline levels by 30 minutes in limit fed pigs after the initial dose, while glucose remained elevated in *ad libitum* pigs, with values not returning to baseline levels until 180 minutes post dosing. Overall, the glucose AUC was increased by 42% for the *ad libitum* Mangalica pigs compared to limit fed counterparts ($p < 0.001$; Figure 2.1A, Table 2.3).

Table 2.2. Body composition of initial cohort of adult pigs demonstrating lean and obese phenotypes[1]

Variable	Limit Fed	Ad Libitum	P-value
Number of pigs	5	5	NA[2]
Body weight, kg	110.1 ± 1.9	167.8 ± 4.9	0.001
Subcutaneous fat, mm	36.1 ± 3.2	61.0 ± 5.2	0.001
Longissimus dorsi, mm	47.3 ± 1.3	50.5 ± 2.1	0.73

[1]Values are means ± standard errors

[2] NA=not applicable

Table 2.3. Effect of adiposity on indexes of insulin sensitivity during OGTT challenge in adult lean and obese pigs[1]

Variable	Limit Fed	Ad Libitum	P-value
Number of pigs	5	5	NA[2]
Glucose AUC[3]	23,400 ± 1,910	33,280 ± 2,140	0.001
Insulin AUC[3]	5,010 ± 599	8,955 ± 674	0.001
QUICKI[4]	.35	.28	0.05
HOMA-IR[5]	1.50	6.25	0.001
HOMA-B[6]	149	122	0.05
HOMA-S[7]	60	36	0.01
Matsuda Index[8]	4.54	1.70	0.001
Insulinogenic Index[9]	.46	.20	0.001
Disposition Index[10]	2.1	.34	0.001

[1]Values are means ± standard errors or simple means
[2] NA=not applicable
[3] AUC= area under the curve
[4]QUICKI= Quantitative Insulin Sensitivity Check Index Visual; normal ranges between .3-.45 and insulin resistance is <.3; lower numbers reflect greater insulin resistance.
[5] HOMA-IR = Homeostatic model assessment- insulin resistant; normal is indicated by values lower than ≤ 1.8.
[6] HOMA-B = Homeostatic model assessment- insulin resistant-β-cell function
[7] HOMA-S = Homeostatic model assessment- insulin resistant-insulin sensitivity
[8] Matsuda Index = whole body insulin resistance is indicated for values ≤ 2.5
[9] Insulinogenic Index = defects in insulin secretion are indicated for values < 0.4
[10] Disposition Index = (Insulinogenic index)*(Matsuda index); normal is indicated for values > 1

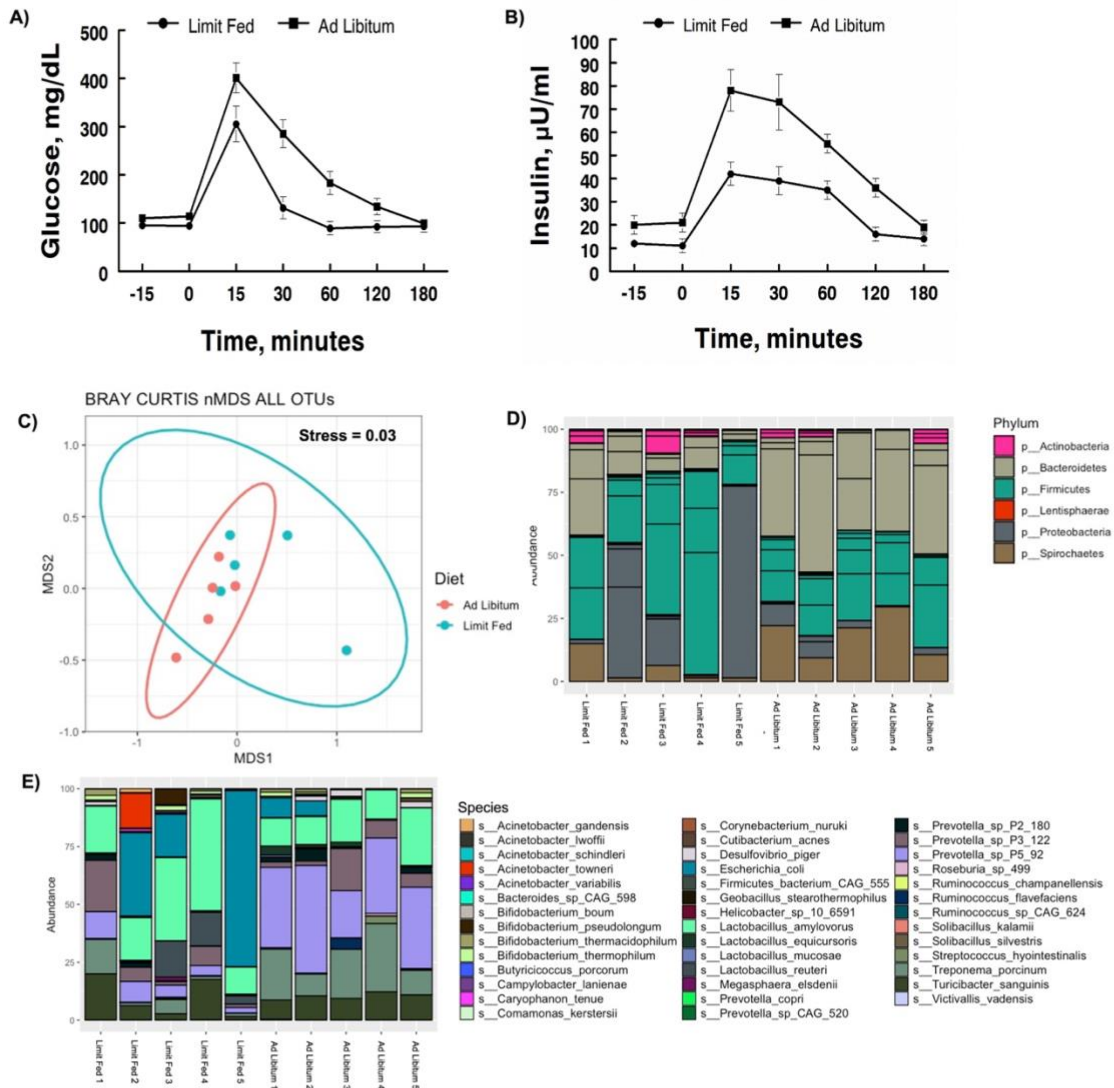

Figure 2.1. Characterization of metabolic parameters and intestinal microbiota in *ad libitum* and limit fed Mangalica pigs. Oral glucose tolerance test (OGTT) conducted on lean and obese pigs. **A)** Plasma glucose and **B)** insulin levels were measured following administration of an oral dose of glucose (2 g/kg BW) to fasted pigs. **C)** nMDS ordination plot was generated using a Bray-Curtis dissimilarity index. The stress associated with this ordination is 0.003. Bacterial **D) Phylum,** and **E) Species** level, composition in Mangalica pigs on either a limit fed or *ad libitum* feeding.

Consistent with the glucose data, fasting insulin levels were significantly higher in *ad libitum* versus limit fed pigs ($p < 0.05$; Figure 2.1B). Like the glucose response, plasma insulin levels rose significantly from baseline by 15 minutes in both limit fed and *ad libitum* pigs, with peak insulin values being almost twice as high in *ad libitum* versus limit fed pigs. Insulin values returned to baseline levels by 120 minutes in both limit fed and *ad libitum* pigs. Overall, the insulin AUC was increased 79% for *ad libitum* Mangalica pigs compared to limit fed counterparts ($p < 0.001$; Figure 2.1B, Table 2.3). Several measures of insulin sensitivity were then utilized to assess plasma glucose and insulin values from an oral glucose tolerance test (OGTT) to determine if the *ad libitum* pigs developed insulin resistance (Table 2.3). Utilizing fasted values, HOMO and QUICKI indexes both indicated that pigs fed an *ad libitum* diet displayed impaired insulin sensitivity (Table 2.3). Using peak curve values compared to fasted baseline, the Masuda index likewise indicating an insulin resistant state in *ad libitum* fed pigs compared to their limit fed counterparts (Table 2.3). As expected, the mature *ad libitum* cohort developed many of the phenotypic and metabolic hallmarks of obesity in humans.

Shotgun metagenomic analysis of fecal microbiota demonstrate altered microbial composition in ad libitum fed pigs vs. limit fed

To characterize the intestinal microbiota of mature limit fed and *ad libitum* pigs, shotgun metagenomic analysis was conducted on fecal samples. Rarefaction curves were generated to assess sequencing depth. Each sample reached a plateau, indicating the presence of more sequences than OTUs, signifying adequate sequencing depth was achieved (Figure S2.1). To assess the microbiota of our limit fed and *ad libitum* cohorts holistically, we performed non-metric dimensional scaling (nMDS) using a Bray-Curtis dissimilarity matrix to determine the

degree of dissimilarity between each sample. The stress, or "fit" of the model was 0.03, which is within the acceptable range (fit < 0.3) indicating that this is an appropriate representation of the dissimilarity of each sample in 2D space. Most individuals clustered with their cohort, indicating congruency between cohort members. Although the clusters were distinct, limit fed and *ad libitum* microbiota samples plotted close to each other (Figure 2.1C). Diet did have a significant influence on the dissimilarity of our treatment groups (PERMANOVA, $p = 0.012$, $R^2 = 0.37182$). Thus, the limit fed and *ad libitum* cohorts had a somewhat similar gut microbiota bacterial consortia, likely due to exposure to identical feed and adjacent housing. However, the clustering seen between these groups points to the *amount* of feed received as being a significant driving factor in the structuring of the microbiota.

Next, we wanted to determine the compositional changes that were driving the dissimilarity between our two cohorts. To do this, we calculated the relative abundance at the taxonomic level of bacterial phyla and species (Figure 2.1D). The major bacterial populations present within the Mangalica intestinal microbiota were consistent with the well-documented, healthy consortia of microorganisms reported in the literature in multiple models, such as humans, mice, and pigs[6,10,25]. The limit fed cohort's intestinal microbiota was dominated by 4 main phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes. (Figure 2.1B). We observed a higher abundance of Spirochaetes ($p = 0.02$) and Bacteroidetes, and a lower abundance of Proteobacteria ($p = 0.0009$) and Firmicutes (0.016) in our *ad libitum* fed pigs. To further resolve these differences, we evaluated bacterial composition at the level of species in our 2 cohorts.

Though there was variation from animal to animal, the limit fed cohort was dominated by *Lactobacillus amylovorus*, *Escherichia coli*, *Treponema porcinum*, *Turicibacter sanguinis*, and multiple Prevotella spp. such as *Prevotella spp P3-122* and *Prevotella sp P5-92* (Figure 1E). The *ad libitum* cohort harbored similar species; however, we observed an increase in *Treponema porcinum*, *Prevotella sp P5-92* and *Bifidobacterium boum* (p = 1.16E -14) in and a decrease *Lactobacillus amylovorus*, and *Lactobacillus reuteri* (p = 0.02) (Figure 2.1E). To our knowledge, this is the first report to define the microbial populations within the Mangalica gut microbiota. Additional taxonomic classification at the level of family and genus is available in Supplementary Figure 2.2. Data presented in these additional figures agree with and support the description of the microbiome given above.

An advantage of our shotgun metagenomics approach was the ability to monitor all constituents of the gut microbiota, particularly bacteriophage. Given that these viruses prey on bacteria, they represent a key selective force in regulating the bacterial composition of the microbiome[42–45]. Overall, individual variability was much greater within bacteriophage populations as compared to bacterial populations in this cohort. Given the highly variable nature of our samples, especially at the taxonomic level of species, we were not able to detect any meaningful shifts within bacteriophage populations at this final endpoint of obesity (Figure S2.3).

Taken together, we observed significant shifts in the intestinal microbiota in the natural feeding model of the Mangalica pig. These disruptions within the *ad libitum* group's gut microbiota were seen in parallel with the phenotypic and metabolic symptoms indicating that like obesity in humans, obesity-associated conditions are associated with shifts in microbial populations within the gut.

Juvenile Mangalica pigs exhibit different body compositions when fed divergent diets during a longitudinal analysis across 18 weeks

As expected of growing pigs, all groups increased in body weight over the 18 weeks of the experiment (Figure 2.2A). However, the *ad libitum* (AL) and high fat (HF) piglets gained weight rapidly compared to limit fed (LF) counterparts and body weights between the three cohorts significantly diverged starting at 9 weeks on the diet ($p < 0.05$). Further, animals on the high fat diet gained significantly more weight than both the *ad libitum* and limit fed groups starting at 13 weeks ($p < 0.05$). Both the *ad libitum* and high fat fed groups exhibited significantly greater adiposity compared to the limit fed piglets beginning at 5 weeks on the diet ($p < 0.01$, $p < 0.001$, respectively) (Figure 2.2B). High fat fed piglets exhibited significantly greater adiposity than the *ad libitum* group starting at 16 weeks ($p < 0.01$). In contrast, the limit fed animals showed no significant increase in back fat accumulation over the course of the experiment (Figure 2.2B). These data indicate that diet-induced differences in adiposity were achieved between dietary treatments.

Mangalica pigs exhibit progressive changes in the gut microbiota influenced by age and diet

We next wanted to determine how the microbiota composition changed over time in response to our respective diets. To do this, fresh-catch fecal samples were collected at 1 Week, 10 Weeks, 14 Weeks and 18 Weeks post-dietary exposure (PD), and shotgun metagenomic sequencing was performed. Sequencing depth was assessed through rarefaction curves (Figure S2.4). Each sample reached a plateau, confirming adequate sequencing depth.

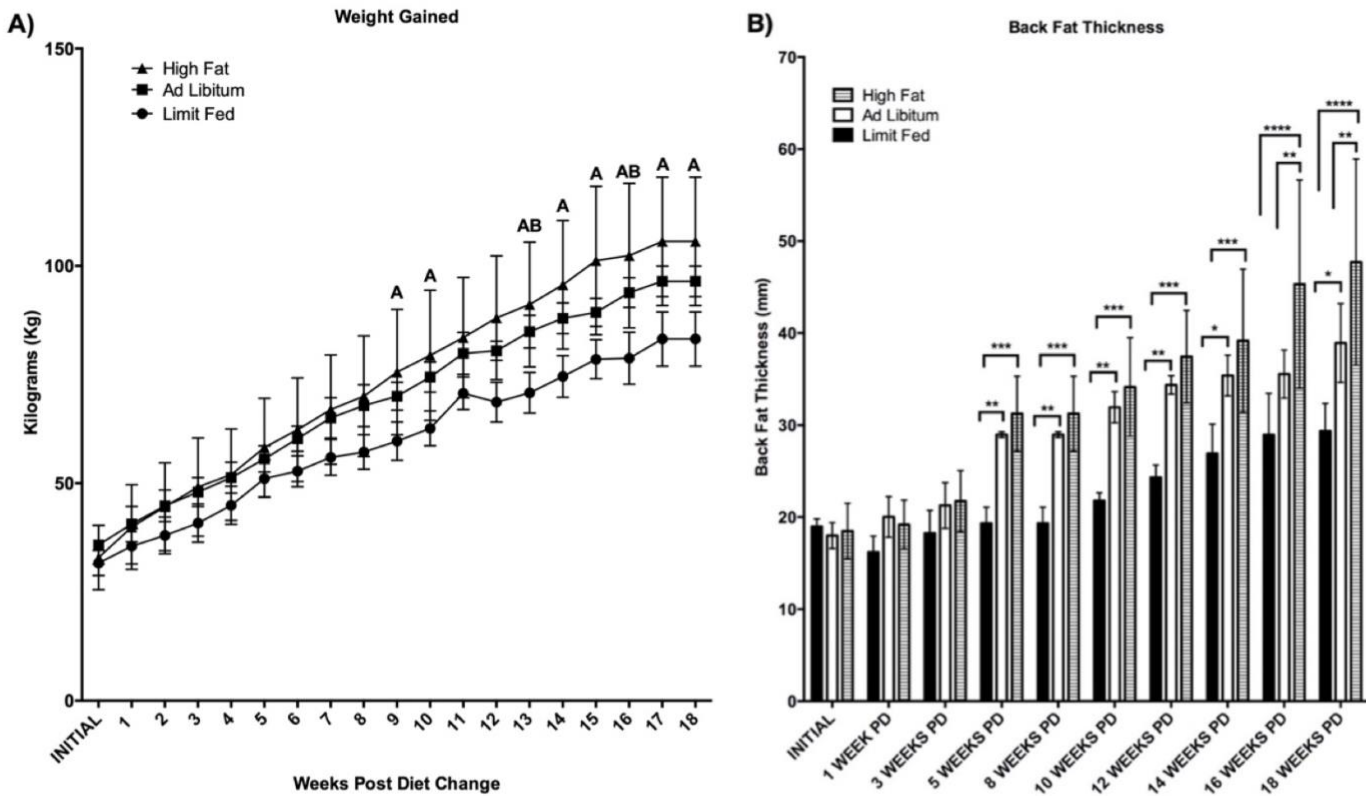

Figure 2.2. Body weight A) and Back Fat B) change over time. Weekly weight change and biweekly backfat change over time is shown for the limit fed, *ad libitum*, and the *ad libitum* + high fat groups. Group differences over the course of dietary treatment were analyzed by ANOVA. All data points are shown as group mean ± SD. For **A)**, Tukey's post hoc test results are described as A (represents a $p < 0.05$ for *ad libitum* + high fat compared to limit fed) or B (represents $p < 0.05$ for *ad libitum* compared to limit fed groups)). For **B),** *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

To first assess overall differences in the microbiomes between our diet groups, we again performed non-metric dimensional scaling (nMDS) using a Bray-Curtis dissimilarity matrix (Figure 2.3A). We obtained a stress of 0.13, indicating that our model was a good representation of the dissimilarity between samples. Both diet and time had a significant effect on the differences between group (p =0.003; p=0.0010), with diet explaining 15% of the variation and time explaining 41% of the variation. Additionally, there was no interaction between time and diet (p=.201) It's important to note that the diets were fundamentally the same (only different in quantity), with the exception of the high fat group which were given an additional component of high dietary fat. In addition, it is known that the intestinal microbiome responds to aging of the host [46] Consistent with this, both diet and time were major drivers of dissimilarity in our system. Having assessed overall differences between the groups throughout the experiment, our next goal was to determine how the composition of the gut microbiota corresponded to dissimilarity. We also sought to compare the microbial composition from this longitudinal study with the results of the initial study (Figure 1). We observed similar overall microbial profiles as in our initial study utilizing limit fed and *ad libitum* cohorts. The intestinal microbiota of all groups was dominated by 4 major phyla: Firmicutes, Bacteroidetes, Proteobacteria and Spirochaetes (Figure 2.3B). We saw significant, meaningful changes in the *ad libitum* (AL) animals, and more significantly in the high fat (HF) animals, as compared to our limit fed (LF) pigs starting at 10 Weeks post-dietary intervention (PD). Specifically in our *ad libitum* group, we observed a decrease in the phylum Bacteroidetes at 10 weeks (p = 0.03) and a decrease in the phylum Firmicutes at 14 weeks (p = 0.01), as compared to our limit fed animals. More significant changes were observed in our high fat group.

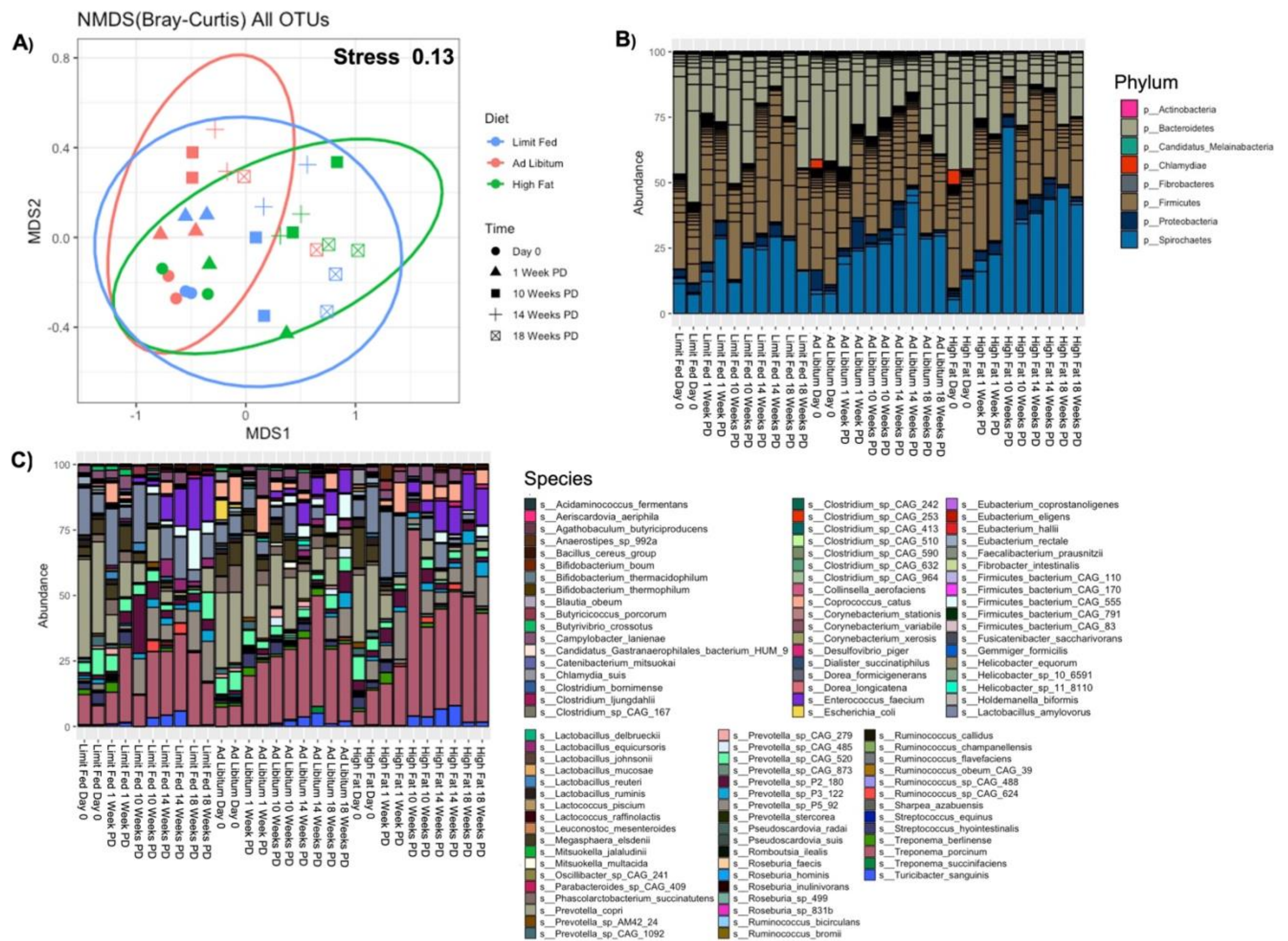

Figure 2.3. Longitudinal evaluation of bacterial composition following dietary exposure in weaned Mangalica Pigs. A) An nMDS ordination plot was generated using a Bray-Curtis dissimilarity matrix. The shapes represent the time point in which the pigs were sampled, while the color represents the diet administered. The stress for this plot was 0.134. **B)** Phylum, **C)** an**d Species** level bacterial composition in Mangalica pigs fed either a limit fed, *ad libitum*, or *ad libitum* + high fat diet prior to and after 1, 10, 14, and 18 weeks of dietary exposure.

Specifically, we observed again a decrease in Bacteroidetes starting at 10 weeks (p = 0.0009), that remained in lower abundance for the duration of the experiment as compared to our limit fed group. We observed this trend within the phylum Firmicutes as well. We saw an increase of Proteobacteria (p = 0.002) and Spirochaetes (0.02) only in our high fat animals, both becoming significant at 10 weeks PD as compared to our limit fed group. Though all groups displayed an increase in Spirochaetes over time, we observed that this phylum was elevated in our high fat animals for the duration of the experiment. Overall, we observed meaningful shifts in microbial composition over time in our 3 groups starting at 10 weeks, with the most significant changes occurring in our high fat animals.

To more specifically identify bacteria undergoing the greatest changes in abundance, we evaluated the relative abundance at the taxonomic level of species in the growing pigs. Though some expected individual variation was observed, the microbiome of our growing pigs in all treatment groups were remarkably resilient towards dietary intervention. However, we did observe significant changes in bacterial species within the high fat group that were consistent with the composition of our *ad libitum* cohort in the first study (Figure 2.3C). For example, we observed an overall increasing trend in multiple *Treponema spp*, such as *Treponema porcinum* (p = 0.03 LF-HF) and *Treponema succinifaciens* (p = 4.60^-5 AL-HF), becoming significant at 10 weeks, and continuing throughout the duration of the experiment (*T. porcinum* [p = .04 LF-HF]). Additionally, we observed multiple Prevotella spp. change in abundance in response to the high fat diet. For example, *Prevotella sp P5-92* was increased in our high fat animals as compared to both our limit fed and *ad libitum* (p = .048) piglets and became increasingly significant over time up to 14 weeks (p = 1.01^-6). Interestingly, *Prevotella copri* decreased over time in response to a high fat diet. Starting at 1 week PD, *Prevotella copri* was depleted in the high fat fed piglets.

Finally, as in our endpoint *ad libitum* model, the high fat fed piglets displayed an overall decrease in *Lactobacillus amylovorus* as compared to the limit fed group, becoming significant at 18 weeks PD ($p = 0.038$). The high fat fed group also displayed changes in microbial abundances that were not detected in our initial study. For example, the high fat fed piglets underwent a depletion of *Streptococcus hydrointestinalis* starting at 10 weeks ($p = .03$ LF-HF; p = 7.17^ -6 AL-HF) which remained almost undetectable for the duration of the experiment. This trend was observed to a lesser extend in our limit fed piglets as compared to our *ad libitum* piglets starting at 10 week PD as well ($p = .046$). Overall, we observed multiple changes within our high fat piglets that closely resembled the microbial composition seen in our fully developed obese pigs. As in our preliminary study, bacterial taxonomic classification at the family and genus level is shown in Supplemental Figure 2.5 and supports the changes in the microbiome due to diet perturbation described above.

The microbiota composition was altered in Mangalica pigs fed a high fat diet, reflecting an obesity-associated profile

To obtain a more holistic picture of how fluctuations in the intestinal microbiota related to the pig physiological phenotype, we analyzed the correlations between specific bacterial taxa and metabolic measurements at 18 weeks post dietary intervention (PD). Interestingly, the bacterial species that were more abundant within the intestinal microbiota of the high fat-fed animals also demonstrated a positive relationship with adipose accumulation and weight gain (Figure 2.4). In contrast, microbial constituents that were seen to dominate the intestinal microbiota profile of our limit fed group revealed an inverse relationship to adipose accumulation and weight gain (Figure 2.4).

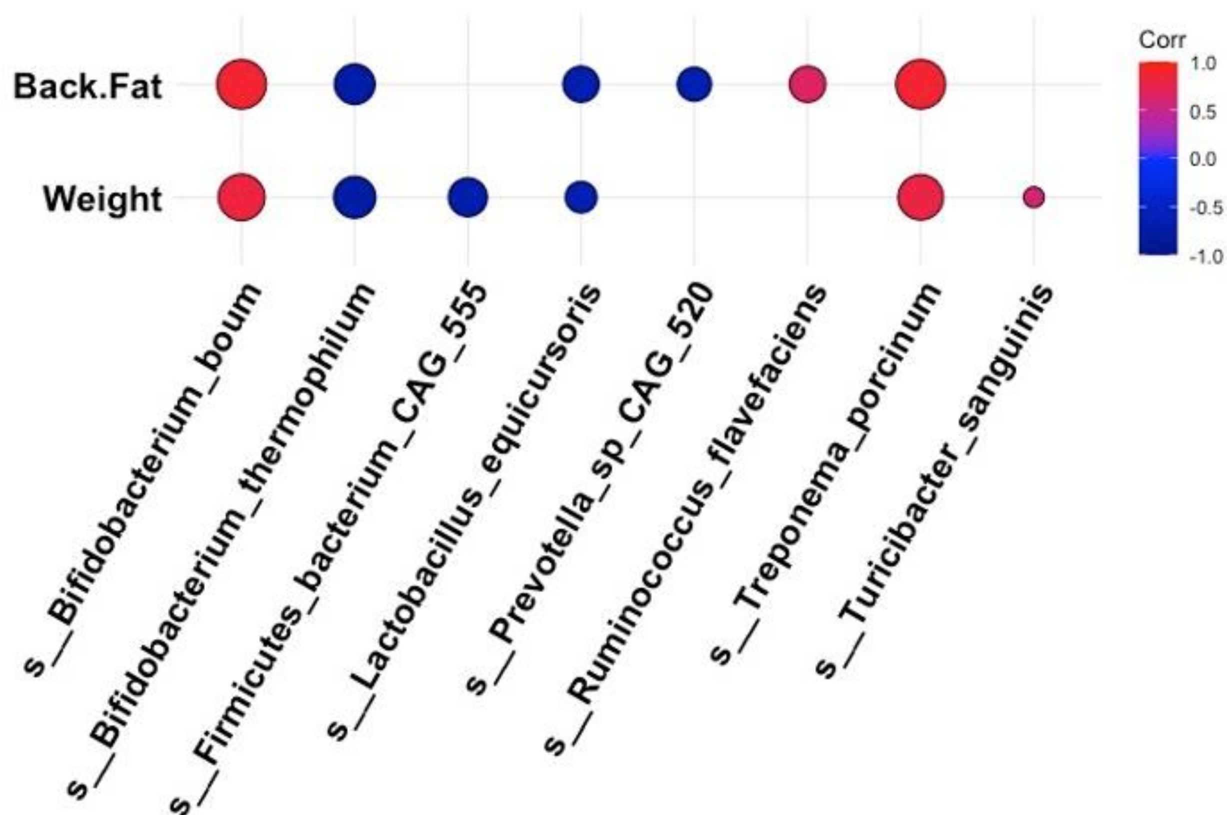

Figure 2.4. Correlations between bacterial species and metabolic phenotype in Mangalica pigs following 18 Weeks of dietary exposure. Pearson's correlation plot of bacterial species and phenotypic data for Mangalica pigs fed a limit fed, *ad libitum,* or *ad libitum* + high fat diet. Data shown is after 18 weeks of dietary exposure. Statistical significance was determined for all pairwise comparisons. Positive values (red circles) indicate positive correlation coefficients above 0.6, and negative values (blue circles) indicate inverse correlation coefficients below -0.6. The size and shading of the circles indicate the magnitude of the correlation, where larger circles indicate a stronger correlation than smaller circles. Correlation coefficient values outside of |0.6| are not included in this plot.

For example, we observed strong positive correlations between *Treponema porcinum* and *Bifidobacterium boum* with back fat and weight. This would indicate that an increase in weight due to increased adiposity is accompanied by higher abundance of these intestinal microbiota constituents. This is consistent with elevated representation of these species, or species belonging to the same genus, within the *ad libitum* intestinal microbiota profile in the preliminary study. On the other hand, *Bifidobacterium thermophilum* and *Lactobacillus equicursoris* were both negatively correlated with back fat thickness and weight gain. *Lactobacillus* species were seen to be depleted in both our growing piglets and our fully developed obese pigs, which would indicate they are associated with leanness (Figure 2.4). Other species were found to be either weakly positively (*Ruminococcus flavefaciens* and *Turicibacter sanguinis*) or negatively (*Firmicutes bacterium CAG 555* and *Prevotella sp CAG 520*) correlated with our phenotypic markers. Taken together, the bacterial microbiota of developing Mangalica pigs was remarkably resilient to diet change. However, we did observe shifts within the high fat fed animals, resembling the *ad libitum* cohort in the initial study. Our data suggest that high dietary fat expedites changes within the bacterial constituents of the microbiota generating an obesity-associated microbiota profile.

Bacteriophage dynamics were altered by age and diet in Mangalica Pigs.

Next, we wanted to define viral composition within the intestinal microbiota of the Mangalica piglet in response to diet change. To do this, we calculated relative abundance of viruses at both the genus and species level. The gut was dominated by bacteriophages within the order Caudovirales, so our analysis was focused on looking specifically at these agents.

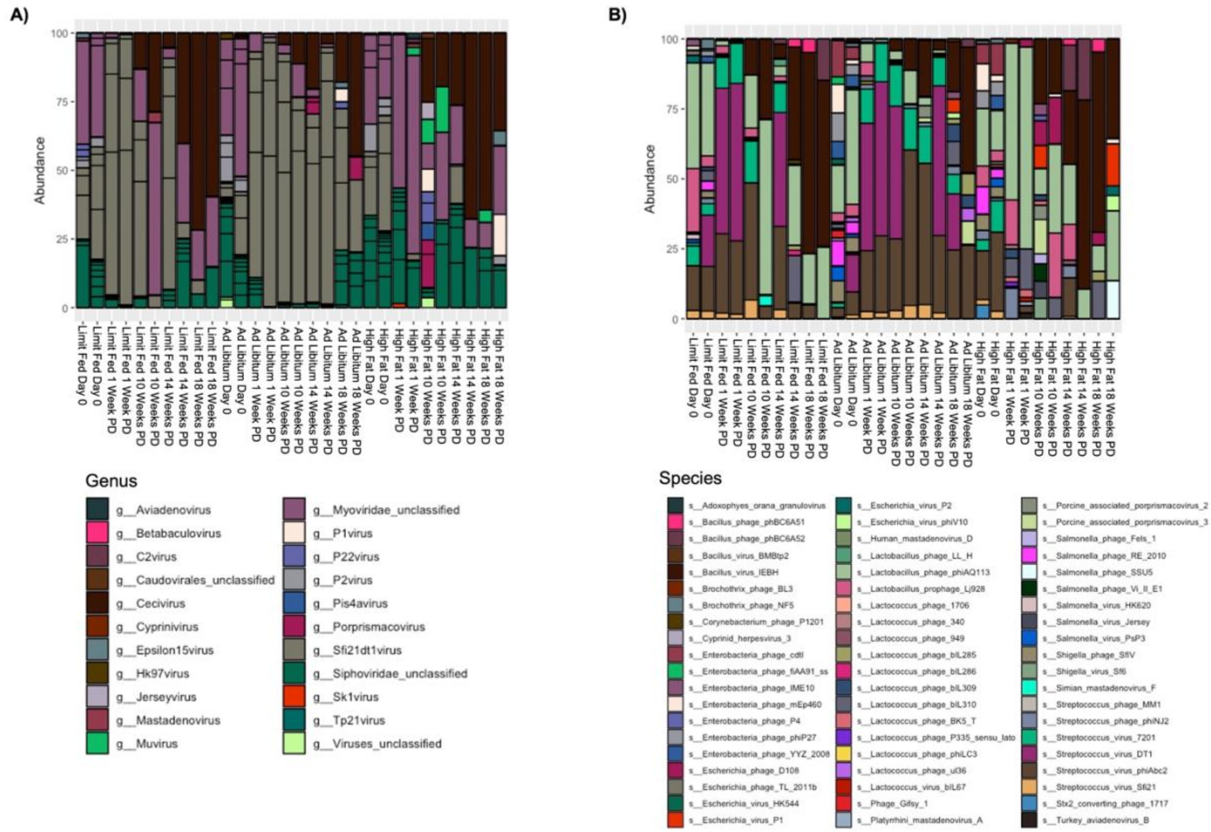

Figure 2.5. Changes in bacteriophage composition after dietary exposure in weaned Mangalica pigs. A) Genus and **B) Species** level viral composition in Mangalica pigs fed either a limit fed, *ad libitum*, or *ad libitum* + high fat diet after 1, 10, 14, and 18 weeks of dietary exposure.

As with the bacterial component of the intestinal microbiota, the *ad libitum* (AL) and limit fed (LF) piglets bacteriophage profiles were very similar in composition (Figure 5A, Supplementary Table 8 and 9). In stark contrast to bacterial populations, we observed rapid and significant restructuring of bacteriophage composition in the piglet gut in response to the high fat diet (HF). These changes were visible first at the genus level (Figure 2.5A). Specifically, we saw a rapid and significant depletion of *Sfi21dt1* viruses in the high fat animals starting at 1 Week post dietary intervention (PD) (p = 3.99^-5 LF-HF; p = 2.09^-5 AL-HF), and this trend continued for the duration of the experiment (p = .02 AL-HF). Though not significant, we saw an overall increase in bacteriophage belonging to the genus *Myoviridae unclassified*, especially at later time points: 14 and 18 weeks PD.

To gain better resolution on phage dynamics in our growing piglets, we next characterized the relative abundance of phage populations at the level of species (Figure 2.5B). As expected, some bacteriophage followed the patterns of their host. For example, the virulent phage *Lactobacillus phage phiAQ113* and the temperate phage *Lactobacillus prophage Lj928*, which target *Lactobacillus* spp., followed a similar abundance pattern over time as their host. Additionally, consistent with the level of genus, we observed rapid changes in the bacteriophage populations at the taxonomic level of species in response to the high fat diet. Specifically, we saw rapid depletion of 4 of 6 annotated bacteriophage that target *Streptococcaceae* starting at 1 week. These 4 viruses belong to the genus *Sfi21DT1 viruses* and include the temperate bacteriophage *Streptococcus virus Sfi21* (p = .0012 LF-HF; p = .0053 AL-HF), and the virulent bacteriophages *Streptococcus virus 7201* (p = 5.32^-5 LF-HF; p = .0004 AL-HF), *Streptococcus virus DT1* (p = 5.32^-6 LF-HF; 6.26^-5 AL-HF), and *Streptococcus phiABC2* (p =.0015 LF-HF; p =.0053 AL-HF). These phage remained mostly undetectable in our high fat fed piglets for the duration of the

experiment, while remaining fairly constant in our limit fed and *ad libitum* fed piglets. Interestingly, *Streptococcus spp.* were detectable in the high fat fed piglets at 1 week PD, yet by 10 weeks were significantly depleted. Collectively, numerous bacteriophage genera, specifically those who target Streptococcaceae, were reduced in abundance and their putative hosts also became depleted, yet more slowly.

Finally, to gain a better understanding of phage-host dynamics in our growing pigs, we analyzed the correlative relationships between bacteria and bacteriophage species at 18 weeks post dietary intervention (PD) (Figure 2.6). Strong positive correlative patterns were observed between many bacteria and phage species. Of note, many strong positive correlations were detected between a wide variety of bacterial species and *Streptococcus* – targeting phage, such as the temperate phage *Streptococcus phage phiNJ2*, and virulent phages *Streptococcus virus 7201* and *Streptococcus virus DT1.* This strong, positive pattern was also observed with the temperate phages *Bacillus virus BMBtp2* and *Lactococcus phage biL309*. Conversely, the *Bacillus* – targeting temperate phage*, Bacillus virus IEBH* was negatively correlated with a wide variety of bacterial species. Overall, this trend was observed in bacterial species that were not affected by diet perturbation and remained consistent between diet groups throughout our experiment. As in our differential abundance analysis (Figures 3 and 5), we noticed that many bacteriophage correlated positively with their putative hosts. We observed this trend across multiple bacteria/phage pairs, including both temperate and virulent relationships. For example, *Streptococcus hydrointestinalis* was positively correlated with *Streptococcus phage phiNJ2, Streptococcus virus 7201,* and *Streptococcus virus DT1*. Additionally, the virulent phage *Lactobacillus phage phiAQ113* was positively correlated with one of its putative hosts, *Lactobacillus amylovorus*.

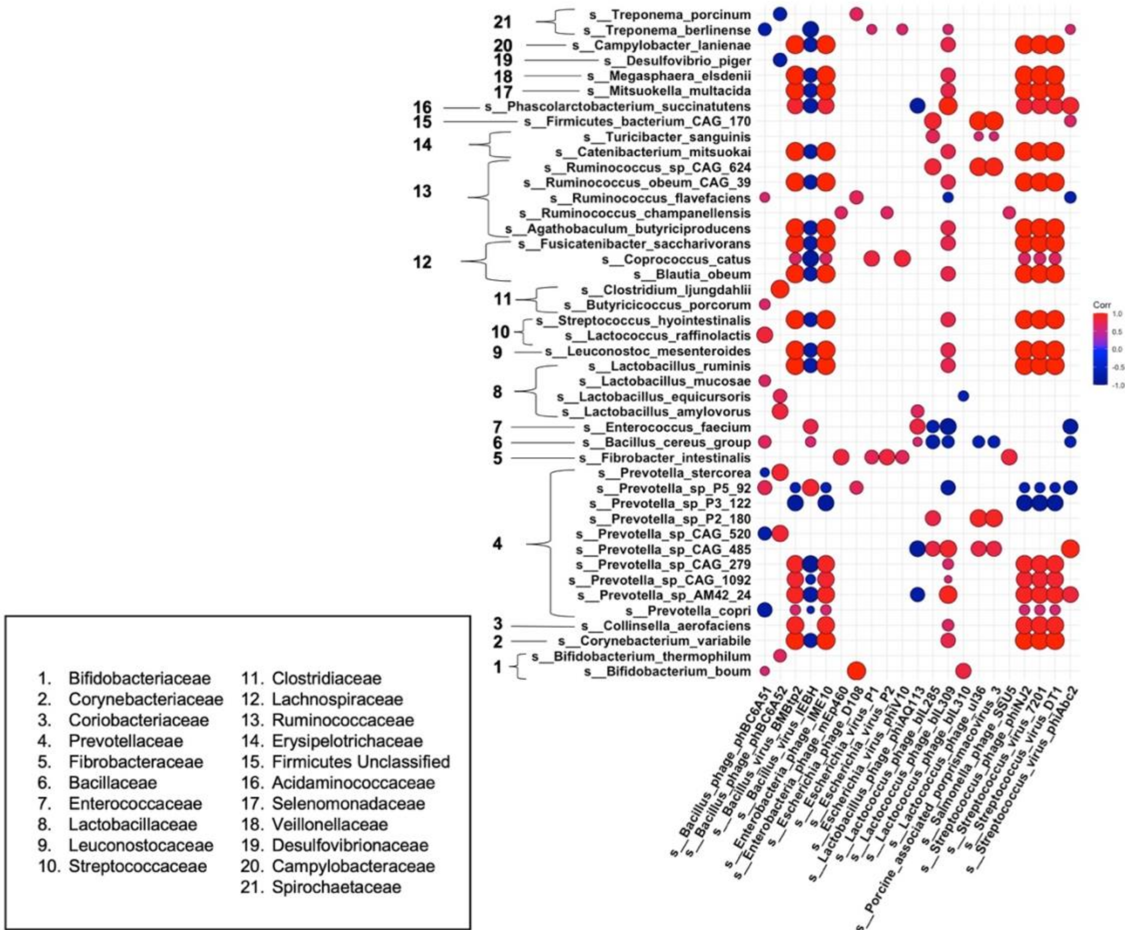

Figure 2.6. Correlations between bacteria and bacteriophage species at 18 weeks post dietary interventions. Pearson's correlation plot of bacterial species vs. bacteriophage species after 18 weeks of dietary exposure. Statistical significance was determined for all pairwise comparisons. Positive values (red circles) indicate positive correlation coefficients above 0.6, and negative values (blue circles) indicate inverse correlation coefficients below -0.6. The size and shading of the circles indicate the magnitude of the correlation, where larger circles indicate a stronger correlation than smaller circles. Correlation coefficient values outside of |0.6| are not included in this plot.

Some bacterial species we noted as being significantly different in our high fat fed pigs correlated uniquely with various bacteriophage species. For example, *Prevotella P5-92* was seen to be significantly elevated within our high fat pigs. We observed a negative correlation of this bacterial species with multiple *Streptococcus*-targeting bacteriophage that were depleted within our high fat fed group. Interestingly, other members of the *Prevotella* genus, such as *Prevotella copri*, were depleted within our high fat group and were positively correlated with *Streptococcus* – targeting bacteriophage. Additionally, *Ruminococcus flavefaciens*, which was positively correlated with back fat thickness and body weight (Figure 4), was weakly negatively correlated with the *Streptococcus* – targeting phage *Streptococcus virus phiAbc2.* Overall, we saw a mostly consistent pattern of correlation between bacteria and bacteriophage species, in which most bacteriophage followed the pattern of their host. However, we observed a deviation from this pattern in bacteria and phage that were significantly changed within our high fat animals, or ones that were positively correlated with phenotypic markers of our growing piglets.

Taken together, we found feeding different amounts of the same diet to young Mangalica pigs had only modest effects on the microbiota within the 18 weeks of this study. In contrast, when fed a diet supplemented with dietary lipids, we observed expedited changes within the gut microbiota that reflected the composition seen at the endpoint of obesity in our novel model, the Mangalica pig. Though significant changes were observed within the bacteriome of our high fat fed piglets, the repercussions of this diet were highlighted by the rapid restructuring of the virome. This also highlights the capability of bacteriophage to contribute to the restructuring of the bacteriome during the development of an obesogenic state, which is promoted by the presence of dietary lipids.

Discussion

The prevalence of obesity is increasing worldwide in adults at an alarming rate given obesity associates with a multitude of comorbidities[1]. Due to its proinflammatory, insulin resistant metabolic phenotype that develops concomitant with its extreme, early onset, morbidly obese body type, the Mangalica pig displays great potential to serve as a relevant animal model of obesity. Such a model of obesity could facilitate novel research aimed at either preventing obesity or uncoupling the obese phenotype from its underlying meta-inflammation (Roberts et al, under review). The aim of this study was to extend such observations by characterizing the intestinal microbiota in mature lean and obese Mangalica pigs and by determining the longitudinal effects of age and diet on the developing gut microbiota in juvenile Mangalica. These studies are the first to describe the overall development of the intestinal microbiome in the Mangalica pig and represent a key first step toward the development of this breed as a useful model to study mechanisms linking the intestinal microbiota and obesity.

Mature *ad libitum*-fed Mangalica pigs in the present study developed a striking degree of adiposity and presented similar characteristics to that of human obesity. These adult, obese pigs developed hyperglycemia and hyperinsulinemia suggesting that their obesity manifested a metabolic phenotype like that of humans suffering obesity-induced diabetes. This metabolic dysregulation was absent in leaner age-matched Mangalica pigs. The development of insulin resistance is a key link between obesity and downstream disease in humans and rodent models of obesity[3,5]. While these results confirm previous observations that the Mangalica breed serves as a novel swine model for obesity-induced metabolic disease, importantly, they also provide key context for our characterization of the Mangalica gut microbiota. For instance, the changes observed within the gut microbiota in response to differences in body composition, age, and diet in the current study occurred amidst a backdrop of obesity-induced metabolic perturbations

suggesting these changes in the microbiota were part of a sequence of events that are faithful to the etiology of human obesity.

The growth responses to dietary treatments in the present study were consistent with expectations for dietary manipulation of body composition in pigs. Importantly, limit fed pigs achieved a similar muscle mass as their *ad libitum* counterparts indicating the striking differences in their body weights were largely driven by differences in adiposity. This suggests that the differences observed in glucose and insulin levels were not confounded by differences in skeletal muscle mass but rather may have been a function of adiposity or due to changes in the gut microbiome as current models of obesity-induced insulin resistance maintain[3–8]. Interestingly, the distinct differences between the gut microbial composition of the limit fed (lean) and *ad libitum* (obese) pigs observed in the first study are consistent with a role for the gut microbiota. Such changes mirror the evolution of the human gut microbiota during development of human obesity as obese humans experience enrichment of bacteriophage concomitant with abundance changes in numerous bacterial phyla[47–49]. This would be expected to both promote a more positive energy balance due to more efficient energy harvest from the diet as well as reflecting a more proinflammatory state[9].

Juvenile pigs that were limit fed, fed *ad libitum* or fed ad libitum + high fat diets during the second (longitudinal) study displayed the expected continuum in growth and body composition. While the gut microbiota was resilient to dietary treatment for pigs in the non-supplemented groups, juvenile pigs fed the high fat diet *ad libitum* displayed shifts in their microbiota that reflected a similar profile to that seen in our mature obese, *ad libitum*-fed pigs during the first study. It is unclear if this was due solely to the high fat component of the diet or it these changes in the microbiota were associated with changes in body composition as pigs fed

the fat supplemented diets were also significantly fatter than pigs in the other dietary treatment groups. Nonetheless, results from the second (longitudinal) study suggest that it takes time for changes in intestinal microbiota within growing Mangalica pigs to reflect the lean or obese phenotype and this is likely due to age-related effects on adipose tissue development. The fattest pigs in the longitudinal study were still much leaner even at 18 weeks than the mature, obese pigs characterized in the first study (subcutaneous fat thickness of 46 mm vs 61mm respectively). The fact that fat supplementation expedited shifts in the gut microbiome might reflect the greater adiposity in this group.

To our knowledge, this study is the first to characterize the intestinal microbiota of the adult Mangalica pig in response to age and diet. The overall composition of the Mangalica gut microbiota was similar to that seen in human, mice, and other mammalian models[6–8]. As in other pig models, we observed a domination of 4 main phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Spirochaetes. The overall consortia seen in our pigs was similar to the core porcine microbiota recently proposed[50]. We observed unique differences in the intestinal microbiota of the *ad libitum*, obese adult pig that were supported by other fatty swine models[26,51]. For example, we reported an increase in *Treponema spp*, which have been associated with fattiness in other swine models [52]. We also reported a decrease in some beneficial taxa, such as *Lactobacillus spp.,* which is also consistent with other reports [53]. More specifically, *Lactobacillus amylovorus* has been shown to directly ameliorate obesity [54]. It has been proposed that obese individuals, mice and humans alike, exhibit a higher proportion of Firmicutes to Bacteroidetes. However, previous reports show this is not always the case in human and swine studies involving obese individuals[26,51,55,56]. The microbiome of our fully developed obese pig did not support this notion, in line with recent reports that conclude this ratio is not an indicator

of host disease state [19,57,58] . Host genetics plays a role in shaping the gut microbiota as well as contributing to the onset of obesity[59,60]. Perhaps some of these findings reflect the unique characteristics of the Mangalica microbiota due to host genetics or environmental factors. Future studies could investigate how host genetics of swine breeds influences microbiota composition. Nonetheless, *ad libitum*-fed Mangalica pigs display an obese phenotype and the Mangalica intestinal microbiota responds to an obesogenic state in a somewhat unique manner relative to other models in the literature.

Juvenile Mangalica pigs were used to temporally describe how the microbiota responds to a limit fed, *ad libitum* or *ad libitum* + high fat (high fat) diet. When looking at the dissimilarity between the 3 groups over time, age was a more important factor in driving microbiota diversity and composition than diet. This observation is consistent with previous reports which have shown that a growing pig's microbiota changes as it ages[61,62]. Regardless of diet, the constituents of the microbiota in both juvenile and mature Mangalica pigs were consistent with other reports describing the gut microbiota of piglets[61,62]. In the present study, we did not observe dramatic differences such as the loss of one more constituents of the gut microbiota. Rather, more modest changes in relative constituent abundances were present. This is to be expected, as all groups were fed the same diet and differed only in the amount of feed given, aside from the supplementation of high fat group. Interestingly, the largest changes in the juvenile gut microbiota occurred within our high fat group, indicating that macronutrient content might be more important than differences in caloric intake alone. Within our high fat samples, we reported changes in *Treponema spp*. and *Prevotella* spp. to be the most notable. *Prevotella spp.* have been reported to be associated with both lean and obese individuals, depending on the study[63]. Further, recent reports by Ley, et al highlighted the genetic diversity within the genus *Prevotella,*

indicating that the complete function of bacteria within *Prevotella* and their function in the gut microbiota might not yet be fully understood[64]. This may explain why we observed opposing shifts in microbial abundance withing the genus *Prevotella*. Additionally, the presence of *Prevotella,* regardless of abundance, may be linked to its ability to metabolize complex carbohydrates, which were present in all respective diets [12,13]. Taken together, however, these data indicate that the juvenile pig's gut microbiota is remarkably resilient to diet change.

Using shotgun metagenomic sequencing, we were able to describe populations in the gut microbiota other than bacteria, namely bacteriophage. Bacteriophage have recently been implicated as important modulators of the gut microbiota in health and disease[42–45,65]. We found that unlike the bacteriome, the virome was much more sensitive to diet change. In the high fat fed juvenile pigs, there was a rapid depletion of virulent Streptococcaceae-targeting viruses – specifically viruses that belonged to the genus *Sfi21dt1* viruses, both temperate and virulent. Interestingly, we observed that *Streptococcus* spp. were not significantly depleted until 10 weeks post dietary intervention. Possible explanations for these seemingly contradictory trends include development of phage resistance by host bacteria or increased sensitivity of the phage to the gut environment. The notion that bacteriophage contribute to the restructuring of the bacterial community within the gut microbiota has been hypothesized before [42]. Our study provides support for this idea, as changes within the virome preceded that of the bacteriome. We have also provided evidence for this in a previous study using a mouse model of obesity [66].

Though we annotated bacteriophage populations within our fully-developed obese pigs, variation between individuals was very high and we were not able to discern specific trends that corresponded with either fattiness or leanness. This was not completely unexpected however, as the virome has been reported to be one of the most variable parts of the gut microbiota[48].

Moreno-Gallego et al observed variability within the virome of monozygotic twins as the individuals aged, citing environmental variables as one possible explanation[48]. In the present study, the 2 cohorts of pigs were housed in different environments, had different maternal lineages, and were different ages; all of which can impact the virome and gut microbiome in general. In addition to this, these pigs had been fed an *ad libitum* or a limit fed diet for a longer period of time as compared to our piglets. This gave time for increase adiposity and metabolic symptoms resulting from obesity. Perhaps the presence of these two factors contributed to the variability seen between the viromes of fully developed, obesogenic Mangalica pigs and growing Mangalica piglets.

One explanation for elevated bacteriophage abundance following dietary change could be that viruses replicate at a much faster rate than bacteria. Bacteria typically produce one daughter cell per replication cycle where one bacteriophage can give rise to hundreds of new virions within one host cell per replication cycle. Additionally, bacteriophage require less resources for production of progeny than bacteria. When the opportunity arises, such as a bloom in target bacteria following changes in nutrient availability, bacteriophage can benefit from the increase in viable host bacteria. In this way, a small bloom of bacteria could give rise to a rapid bloom of bacteriophage that target this host. Bacterial abundance levels could appear reduced or stagnant as bacteriophage progeny are infecting new daughter bacterial cells. Taken together, these data highlight the duality of the piglet microbiota. On one hand, the bacteriome was generally resilient to dietary change, while bacteriophage community rapidly restructured in the presence of dietary lipids. With recent reports indicating that bacteriophage can alter not only the abundance of bacterial constituents in the microbiota, but also metabolites they produce, it has

become clearer that bacteriophage need further investigation as to their contribution to the overall structure and function of the gut microbiota[42].

Though our study extensively characterized the metabolic and microbial characteristics of a novel swine model of obesity, it does not come without its limitations. First, because of the scarce availability and the extensive housing used for this pig model, we were only able to include a limited number of animals in the study. Additionally, a more common dietary fat could be used and compared to the results of our study. Here, we utilized soybean oil, as it is a common addition to pig feed [67,68]. In terms of microbial assemblages, there are nuanced differences between a ‘typical’ human and pig microbiome, one of them being the presence of Spirochaetes, specifically *Treponema* spp. However, these microbes have been found in the gut microbiome of rural native individuals, who consume a diet high in plant polysaccharides, which is similar in nutritional composition to the diet administered here[69]. Future studies utilizing this model should include a larger sample size and different high fat additives to confirm the Mangalica pig as a valid model to study human obesity.

In conclusion, these studies provide insight into how the swine intestinal microbiota responds to dietary changes and age. Pigs have been proposed as a more clinically relevant mammalian model to study human obesity compared to rodent models. These studies are the first to describe the progression of the intestinal microbiome composition in the Mangalica pig and are the first to provide evidence that changes in body composition and dietary conditions are associated with alterations in the microbiome of this novel porcine model of obesity.

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Chapter 3

The Effects of Reactive Oxygen Species and Reactive Chlorine Species on the Reproductive Capability of a Temperate and Virulent *Escherichia coli*-targeting bacteriophage

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Abstract

The intestine is home to the largest immune network in the host and is tasked with the unique challenge of maintaining tolerance to commensal microorganisms while also effectively reacting to invading pathogens. The intestine is also home to the gut microbiome- a collection of microorganisms including bacteria, fungi, and viruses such as bacteriophage. During times of intestinal inflammation, the gut microbiome is subjected to a multitude of antimicrobial substances, such as reactive oxygen species produced by neutrophils. While bacterial responses to reactive oxygen species are well defined, how these species affect bacteriophage, prokaryotic-targeting viruses, is less characterized. Thus, our study aimed to determine the impact of hydrogen peroxide and hypochlorous acid, two reactive species generated during the immune response, on reproduction in a virulent (PF2) and temperate (Lambda λLZ613) bacteriophage species. We found that both hydrogen peroxide and hypochlorous acid had dose-dependent effects on the ability of bacteriophage PF2 to adsorb to its host. In contrast, these products had no effect on adsorption of bacteriophage Lambda λLZ613 to its host. Similarly, hydrogen peroxide inhibited progeny production in PF2, while only modest changes in progeny production were detected in the presence of hypochlorous acid. Finally, hydrogen peroxide, but not hypochlorous acid, induced prophage activation in Lambda λLZ613. Our study provides insight

into a potential mechanism by which bacteriophage populations can be altered in times of intestinal inflammation.

Introduction

Within the past decade, bacteriophage have proven to be an integral and necessary part of many microbial communities, spanning a wide variety of environments such as marine, soil, and gut microbiomes[1]. Within the gut microbiome, bacteriophage are numerous, and estimated to be in a 1:1 ratio with their host[2]. Most of our knowledge regarding the gut microbiome revolves specifically around bacteria. However, recently published reports, including our data described in Chapter 2, have shown that intestinal bacteriophage also play a large role in gut microbiome-host-disease dynamics[3–6]. However, the question remains of what specific mechanism is driving changes seen in bacteriophage populations during disease. In this chapter, I explore one of those mechanisms, the influence of the intestinal immune system, on the reproductive capability of bacteriophages.

Because our gut harbors trillions of microorganisms, the intestinal immune system residing in the gut is tasked with the unique challenge of maintaining homeostasis by tolerating commensal organisms, while retaining the ability to mount an immune response to invading pathogens. During a pathogenic invasion or a general breach in the intestinal barrier, such as in Irritable Bowel Disease (IBD), an inflammatory response is initiated through the production of inflammatory cytokines[7]. One role of inflammatory cytokines is to recruit granulocytes, such as neutrophils. These are among the first cell types to infiltrate the intestine during an inflammatory episode[8,9] and they can employ a variety of tactics to destroy bacterial pathogens. Among their defense mechanisms is release of granules containing antimicrobial substances, phagocytosis, and initiation of respiratory burst through NADPH oxidase[10,11]. During a respiratory burst event, neutrophils release multiple reactive oxygen species (ROS), including hydrogen peroxide[10]. To increase the potency of their respiratory burst, neutrophils can release enzymes such as

myeloperoxidase through degranulation, for which hydrogen peroxide is a substrate, to produce other reactive species such as hypochlorous acid (HOCL)[12]. In turn, bacteria can respond and adapt to oxidative stress due to these products. When oxidative stress is sensed by a bacterium, it turns on global regulatory networks, such as the SOS response, to reduce damage vital biomolecules such as DNA[13].

The impact of gut inflammation on bacteria within the microbiome has been well documented. It is well known that bacterial populations are altered in a variety of ways, compared to non-inflamed controls in diseases such as in IBD[14,15]. However, few studies have examined how bacteriophage populations, both free phage in the environment as well as integrated prophage, are altered during times of intestinal inflammation. In 2015, Norman et al. was the first to observe changes in intestinal phage populations during colitic episodes in mice[6]. Specifically, they saw an overall increase in bacteriophage diversity and the relative abundance of the viral family *Caudovirales*[6]. These findings have been recently validated in both child and adult subjects with ulcerative colitis[14,15]. However, the exact mechanisms through which intestinal inflammation impacts bacteriophage populations remains elusive.
Bacteriophage are prokaryotic-targeting viruses, with most belonging to the class Caudovirales. Viruses within this class are tailed with a dsDNA genome, and account for a large majority of the bacteriophages associated with the intestine. Bacteriophages containing a ssDNA and RNA genomes exist, but are less well understood[16,17]. Generally, bacteriophage can replicate using one of 2 life cycles, the lytic and lysogenic cycle, though other mechanisms of reproduction exist[18]. The specific life cycle a phage species utilizes is used to categorize them.

In order to initiate replication, regardless of the mode used, a bacteriophage must first attach to its host through a mechanism known as adsorption. During this step, a virion will

interact via binding proteins with a receptor on a sensitive host bacterium. This process also positions the virion for DNA injection into the host[19]. It consists of a reversible step, in which binding to the host is not definitive, and the virion retains the ability to desorb from the host. This reversible action might aid in the virions ability to scavenge for its receptor on the bacterium[19,20]. Then, once the receptor is found, the virion irreversibly binds to the host in preparation for DNA ejection. Adsorption also determines the host range of the bacterium; the specific phage receptor could be a protein, sugar, or other structure located on the cell surface[19,21]. Depending on how widely expressed the receptor is in each clade of bacteria, it will dictate what the host range for that phage is. Environmental cues and host physiological status can also play a role in whether a specific phage receptor is expressed[19,21]. Once adsorbed to its host, the phage can then employ a variety of techniques to break through the cell barrier of the host and inject its genome[22].

After adsorption, virulent bacteriophage, those that replicate through a strictly lytic cycle, move directly into replicating its genome, transcribing and translating proteins, and assembling nascent virions. Collectively, the time from adsorption until assembly of the virions is known as the eclipse period, as no progeny have exited from the host yet. Subsequently, the virions produced during infection are released from the host cell. Progeny are then free to infect neighboring sensitive hosts in the environment. Temperate bacteriophage can utilize the lytic infection cycle to produce progeny, but also can undergo the lysogenic cycle. Lysogeny allows the bacteriophage to establish a latent infection and lay dormant in its host until an induction event occurs and cues the bacteriophage to enter the lytic cycle. Once integrated into the host genome or existence as an extrachromosomal element is established, the phage is now known as a prophage. The prophage is then maintained through molecular mechanisms that repress lytic

genes. For example, in bacteriophage Lambda, the *cI* and *cII* repressors are responsible for establishing and maintaining the prophage state[23]. Specifically, *cII* is responsible for initially stimulating expression of the *cI* repressor, amongst other proteins[23,24]. Then *cI* will repress promoters P_L and P_R, which are needed to express genes that encode for lytic functions[23,25]. When *cI* is inactivated or inhibited, this allows for the expression of lytic genes and active viral replication occurs. Inactivation of the *cI* protein can occur through multiple avenues, such as the bacterial stress response to oxidative stress. Though much is known about the molecular mechanisms that drive both lytic replication and lysogeny, how these mechanisms fit into a larger context, such as how they are impacted by the gut environment, is ill-defined. Further, how products of the host intestinal immune system, an important modulator of the gut microbiome, impact the replication of bacteriophage, is not known.

The purpose of this study was to elucidate how certain antimicrobial substances, such as reactive oxygen and chlorine species, specifically hydrogen peroxide and hypochlorous acid, affected the reproduction of virulent and temperate bacteriophage. To this end, we performed a series of assays to assess the infectivity potential and progeny production of a virulent and temperate bacteriophage in the presence of these reactive products. We observed a dose-dependent inhibition of adsorption in the virulent phage PF2 when in the presence of increasing amounts of hydrogen peroxide and hypochlorous acid. The delay in adsorption due to hydrogen peroxide was translated to a decrease in progeny production. However, this was not observed with hypochlorous acid. In contrast, these reactive species had no effect on the adsorption kinetics of temperate bacteriophage Lambda, regardless of dose. However, hydrogen peroxide, but not hypochlorous acid, induced prophage activation. Our study provides insight on how

immune products present in the gut during times of inflammation can potentially influence bacteriophage function and reproduction.

Materials and Methods

Bacteriophage and Bacterial Isolation and Strains

Bacteriophage PF2, the virulent phage used for our study, was isolated from porcine fecal samples. We first isolated an *E. coli* species from a pig fecal sample that was identified through phenotypic and metabolic characteristics. This *E. coli* strain was used as an indicator strain in the isolation of PF2. We then incubated an aliquot from the same fecal sample in salt-magnesium buffer containing 10% beef extract. Samples were then filtered using a 0.45-micron filter and plated using the double agar overlay method. Plaques found on resulting plates were cored and placed in a 10% chloroform solution and for 4 hours at room temperature. The upper phase was obtained and again plated with our indicator strain for subsequent isolation. Several rounds of isolation were performed to ensure a monoculture of our specific bacteriophage strain. PF2 was found to be a virulent phage and was determined to belong to the family *Podoviridae* through electron microscopy.

For our temperature phage, a mutant strain of bacteriophage Lambda was used. Specifically, we utilized the ƛLZ613 strain, a generous gift from the Lanying Zeng lab. This strain contains a temperature-sensitive *cI*, where growing the phage along with its host bacterium at 37C forces an obligatory lytic reproductive pattern. Additionally, when this bacteriophage integrates as a prophage, it makes the host resistant to the antibiotic Kanamycin. To ensure a free lytic/lysogeny decision, all experiments were performed at 30C. The host *E. coli* strain, *E. coli* C600 was obtained from ATCC (ATCC 23724). For prophage induction experiments described below, a Lambda lysogen culture was generated by selection with Kanamycin. To do this, an overnight culture of the sensitive host strain, *E. coli* C600 was diluted 1:100 in Lysogeny Broth (LB) broth containing 0.1232% Magnesium Sulfate and 0.2% maltose to ensure the expression of the bacteriophage Lambda receptor, LamB. The culture was grown to an OD_{600} of 0.4 and

bacteriophage Lambda ƛLZ613 was added at an MOI of 0.1. The co-culture was allowed to incubate at 30C for 4 hours. After incubation, the culture was plated on Kanamycin-containing agar plates and incubated overnight at 30C.

Reagents

To mimic immune products bacteriophage might encounter in the inflamed gut, we selected hydrogen peroxide (reactive oxygen species) and hypochlorous acid (reactive chlorine species), which are produced by neutrophils. For experiments involving hydrogen peroxide, a 3% solution was diluted to concentrations between 0.5mM and 3mM. For experiments involving hypochlorous acid, a 5% solution of sodium hypochlorite, a compound commonly used to represent hypochlorous acid *in vitro*, was diluted to concentrations between 0.004mM and 0.020mM. Reagents were freshly prepared for each experiment.

Adsorption Kinetics Assay

Adsorption kinetics were measured using a protocol described previously[26]. An overnight culture of sensitive bacterial host strains (isolated *E. coli* or *E. coli* C600) were diluted 1:100 in LB media containing 0.1232% magnesium sulfate, with the addition of 0.2% maltose for *E. coli* C600. Cultures were grown to an OD_{600} of 0.4 and infected with the appropriate bacteriophage strain at an MOI of 0.1. Simultaneously, hydrogen peroxide or hypochlorous acid or a negative water control was added to the appropriate co-culture. Every 2.5 minutes for 7.5 minutes, 750ul aliquots of co-cultures were taken, filtered through a 0.2-micron filter and subsequently plated through the double agar overlay method to enumerate free phage in the culture (Figure 4.1). To calculate adsorption slopes and the adsorption constant *k*, a linear regression analysis was

performed using plaque forming unit concentrations (PFU/ml) over time. Significant differences in slopes were determined through an ANCOVA.

One-step growth curves

In order to determine how hydrogen peroxide and hypochlorous acid affected progeny production in our virulent bacteriophage PF2, one-step growth curves were performed as described previously. To do this, co-cultures of PF2 and its sensitive host *E. coli* (MOI 0.1, OD_{600} 0.4) were incubated at 37C for 10 minutes to allow for adsorption. Following adsorption, co-cultures were diluted in LB broth containing 0.1232% magnesium sulfate. Hydrogen peroxide or hypochlorous acid or a water control were added to appropriate flasks. 1ml samples were taken every 5 minutes for 60 minutes and were plated using the double agar overlay method to enumerate plaque forming unit concentration over time (PFU/ml) (Figure 4.1). Burst estimates were calculated by subtracting free PFU/ml prior to initial burst from the total PFU/ml following the burst. Free PFU/ml was determined by filtering a sample using a 0.2-micron filter and enumerated through the double agar overlay method. Significant differences between treatments and controls were calculated using an ANOVA.

Prophage Activation Assays

The impact of hydrogen peroxide and hypochlorous acid on prophage activation was determined by calculating free plaque forming unit concentrations (PFU/ml) over time and by measuring bacterial growth over time by obtaining the OD_{600} of the culture. For bacterial growth curves performed, an overnight culture of Lambda ƛLZ613 lysogen was diluted 1:100 in LB broth containing 0.1232%, 0.2% maltose, and 50µg/ml Kanamycin to ensure a culture with pure

lysogen. The culture was grown to an OD_{600} of 0.1. Once the appropriate OD_{600} was reached, samples were then centrifuged at 1800RPM for 15 minutes to remove the culture from antibiotic-containing media and to remove free, ambient phage in the culture. The supernatant was then removed, and the pellet is resuspended in the same amount of LB media without kanamycin. The sample was then divided into 5ml aliquots, and the appropriate treatment or water control was added to the cultures. 200µl samples were then plated in replicate in a 96-well plate, and the OD_{600} was measured every 20 minutes for 6 hours. To enumerate free PFU/ml over time, cultures were prepared as described above. Then, the culture was divided into aliquots and the appropriate concentrations of treatments, or a water control, is applied. 750µl samples were taken every hour for 6 hours. Each sample was filtered through a 0.2-micron filter and plated via the double agar overlay method. Statistical differences between OD_{600} readings and PFU/ml were calculated via an ANOVA.

Results

Reactive oxygen and chlorine species differentially affect adsorption rates of bacteriophage PF2 and Lambda

To assess the impact of reactive oxygen and chlorine species on the adsorption kinetics of bacteriophage PF2 and Lambda, we utilized an adsorption kinetics assay in the presence or absence of hydrogen peroxide or hypochlorous acid. However, before performing such assays, the impact of our treatments on the bacterial host strains of our bacteriophages needed to be assessed. We wanted to utilize a concentration of hydrogen peroxide and hypochlorous acid that would induce stress in the bacterium, but not completely kill it. To do this, we measured OD_{600} of bacterial cultures over 12 hours. The *E.coli* host strain for PF2 showed a dose-dependent sensitivity hydrogen peroxide at a concentration of 1mM to 3mM. In all treatments, bacteria displayed a delay in growth but were not killed (Figure 3.1A). This trend was also observed in the host of bacteriophage Lambda, *E. coli* C600 (Figure 3.1B). In contrast, hypochlorous acid did not seem to induce a delay in growth. However, concentrations used were consistent with previous literature and shown to cause delay in other experiments described below[27]. In the case of both stressors, PF2 and Lambda were not shown to be directly impacted (data not shown).

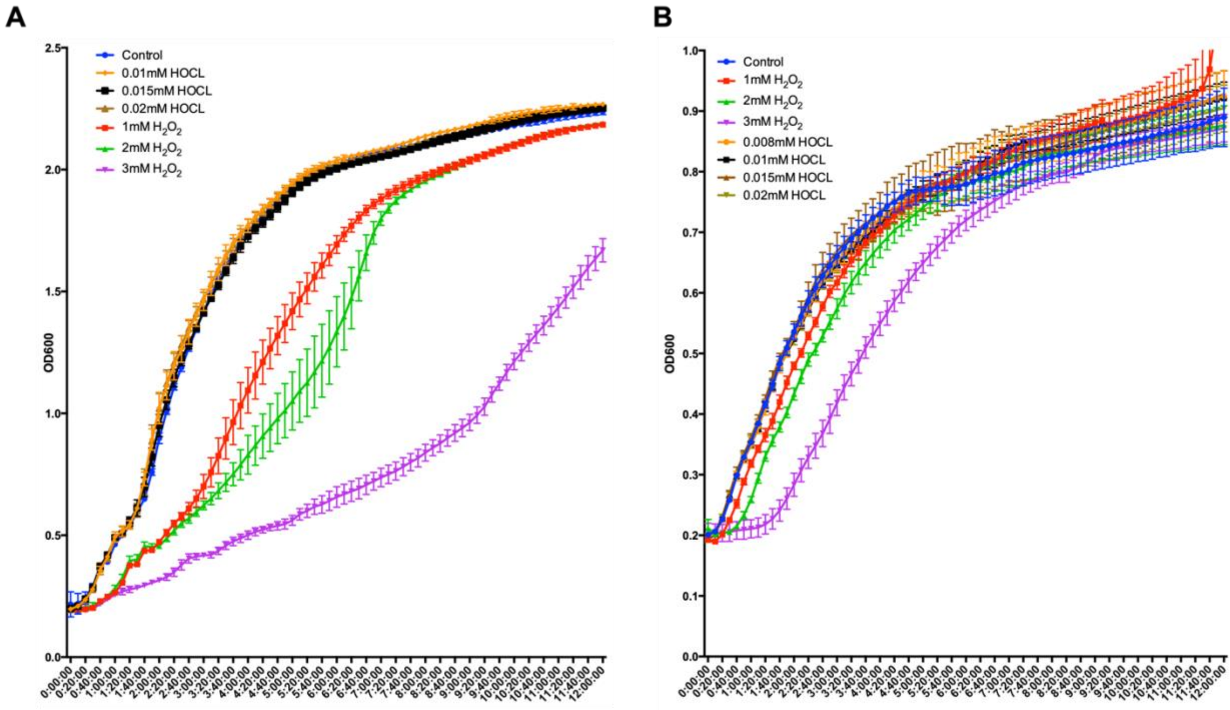

Figure 3.1 Growth of sensitive hosts strains in the presence of hydrogen peroxide or hypochlorous acid. (A) porcine-isolated *Escherichia coli* and (B) *E. coli* C600 cultures were grown to an OD_{600} of 0.1 and varying amounts of hydrogen peroxide (1mM, 2mM, 3mM), hypochlorous acid (0.008mM, 0.01mM, 0.015mM, 0.020mM), or PBS control was added. OD_{600} was measured every 20 minutes for 12 hours.

With this in mind, we first examined the effects of hydrogen peroxide on the adsorption rate of both bacteriophage PF2 and Lambda. It is important to keep in mind that we are not able to measure adsorption directly. However, it is possible to measure the loss of free phage in the culture over time. By measuring this loss over time, we can determine the acceleration at which free phage are lost, as well as calculate the adsorption constant, *k*. By measuring this loss over time, we can determine the acceleration at which free phage are lost, as well as calculate the adsorption constant, *k*. *k* represents the probability of a single phage adsorbing to a single bacterium within a given volume and unit of time. A larger adsorption constant indicates an overall faster adsorption rate.

In our virulent bacteriophage PF2, we found that hydrogen peroxide significantly delayed adsorption to its host in a dose dependent manner. Initially, PF2 was found to have a mean slope of -4.456 and an adsorption rate constant, *k*, of 1.7290×10^{-7}ml/min (Table 3.1). Additionally, we found that ~30% of free phage in the culture adsorbed to a host by 7.5 minutes. When hydrogen peroxide was present in the sample, we found the *k* to be decreased, indicating a slowing of adsorption. At low concentrations of hydrogen peroxide, such as 0.5mM, the loss of free phage over time was slowed, indicated by a slope 1.175 units higher than the control (Figure 3.2A, Table 3.1). In turn, the calculated *k* was significantly smaller than that of the control, indicating an overall slower adsorption ($p=0.022$). 1mM had similar effects as 0.5mM but was not statistically significant ($p=0.154$) (Figure 3.2B, Table 3.1). Finally, 2mM had the most significant effect on adsorption, indicated by a significantly smaller *k* ($p=0.004$), and a 2.464 unit increase in the slope (Figure 3.2C, Table 3.1).

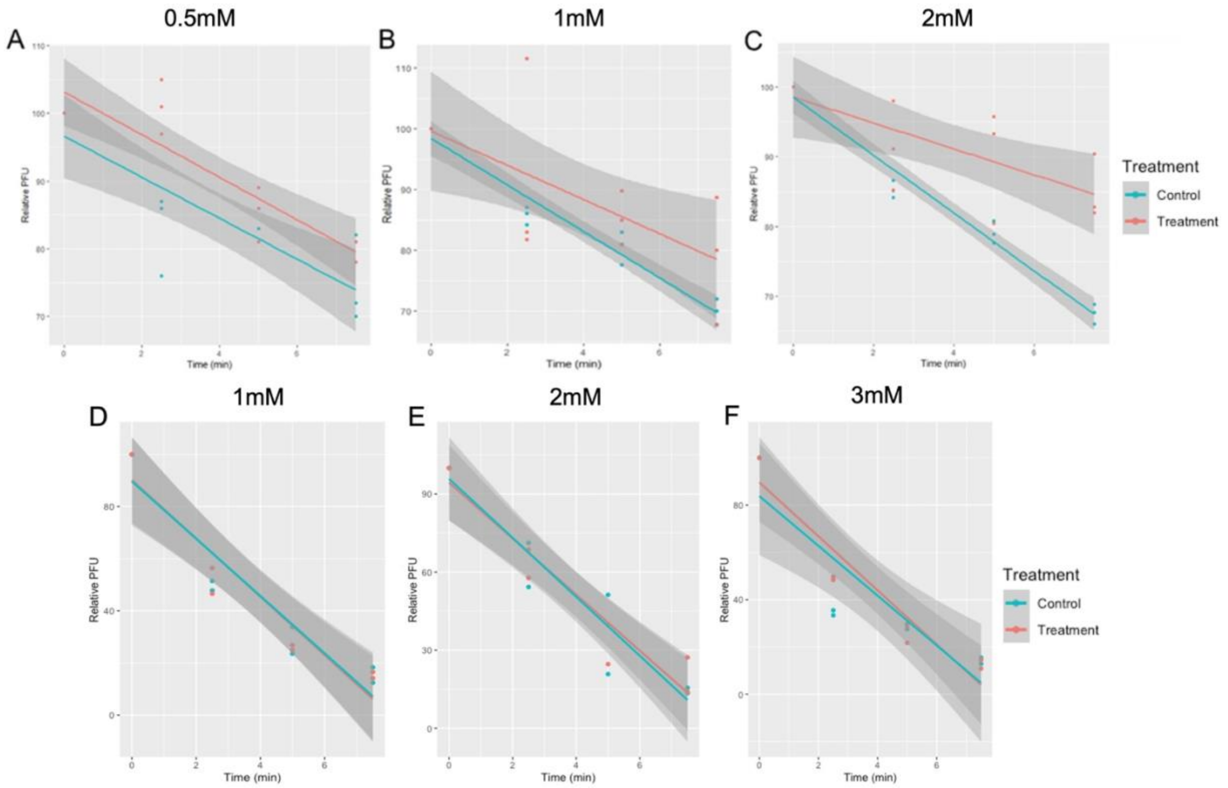

Figure 3.2 Effects of hydrogen peroxide on bacteriophage PF2 and Lambda*. PF2 and Lambda free phage were enumerated over 7.5 minutes in the presence of varying amounts of hydrogen peroxide. For bacteriophage PF2, (A) 0.5mM, (B) 1mM, and (C) 2mM hydrogen peroxide was used. For bacteriophage Lambda, (D) 1mM, (E) 2mM, (F) 3mM hydrogen peroxide was used. Slope, *k* value, and p values are outlined in Table 3.1 and 3.2.

*Figure 3.2 A-C from Keah Higgins' dissertation[28]

Table 3.1. Adsorption kinetics of bacteriophage PF2 following treatment with hydrogen peroxide*

Treatment	Differences in slopes	*k*	*p* value
Control	Mean slope: -4.3226	1.7290×10^{-7} ml/min	
0.5mM H_2O_2	+1.1756	1.2588×10^{-7} ml/min	0.0224*
1mM H_2O_2	+0.8656	1.3828×10^{-7}	.938
2mM H_2O_2	+2.4646	7.4320×10^{-8}	0.00468**

*Table adapted from Keah Higgins' dissertation[28]

Table 3.2. Adsorption kinetics of bacteriophage Lambda following treatment with hydrogen peroxide

Treatment	Differences in slopes	*k*	*p* value
Control	Mean Slope: -10.17	1.017×10^{-6} ml/min	
1mM H_2O_2	+1.41	8.757×10^{-7} ml/min	0.979
2mM H_2O_2	-0.60	1.077×10^{-6} ml/min	0.898
3mM H_2O_2	-1.25	1.142×10^{-7} ml/min	0.752

Bacteriophage Lambda was found to adsorb faster to its host as compared to PF2. Lambda had a mean slope of -10.17, and a *k* of 1.017×10^{-6} ml/min (Table 3.2). Additionally, ~85% of free phage in the culture we adsorbed to its host within 7.5 minutes. Regardless of dose, there was no significant effect of hydrogen peroxide on the adsorption rates of bacteriophage Lambda (1mM $p=0.979$; 2mM $p=0.898$; 3mM $p=.752$) (Figure 3.2D-F, Table 3.2). Overall, hydrogen peroxide had differential effects on adsorption rates in bacteriophage PF2 and Lambda.

Next, we wanted to determine the impact of hypochlorous acid on the adsorption rate of our two bacteriophage strains, virulent PF2 and temperate Lambda. As with hydrogen peroxide, hypochlorous acid caused a significant delay in adsorption of PF2 to its host. At our lowest concentration, 0.004mM, we did not observe any significant differences between adsorption rates or slopes (Figure 3.3A, Table 3.3). At concentration of 0.008mM, we observed a slowed loss of free phage over time, as indicated by a slope 1.742 units higher than the control (Figure 3.3B, Table 3.3). We also observed a significantly smaller adsorption constant, *k* ($p=0.007$) (Table 3.3). Similar trends were observed when a treatment of 0.01mM was applied, though not significant (Figure 3.3C, Table 3.3). Finally, adsorption was delayed most significantly at our highest dose of hypochlorous acid, 0.020mM, indicated by an increase in slope by 3.053 units, and a significantly smaller *k* ($p=0.001$). In contrast, and like hydrogen peroxide, hypochlorous acid had no effect on adsorption rates of bacteriophage lambda. Regardless of dose, no significant differences were seen in slopes or adsorption rate constants when hypochlorous acid was applied (0.008mM $p=0.958$; 0.01mM $p=0.472$; 0.015mM $p=0.718$) (Figure 3.3D-F). Taken together, both reactive oxygen and chlorine species, represented by hydrogen peroxide and hypochlorous acid respectively, have differential effects on adsorption rates of bacteriophage PF2 and Lambda.

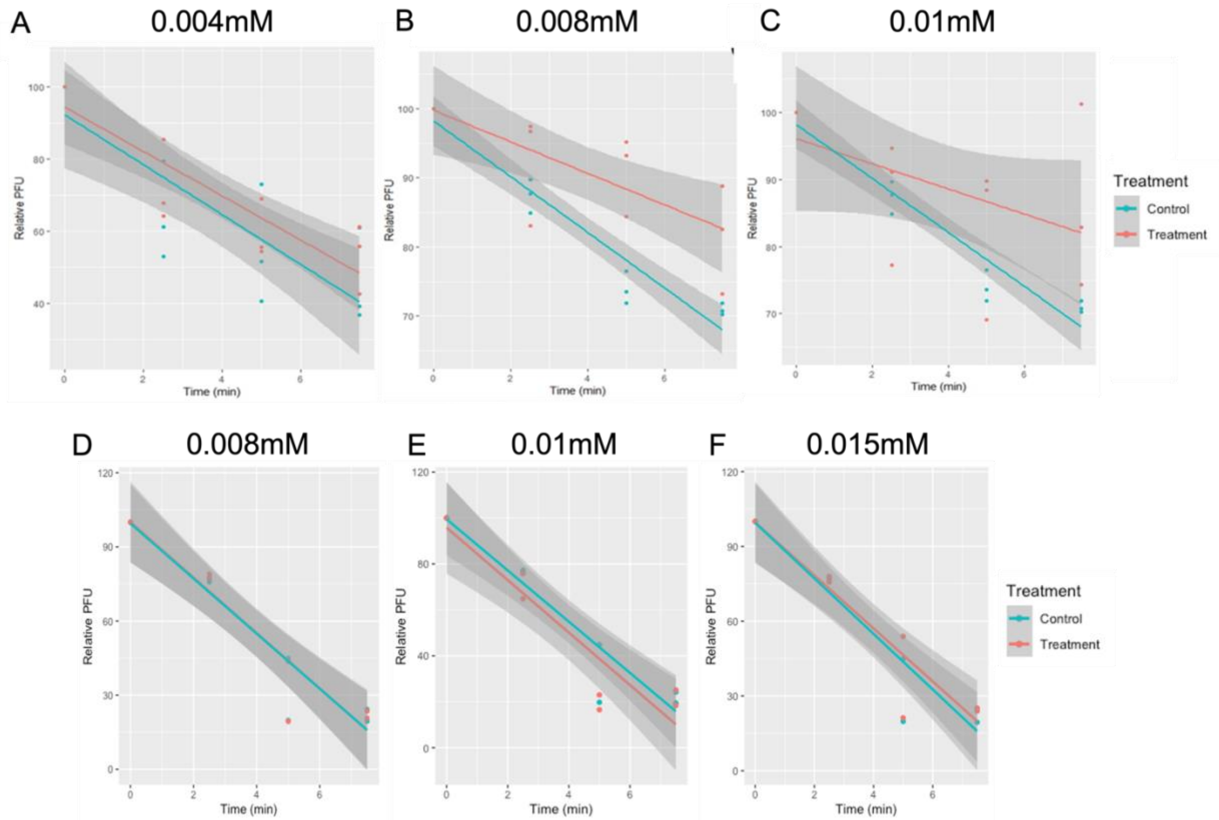

Figure 3.3 Effects of hypochlorous acid on bacteriophage PF2 and Lambda*. PF2 and Lambda free phage were enumerated over 7.5 minutes in the presence of varying amounts of hypochlorous acid. For bacteriophage PF2, (A) 0.004mM, (B) 0.008mM, and (C) 0.01mM hypochlorous acid was used. For bacteriophage Lambda, (D) 0.008mM, (E) 20.01mM, (F) 0.015mM hypochlorous acid was used. Slope, *k* value, and p values are outlined in Table 3.3 and 3.4

*Figure 3.3 A-C from Keah Higgins' dissertation[28]

Table 3.3 Adsorption kinetics of bacteriophage PF2 following treatment with hypochlorous acid*

Treatment	Differences in slopes	*k*	*p* value
Control	Mean Slope: -4.456	$1.8053x10^{-7}$ ml/min	
0.004mM	+ 0.772	$2.4850x10^{-7}$ ml/min	0.377
0.008mM	+ 1.742	$9.2222x10^{-8}$ ml/min	**0.007 ****
0.01mM	+ 2.147	$7.5810x10^{-8}$	0.204

*Table adapted from Keah Higgins' dissertation[28]

Table 3.4 Adsorption kinetics of bacteriophage Lambda following treatment with hypochlorous acid

Treatment	Differences in slopes	*k*	*p* value
Control	Mean slope: -11.47	$1.15x10^{-6}$ ml/min	
0.008mM	- 0.071	$1.12x10^{-6}$ ml/min	0.958
0.01mM	- 0.268	$1.14x10^{-6}$ ml/min	0.472
0.015mM	+ 0.521	$1.03x10^{-6}$ ml/min	0.718

Reactive oxygen and chlorine species inhibit progeny production in bacteriophage PF2

Both hydrogen peroxide and hypochlorous acid displayed a clear inhibitory effect on the adsorption rate of PF2. Because of this, we were interested in whether these reactive species had an impact on progeny production in this bacterial species. To do this, we performed a one-step growth curve in the presence or absence of varying doses of hydrogen peroxide or hypochlorous acid. Overall, PF2 reproduced quickly, with the initial burst of progeny occurred 10 minutes post adsorption, followed by a subsequent 10-minute latent period and additional bursts throughout the rest of the experiment (Figure 3.4A). The burst size, calculated by subtracting the free phage amount prior to the initial burst (5 minutes post adsorption) from the total plaque forming units per milliliter (PFU/ml) during the plateau following the initial burst (25 minutes post adsorption), was estimated to be $1x10^8$ in controls (Figure 3.4B).

With the addition of hydrogen peroxide, we found a dose-dependent reduction in progeny production and overall bacteriophage fecundity. At low concentrations, such as 0.5mM hydrogen peroxide, the initial burst of progeny occurred 15 minutes post adsorption, a 5-minute delay as compared to the control. This 5-minute delay continued through the duration of the experiment, leading to a significant reduction in progeny production at 55 minutes post adsorption. However, no differences in burst size compared to the control were seen. In contrast, 2mM and 3mM resulted in a complete loss of the typical growth pattern for PF2, resulting in a severe reduction in progeny produced as well as burst size (Figure 3.4 A,B). In contrast, hypochlorous acid did not greatly impact progeny production or burst size in PF2. At 0.01mM hypochlorous acid concentration, no significant delays in bursts were observed, as the initial burst occurred 15-minutes post adsorption, as seen in the control (Figure 3.5A).

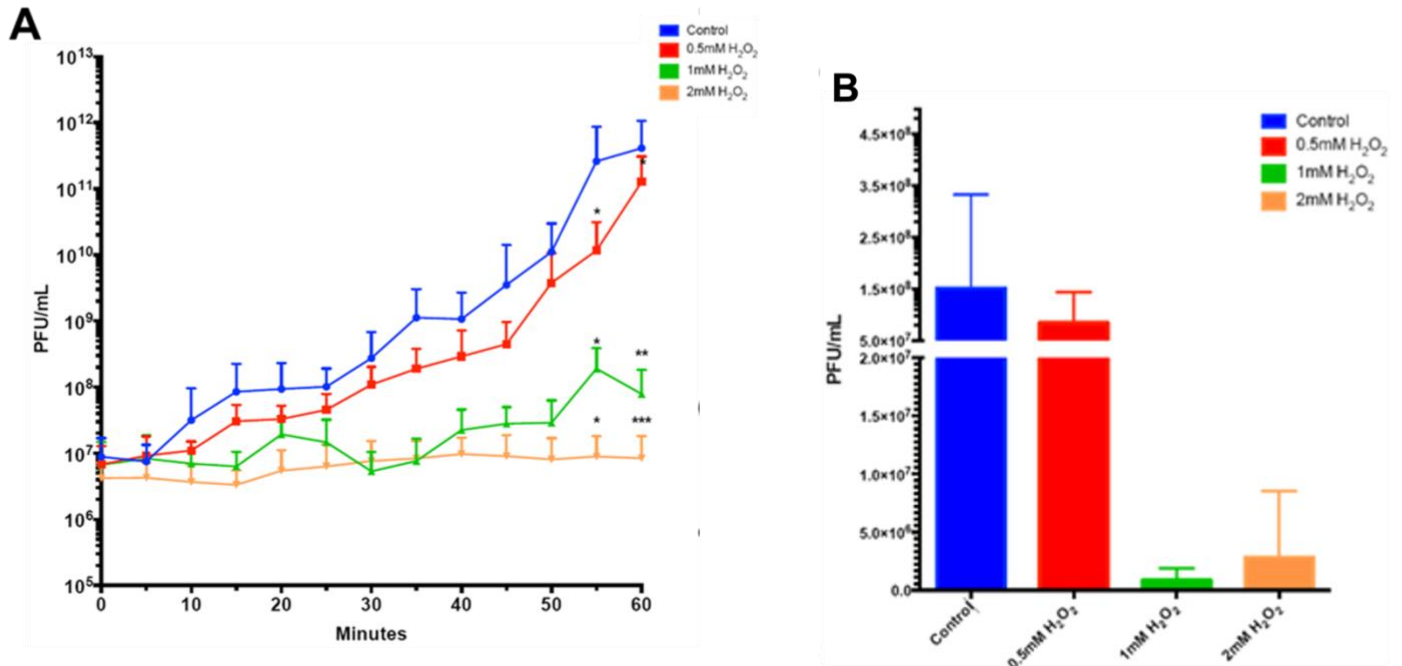

Figure 3.4 Impact of hydrogen peroxide on bacteriophage PF2 progeny production over time*. (A) represents the varying effects of hydrogen peroxide on bacteriophage PF2 progeny production over time. From this, (B) burst estimates for each treatment and negative control were calculated. $*p < 0.05$, $** p > 0.01$, $*** p > 0.001$

*Figure adapted from Keah Higgins' dissertation[28]

Additionally, the treatment did not have any significant effect on burst size or total progeny produced (Figure 3.5 A,B). Similarly, when cultures were treated with 0.020mM hypochlorous acid, no delay in the initial bursts were detected, as seen in lower concentrations of the treatment as well as the control. However, we observed a significant reduction in PFU/ml at 60 minutes, which might indicate that hypochlorous acid affects progeny production in a delayed manner (Figure 3.5 A). Even though this significant reduction was observed, no significant differences in burst sizes were observed as compared to the control (Figure 3.5 B). Overall, both hydrogen peroxide and hypochlorous acid influenced overall progeny production. Cultures treated with hydrogen peroxide showed a significant decrease in burst size as well as a delay, or a complete absence, in progeny production.

In contrast, hypochlorous acid showed little impact on progeny production or burst size as compared to the control. However, at higher concentrations, hypochlorous acid impacted progeny production in a delayed manner. This could indicate that virulent bacteriophage reproduction is inhibited during intestinal inflammation (Figure 3.5A,B).

Both hydrogen peroxide and hypochlorous acid lead to activation of prophage

Temperate bacteriophages can reproduce utilizing lytic replication, as explored above, as well as undergo lysogeny. Therefore, we next wanted to explore how hydrogen peroxide and hypochlorous acid influenced prophage activation. To do this, we generated a Lambda ƛLZ613-containing lysogen by incubating a sensitive host with bacteriophage Lambda ƛLZ613 at an MOI of 0.1 at 30C for 4 hours to allow for lysogeny. The co-culture was then plated onto kanamycin-containing agar plates and grown at 30C. This generated lysogen was used for all prophage experiments.

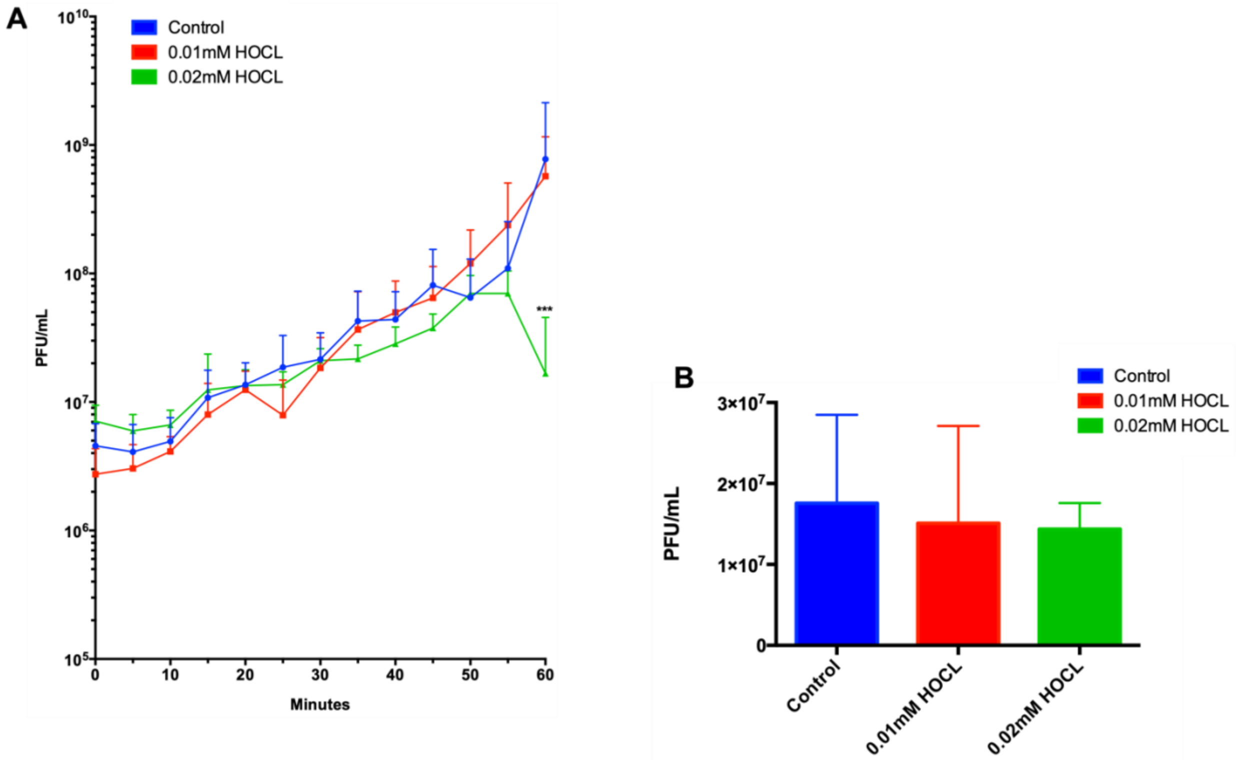

Figure 3.5 Impact of hypochlorous acid on bacteriophage PF2 progeny production over time.* (A) represents the varying effects of hypochlorous acid on bacteriophage PF2 progeny production over time. From this, (B) burst estimates for each treatment and negative control were calculated. $*p < 0.05$, $** p > 0.01$, $*** p > 0.001$

*Figure adapted from Keah Higgins' dissertation[28]

We utilized two approaches to determine how our treatments influenced prophage activation. First, we determine the overall growth of the culture by measuring the OD_{600} every 20 minutes for 12 hours in the presence of absence of our stressors. Second, we determined how free plaque forming units (PFU/ml) changed over time in presence of our stressors. Looking at these results together can allow us to gain an understanding of how hydrogen peroxide and hypochlorous acid influence prophage activation. As in other experiments performed, we found that hydrogen peroxide had a dose dependent effect on prophage activation. Under normal, homeostatic conditions, our lysogen entered log phase rather quickly (20 minutes) (Figure 3.6 A). Our lysogen stayed within log phase for approximately 10 hours and started to plateau. At our lowest concentration of hydrogen peroxide used in this experiment, 1mM, we noticed a slight lag in initiation of log phage of 20 minutes, and a significant lower OD_{600} starting at 40 minutes and remaining significant until 10 hours post administration (Figure 3.6A, $p= <0.01$). At 2mM, we noticed a slightly longer delay in the initiation of log phage of about 60 minutes. Cultures treated with 2mM hydrogen peroxide also maintained a significantly lower OD_{600} as compared to the control starting at 40 minutes post administration and continued through the duration of the experiment (Figure 3.6A, $p= <0.0001$). Our 2mM treated cultures also displayed a significantly lower OD_{600} as compared to our 1mM treatment starting at 80 minutes post administration to 9 hours and 40 minutes (Figure 3.6A, $p= <0.01$). Finally, our highest concentration of 3mM hydrogen peroxide, we saw a more severe 2 hour delay in the initiation of log phase (40 minutes, $p= >0.0001$). Starting at 40 minutes during this delay, 3mM treated cultures had a significantly lower OD_{600} as compared to the control ($p = <0.0001$) that lasted the duration of the experiment.

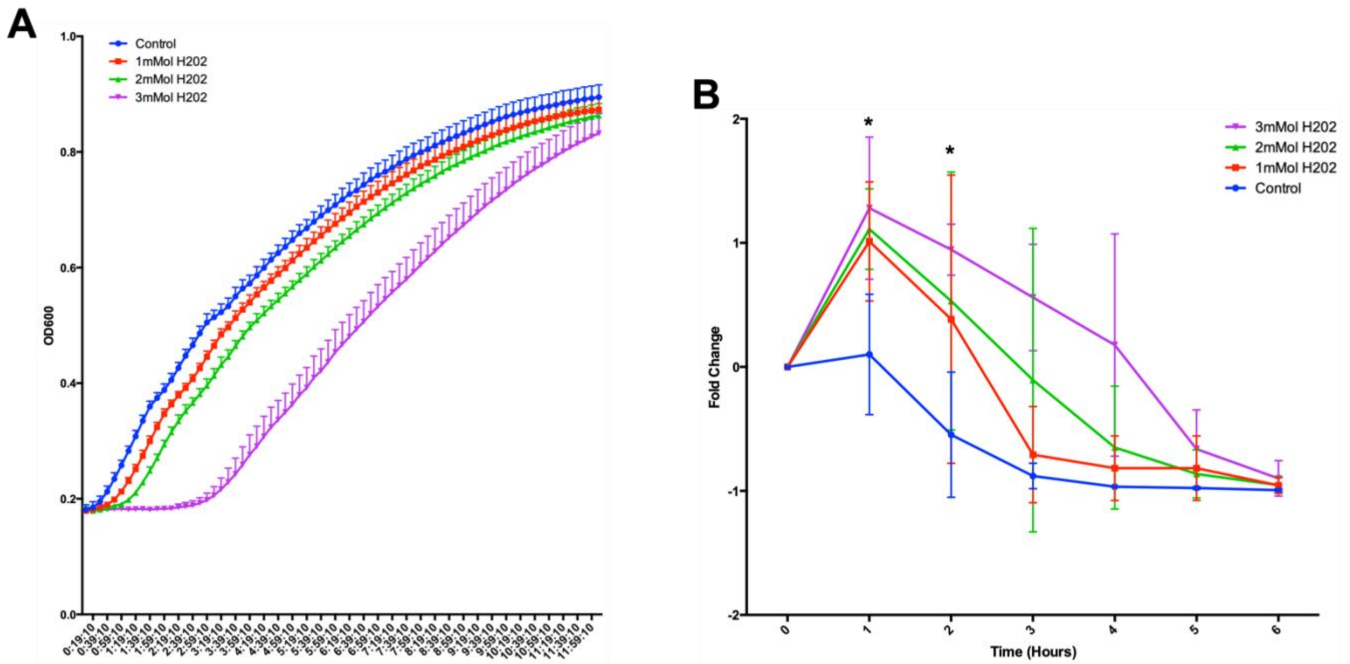

Figure 3.6 Effects of hydrogen peroxide on Bacteriophage Lambda prophage induction. Cultures of Bacteriophage Lambda ƛLZ613 lysogen were grown to an OD_{600} of 0.1. Cultures were then incubated with varying concentrations of hydrogen peroxide (1mM, 2mM, or 3mM) or a negative control. (A) Represents OD_{600} measurements over time of the Lambda ƛLZ613-containing lysogen. Significant differences between groups are explained in detail within the manuscript. (B) Free phage concentration was measured over the course of 6 hours, and fold change from initial time point was calculated. Statistical differences in both graphs were determined using an ANOVA. *$p < 0.05$

Additionally, starting at 60 and 80 minutes respectively, 3mM had a significantly lower OD_{600} as compared to our 1mM and 2mM treated groups ($p= <0.0001$) that lasted the duration of the experiment. Overall, this indicated to us that the hydrogen peroxide was causing delays in the growth of our lysogen, more so than what was observed in our sensitive host (Figure 3.1A).

Next, we wanted to quantify the amount of progeny produced over time due to activation of prophage in the presence of varying amounts of hydrogen peroxide. To do this, we sampled cultures spiked with varying concentrations of hydrogen peroxide, 1mM, 2mM, and 3mM, or a negative control every hour for 6 hours. Free phage was then quantified and plotted as fold-change over time. In our negative control, we observed a 0.1 fold-increase in free phage at 1 hour post treatment administration, followed by a 1 fold decrease in free phage starting at 3 hours post administration that stayed consistent for the duration of the experiment. All 3 doses of hydrogen peroxide induced activation of prophage approximately 1-fold higher than in the control at 1 hour post administration. 1mM increased by 1.012 fold by 1 hour, followed by a sharp decline in free phage count similar to the control. The 1mM treatment reached similar levels as the control 3 hours post administration. This trend was also seen in our 2mM treatment, however the sharp decline in free phage abundance was more gradual over time, reaching similar levels as the control at 5 hours post administration. In our highest treatment, 3mM hydrogen peroxide, we saw a significant 1.28 fold-increase in free phage numbers at 1 hour post administration ($p = 0.0258$), and remained significantly elevated 2 hours post administration as well ($p = .0329$). Like our other treatments and negative control, we observed a decrease in free phage over time in our 3mM treatment, however this was decline was even more gradual, and did not reach the level of the control until 6 hours post administration. Taken together, hydrogen peroxide is a potent inducer of prophage in a dose dependent manner.

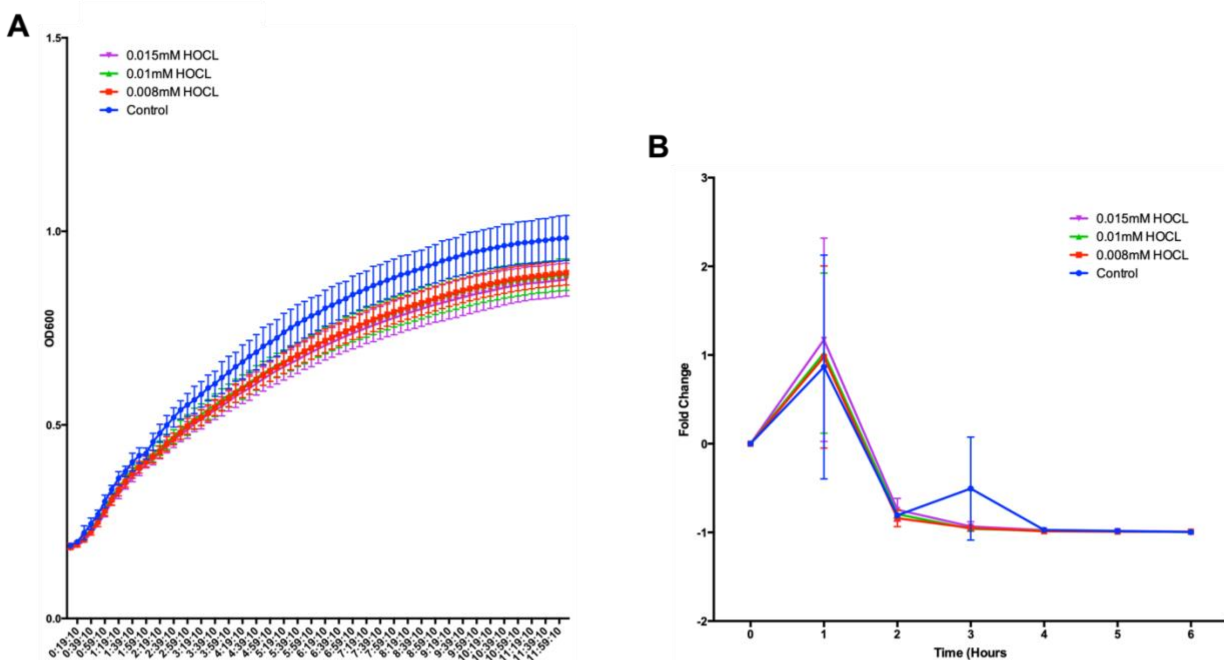

Figure 3.7 Effects of hypochlorous acid on Bacteriophage Lambda prophage induction. Cultures of Bacteriophage Lambda ƛLZ613 lysogen were grown to an OD_{600} of 0.1. Cultures were then incubated with varying concentrations of hypochlorous acid (0.008mM, 0.01mM, 0.8 mg/L) or a negative control. (A) Represents OD_{600} measurements over time of the Lambda ƛLZ613-containing lysogen. Significant differences between groups are explained in detail within the manuscript. (B) Free phage concentration was measured over the course of 6 hours, and fold change from initial time point was calculated. Statistical differences in both graphs were determined using an ANOVA.

In contrast to hydrogen peroxide, we found the hypochlorous acid had little effect on prophage activation. When looking at growth of the lysogen over time, our control again entered log phase rather quickly (~20 minutes post administration) and reached stationary phase around 10 hours post administration. Unlike hydrogen peroxide, cultures treated with hypochlorous acid did not have a delay in log phase. However, we did a significant inhibition in growth in a non-dose-dependent manner. Starting at 2 hours and 50 minutes post administration, cultures treated with 0.008mM, 0.01mM, 0.015mM hypochlorous acid had a significantly lower OD_{600} as compared to the control ($p= <0.05$), but not significantly different from each other. This trend continued through the rest of the experiment, and all treatments displayed a more significant delayed growth pattern as time progressed (3 hours and 30 minutes $p= <0.01$; 4 hours and 10 minutes $p= < 0.001$; 7 hours $p= <0.0001$). This trend was not observed in the sensitive host alone. Interestingly, though delays in growth were observed, there was no difference in progeny production over time. This would suggest that the mechanism driving delays in growth within our lysogen are not ones that induce prophage activation.

Discussion

The work presented here aimed to determine the impact of reactive oxygen and chlorine species generated by immune cells on bacteriophage reproduction. First, we examined the effects of hydrogen peroxide and hypochlorous acid, representatives of reactive oxygen and chlorine species respectively, on the adsorption kinetics of virulent (PF2) and temperate (Lambda ƛLZ613) bacteriophage. We found that hydrogen peroxide and hypochlorous acid significantly slowed the adsorption of bacteriophage PF2 to its host. Additionally, hydrogen peroxide displayed a dose dependent, negative effect on progeny production in PF2. Interestingly, hypochlorous acid had little effect on the ability of PF2 to reproduce. In contrast, these reactive species had no effect on bacteriophage Lambda adsorption. However, hydrogen peroxide and hypochlorous acid both significantly induced prophage induction in our Lambda ƛLZ613-containing lysogen. This data highlights the differential effects of immune products on bacteriophage reproduction.

Broadly, this study aimed to determine a potential mechanism for alterations in intestinal bacteriophage populations during times of inflammation. To do this, we utilized reagents that are produced by the innate immune system during intestinal inflammation, hydrogen peroxide and hypochlorous acid. In addition, we isolated a bacteriophage:host pair, virulent phage PF2 and its *E. coli* host, from a porcine fecal sample. This allowed us to utilize a bacteriophage strain that was adapted to the gut environment, making the data more relevant to the question. For the temperate bacteriophage strain, we utilized the mutant Lambda strain, ƛLZ613. This allowed us to easily generate Lambda ƛLZ613 – containing lysogens to study the impact of the treatments on prophage induction. Before measuring the impact of hydrogen peroxide and hypochlorous acid on reproduction in the bacteriophage strains, it was important to determine if these reactive

species had any effect on the bacteriophage themselves. As expected, our treatments had no effects on plaque forming units in a culture containing only our bacteriophage strains (data not shown). Meaning, the effects we observed in the study was due to changes in the bacterial host. *Escherichia coli*, the bacterial species utilized in this study, has a well-defined response due to oxidative stress when in the presence of hydrogen peroxide. In the presence of hydrogen peroxide, the OxyR transcriptional repressor is oxidized, causing a confirmational shift and in turn induces the OxyR regulon[29,30]. Genes under the control of this regulon are involved in the detoxification of hydrogen peroxide[29,30]. Along with this, reactive oxygen species, such as hydrogen peroxide can induce DNA damage[13]. When this occurs, DNA breaks stimulate the induction of the SOS response, mediated by RecA and LexA[13]. In contrast, much less is known about the response to hypochlorous acid by *E. coli*. Hypochlorous acid seems to also induce expression of the OxyR regulon, but in a different manner than hydrogen peroxide. Additionally, hypochlorous acid was found to induce the SOS response, but less profoundly than hydrogen peroxide. The specific genes involved in defense against stress caused by hypochlorous acid remain elusive. Nevertheless, both hydrogen peroxide and hypochlorous acid can damage and react with multiple cellular components including DNA and proteins. Because we saw no effect of hydrogen peroxide and hypochlorous acid on the bacteriophage itself, the effects that were observed in our study are assumed to be due to the induction of stress responses by the host. We did not measure expression of predicted induced genes. However, the data warrants more investigation on what specific inducible defenses are responsible for the aberrant replication cycles observed.

Interestingly, we observed differential effects of hydrogen peroxide and hypochlorous acid on the adsorption kinetics of bacteriophage PF2 and Bacteriophage Lambda ƛLZ613. A

bacteriophage's ability to adsorb to its host is an important step in their replication cycle; if adsorption in inhibited, it will inhibit progeny production as well. In this study, we observed that our treatments inhibited the ability of PF2 to adsorb to its host in a dose-dependent manner. We hypothesize this could be due to two reasons. First, hydrogen peroxide and hypochlorous acid could directly alter or damage the receptor itself. Second, the stress responses due to the presence of our treatments could in turn downregulate the expression of PF2's receptor. Unfortunately, we do not know what receptor is used by PF2 to adsorb. In contrast, hydrogen peroxide and hypochlorous acid had no effect on adsorption kinetics in bacteriophage Lambda ƛLZ613. Perhaps these stressors cannot alter the receptor directly, or the stress response caused by these treatments do not alter the expression of the Lambda receptor. The LamB receptor is used by Lambda to adsorb to its host, and functions as a maltose transporter[31]. Because it is not readily expressed, maltose must be supplemented into the media. This sensing of maltose drives the expression of LamB and might be unaffected during times of oxidative stress. In the setting of the inflamed gut, our results suggest that some bacteriophage are inhibited from adequately adsorbing to their host. This could contribute to alterations in the intestinal phage populations during inflammatory episodes such as ones seen IBD[6]. Taken together, our data warrants investigation into the receptor utilized by PF2 as well as how gene expression and oxidative damage can alter the receptors utilized by these bacteriophages.

Finally, we observed alterations in progeny production in both PF2 and Lambda in the presence of hydrogen peroxide. Hydrogen peroxide is a known inducer of prophage, and more specifically in bacteriophage Lambda, and these results confirm previously published literature [32]. Interestingly, hydrogen peroxide had a deleterious effect on progeny production in PF2. Because there was no direct effect of hydrogen peroxide on PF2, this decrease in reproduction

might be due both a decrease in bacteriophage adsorption and repercussions of the induced stress response by the host. In contrast, we saw little effects of hypochlorous acid on progeny production in PF2. Unlike hydrogen peroxide, hypochlorous acid does not easily diffuse across the membrane[27]. This may explain why we observed an inhibition of adsorption but not on progeny production. This might also explain why hypochlorous acid did not induce prophage production.

Overall, this study provided evidence of a potential mechanism that could account in part for the changes in intestinal bacteriophage composition during inflammatory diseases, such as IBD and obesity. Taken together, alterations and the intestinal virome could be due an inhibition of virulent bacteriophage reproduction, specifically from the inhibition of progeny production, and an increase in prophage activation. Along with this, select bacteriophage whose receptors can be oxidized by hydrogen peroxide and hypochlorous acid, along with ones who are sensitive to host stress responses, could have a decreased ability to adsorb to their host, leading to a decrease in their overall population. Though this study provided clear evidence of immune modulation of bacteriophage replication, other questions remain. Hydrogen peroxide and hypochlorous acid are potent immune products, but many others, such as Lipocalin-2 and LL-37, are produced in response to intestinal inflammation[33,34]. Further study into how other immune products such as these influence bacteriophage reproduction are warranted. Additionally, it is known that the metabolome of the microbiome is altered during intestinal inflammation that have consequences on the host and other bacteria within the gut[35]. However, how these metabolites might influence bacteriophage populations, more specifically replication, remains uncharacterized. Finally, this study utilized one virulent and one temperate strain of bacteriophage. Because of the sheer

magnitude in the diversity of bacteriophages, many more gut-derived bacteriophage strains need to be examined to determine whether the effects described above are strain-dependent.

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Chapter 4

Discussion and Conclusions

Summary of Work

Within the past two decades, the scientific community has begun to appreciate the gut microbiome and its role in host health and disease – one of these diseases being obesity. Recently in Western civilizations, obesity has become an epidemic[1]. Though obesity is rapidly on the rise, the exact etiology is complex and still not completely understood[2]. There seems to be a strong connection between the gut microbiome and obesity, but the exact interplay the exists between these two entities is not fully elucidated. This could be due in partly to the models used to study this interaction. Generally, studies utilize mouse models or human subjects, both of which come with limitations.

Much of what we know about the interaction between an obesogenic state and the gut microbiome comes from changes in bacterial populations, which neglects other meaningful populations within the intestine, such as bacteriophage. Most of these studies only observed the bacterial consortia at the end point of obesity when the development of a chronic inflammatory state over time is an important factor of obesity etiology[2]. Further, many of these studies are a characterization of the composition of the microbiome, which creates a need for more mechanistic studies to determine *why* constituents of the microbiome respond to the development of obesity.

In this dissertation, I explored how intestinal bacteriophage respond to the development of obesity and a potential mechanism of how this response might be mediated. I demonstrated that the intestinal microbiome is responsive to the development of obesity on a high fat diet in a

novel model for obesity, the Mangalica pig. Further, I observed that intestinal bacteriophage populations are much more responsive to diet change, and in turn obesity, as compared to their bacterial counterparts. Additionally, I explored a potential mechanism for these changes observed in bacteriophage populations, immune stressors. I observed differential effects on hydrogen peroxide and hypochlorous acid, products of neutrophils during the inflammatory response, on the replication of a virulent and temperate bacteriophage. My work sheds light on the intricate dynamics of the microbiome during the development of a disease state, as well as a potential mechanism by which these changes occur.

Dynamics of the intestinal microbiome during the development of obesity in a novel swine model

As mentioned above, the connection between bacterial constituents of the gut microbiome and obesity is well established, though specific bacterial species important for this interaction remain elusive[3–5]. However, much of the work connecting the microbiome and obesity was done in fully developed obese mice or obese human subjects. Along with the work reported in Chapter 2, our lab recently explored the interaction between the microbiome and the development of obesity in a mice model as well. Higgins et al found that intestinal bacterial populations displayed rapid shifts in as little as 2 weeks during the development of diet-induced obesity, which is in contrast with data reported in Chapter 2[6]. In the Mangalica pig model, I found that intestinal bacterial populations were quite resilient, even after 18 weeks post-dietary intervention with a high-fat diet. Disparities between these two studies might be due to the differences in the models utilized or feed given. Mice and pigs are physiologically distinct, including their disposition to obesity[7,8]. This, along with other physiological and environmental differences might have led to the resiliency of the pig microbiome to dietary intervention. Additionally,

introduction of microbes through their food might have played a role in their resiliency. The food given to animals to induce obesity in Higgins et al was sterile, while pig feed used in my study was not able to be sterilized. Similar to the data I reported in Chapter 2, Higgins et al found that bacteriophage populations in the gut changed more quickly in response to dietary intervention as compared to bacterial populations[6].

In general, the virome of individuals display high interindividual variability, but is stable over time[9]. Additionally, phage populations can coexist with their host in the intestine in steady-state conditions[10]. In times of disease, such as in IBD, the intestinal phage composition has been shown to not only expand, but enrich temperate bacteriophage species[11,12]. Because of this, it was not far-fetched to propose changes in bacteriophage populations in obesity. Though IBD and obesity have different clinical manifestations, at their core they are both inflammatory diseases. IBD is characterized by strong bouts of inflammation[13], while obesity is associated with chronic, low-grade inflammation that develops over time[14]. Before the publication of the data in Chapter 2, little work had been done to characterize changes in intestinal bacteriophage populations during obesity. Recently, a human clinical trial reported differences in the gut virome over time in individuals with and without obesity and, in some cases, Type II Diabetes Mellitus.[15]. They found an enrichment of bacteriophage that target specific hosts, such as ones that belong to the *Escherichia* and *Lactobacillus* genus. In the study presented in Chapter 2, I also observed an enrichment of such bacteriophage, such as the *Lactobacillus*-targeting viruses *Lactobacillus phage phiAQ113* and *Lactobacillus prophage Lj928*. Interestingly, Yang et al also reported a decrease in positive correlations with *Streptococcus*-targeting bacteriophages and their host in obese individuals, similar to what I observed in Chapter 2[15]. Collectively, these results suggest

that changes are occurring within bacteria during obesity that prohibits certain bacteriophage species to interact with their host.

Interestingly, I, along with Higgins et al, observed rapid shifts in intestinal bacteriophage populations during the development of obesity as compared to bacterial populations[6]. To our knowledge, these are the first reports characterizing the dynamics phage and bacterial populations during the development of disease. Though phage populations can co-exist with their host in steady state, their predation on or integration into their host can have rippling effects on both the abundance of bacterial populations and the metabolome[10]. With our base knowledge of how bacteriophages interact with their host during steady-state, and now during disease, we can infer mechanisms by which bacteriophage can contribute to the restructuring of bacterial populations during the development of disease.

In a simplistic model, bacteriophage will predate on their sensitive target host, which will in turn lead to a reduction in that specific bacterial population in the intestine, along with its metabolic byproducts. During the development of disease, factors that exist within that disease state, or the development of it, could lead to alterations in this model. For example, it's possible that nutritional components of the diet, such as a high fat component that directly contributes to the onset of obesity, are directly altering gene expression of bacterial hosts. This change in gene expression could either increase or decrease the expression of a specific bacteriophage receptor, both leading to a change in bacteriophage reproduction as well as their interaction with their target hosts. It is well established that bacterial gene expression is altered by sensing nutrients in their environment. More specifically, there is evidence for dietary fat to directly alter bacterial gene expression in an intestine[16]. In Chapter 2, I reported a rapid depletion in *Streptococcus*-targeting bacteriophages that preceded the depletion of their host within our high-fat fed animals.

Perhaps the dietary fat was downregulating the expression of their receptor on the host and rendering their target population inaccessible. In addition, it's possible that the dietary fat was directly impacting the bacteriophage themselves, though I believe this to be unlikely. If the opposite is observed, specifically if a nutritional component upregulates the expression of a bacteriophage receptor, the depletion of the that bacterial population, along with their metabolites, opens a niche to allow other bacterial species to bloom. Taken together, even small effects on host bacterial populations can have profound implications on their bacteriophage predators and could influence overall phage-host dynamics during the development of disease from steady state.

Impacts of immune stressors on the replication efficiency of bacteriophage

In addition to nutritional components leading to the modulation of intestinal phage populations, products generated during immune activation could impact the reproductive capability of bacteriophage. In Chapter 3, I explored the impact of two immune stressors, hydrogen peroxide and hypochlorous acid, on bacteriophage reproduction in a virulent and temperate bacteriophage. To examine this, we tested how these products influence the rate of bacteriophage adsorption to its host as well as progeny production. While we saw a dose-dependent effect of our treatments on bacteriophage adsorption in the virulent phage PF2, we observed no effect in temperate phage Lambda. This supports the notion that the immune system's effects on bacteriophage population in the intestine is not "one size fits all". This differential response could be due to differences in receptors, the bacterial response towards the stressor, and the effects on the virion itself. Bacteriophages utilize a wide range of receptors to enter their host. Some are widespread, such as in the case of T4 and T5 phage who utilize LPS as their receptor, while some are niche, such

as bacteriophage Lambda who utilizes the LamB porin expressed in *Escherichia coli*[17]. Therefore, if immune products inhibit the adsorption of T4 bacteriophage, it could have more profound impacts on bacterial populations as compared to bacteriophage Lambda. Rather than interacting with the receptor itself, immune stressors could influence the gene expression of bacteria, leading to either an up- or downregulation of a specific bacteriophage receptor. Depending on this increase or decrease in expression, it could also influence the ability of bacteriophage to adsorb to its host and subsequently reproduce. Finally, the immune stressors could impact the bacteriophage virion directly. Though I did not observe direct evidence for this in my study, the vast diversity of bacteriophage structure leaves this up for debate. Broadly, the inhibition or acceleration of bacteriophage adsorption can lead to a marked bloom or depletion of the bacteriophage species, highlighting bacteriophage adsorption as an important step in the life cycle.

Because I utilized both a virulent and temperate bacteriophage strain in my study, I decided to measure progeny production in two different ways. First, to measure progeny production in the virulent bacteriophage PF2, we characterized its growth kinetics in the presence or absence of the immune stressors. Interestingly, hydrogen peroxide displayed a dose-dependent effect on progeny production while hypochlorous acid displayed modest effects. *E. coli*, the target host for PF2, broadly responds to hydrogen peroxide and hypochlorous acid in a similar way by inducing expression of the OxyR regulon[18,19]. Though both induce this regulon, differential gene expression from the regulon is seen when the different stressors are applied to *E. coli*[20]. However, the full response of *E. coli* to hypochlorous acid has not been elucidated[20]. Perhaps this in conjunction with hypochlorous acid not readily diffusing through bacterial membranes, leads to no effect of hypochlorous acid on progeny production[20]. Rather than causing oxidative

damage inside the bacteria while PF2 is actively replicating, it could directly damage the receptor itself, still leading to delayed progeny growth over time. This idea is supported in our data, as we saw no effect of hypochlorous acid on progeny production in PF2 until 60 minutes post-administration.

Bacteriophage Lambda is a temperate phage, which means it possesses the ability to replicate using the lytic cycle or undergo lysogeny and integrate into the host genome as a prophage. Therefore, I was interested in how our immune stressors would impact prophage induction. It is known that hydrogen peroxide is a potent inducer of prophage activation, due to activation being tightly linked in the expression of the OxyR regulon as well as the SOS response[21,22]. However, before my study, it was not known if hypochlorous acid could induce prophage activation. I found that hydrogen peroxide induced prophage activation in a dose dependent manner, while hypochlorous acid had no effect. I hypothesize that hypochlorous acid had no effect on activation for the reasons listed above. Additionally, hypochlorous acid induces the OxyR regulon, but not the SOS response in *E. coli*, further indicating that activation of the SOS response is necessary for prophage induction in bacteriophage Lambda[23]. Interestingly, hypochlorous acid was shown to induce the SOS response in other bacteria, such as *B. cereus*, indicating the more phage:host pairs need to be examined[24].

In the context of the inflamed gut, my study adds an exciting layer to the complex modulation of bacteriophage populations during the development of disease. As said above, obesity is associated with chronic inflammation, during which these immune products, amongst others, are produced. Additionally, I observed differential effects of these products on two bacteriophage using the same host species but different strains. Typically, the gut microbiome has a high level of diversity in terms of bacteria and phage populations[25]. Given this diversity, it is safe to assume

that immune stressors will differentially affect phage:host pairs, leading to the additional depletion or blooming of various populations.

Implications of these findings

In this dissertation, I aimed to explore how intestinal bacteriophage populations are impacted by the development of obesity as well as investigate a possible relevant mechanism by which they are modulated. Much like the title of this dissertation implies bacteriophage have been undervalued modulators of the gut microbiome until recently. A decade ago, when microbiome research was in its infancy, shotgun metagenomic sequencing was expensive and only beginning to advance. Typically, the gut microbiome was explored through 16S sequencing, which only allowed bacterial and archaeal populations to be identified. More recently, the advances in shotgun metagenomic sequencing have allowed for the characterization of viral populations within the gut during steady-state and disease. However, mechanistic studies exploring how bacteriophage function is impacted during times of disease is lacking. Additionally, functional studies examining phage:host interactions in steady-state and disease need to be performed. Within any given microbial environment, including the gut, bacteria and their predatory bacteriophage are in constant interaction. With this in mind, it begs the question: why don't bacteriophage predate all bacteria within a given environment? The gut microbiome is perfectly poised to allow bacteriophage and bacteria co-exist for multiple reasons. First, bacteria are spatially divided from their phage, and in turn, only allow bacteriophage access to a fraction of bacterial cells from a specific population. This allows the phage populations to actively replicate within sensitive hosts without completely wiping out a species[26,27]. Second, it has been reported that temperate phages dominate the virome of the human gut [11,28,29]. Harboring a prophage can

come with many advantages for the host, such as protection against infection with other similar phages, termed homoimmunity, as well as an increase in overall fitness within complex microbial communities[30,31]. Additionally, bacteriophages can contribute to horizontal gene transfer, which can also be advantageous for the host, such as the introduction of antibiotic resistance genes and virulence factors[32,33]. Almost all gut isolates that have been isolated harbor a prophage, and prophages are extremely numerous in gut metagenomes[34,35]. In addition to the benefits described above, a community comprised of mostly temperate phage in a prophage state lends itself well to maintaining microbial balance. Taken together, bacteriophage and bacteria co-exist within the microbiome in a very intricate and balanced manner. However, when there is a deviation from steady state, this relationship is distorted.

In Chapter 3, I explored how immune products generated during intestinal inflammation might impact bacteriophage reproduction. My results support evidence previously published in descriptive studies determining how bacteriophage populations are impacted during intestinal inflammation[10,11]. These studies observed an expansion of temperate free phage in exchange for a loss of virulent free phage. In light of my results, this makes sense: temperate bacteriophage are expanding due to prophage activation. In addition to immune products, it's been shown that bacterial metabolites and dietary compounds can lead to prophage activation as well[36,37]. In turn, expanded bacteriophage communities can then predate even further specific bacterial populations, opening up niches for other bacteria to bloom. Products like short chain fatty acids, dietary compounds such as fructose, and immune products like hydrogen peroxide are all present in the gut of diet-induced obese mice. Therefore, the expansion and alteration of bacteriophage during obesity could be explained by these factors, along with bacteria-bacteria, bacteria-host, and bacteriophage-host interactions.

Recently, the literature has shed light on how bacteriophage might interact with the immune system in multiple ways[38]. For example, it has been shown that bacteriophage can directly stimulate immune cells. Direct stimulation of immune cells by bacteriophage have classically been studies *in vitro*. However, many of these studies did not clean phage stocks of LPS, which could account for markers of inflammation to be observed[39]. Recently, a study by Gogokhia et al showed that bacteriophage can directly stimulate IFNɣ through TLR9[40]. Additionally, vaccination against the *Pseudomonas aeruginosa* filamentous temperate phage, Pf, aided in wound healing[41]. Together, these results support the idea that bacteriophage can directly modulate the immune response. In this light, perhaps the expansion of certain bacteriophage can aggravate the inflammatory response during obesity or IBD and in turn further changes in bacteriophage populations in the intestine.

Short-comings, Limitations, and Future Work

In recent years, there have been many advancements in metagenomic sequencing, allowing for the characterization and identification of bacteriophage in microbial environments. To that regard, we utilized shotgun metagenomic sequencing to determine the impact of the development of obesity on intestinal phage populations. However, enrichment steps were not taken to sequence free phage samples separately from gut microbiome samples. Additionally, we were not able to identify bacteriophages with RNA genomes. Though bacteriophage genome sequencing has increased in recent years, a large majority of intestinal phages remain uncultured, unclassified, and their target hosts are unknown. Because of this, my studies are biased towards common phages that have been identified, and many important interactions between phage and host that could directly impact the development of obesity remain unknown.

My *in vitro* assessment of immune products and bacteriophage reproduction did provide key information on how bacteriophage reproduction may be altered during times of intestinal inflammation. However, given the vast diversity of bacteriophages, hosts, and their interactions, more work will need to be performed to identify specific trends. Specifically, more phage:host pairs need to be examined, including ones from all major phyla of the intestine. Ideally, these phage:host pairs would be isolated from the intestine, as lab strains of bacteria can differ from ones in their natural environment. Finally, other immune products, as well as other compounds found in the intestine, need to be examined in this context. Phage:host pairs do not exist in a vacuum, they both come in contact with other bacteria and their metabolites, dietary compounds, as well as other host factors.

Future work should be aimed at both increasing our knowledge of bacteriophage diversity as well as their specific functions in the gut. Many questions still exist as to the specific functions of bacteriophage in disease. The foundational information we have currently explains the 'who' though metagenomic sequencing. Additional experiments need to be performed to understand the 'why' and 'how'; why do bacteriophage respond in a specific manner during intestinal inflammation, and through what mechanism is that achieved? Additionally, we know very little about the bi-directional interactions between bacteriophage and the host immune system. The recently published studies discussed above support the idea the bacteriophage are immune stimulating. If this is common with many bacteriophages, why is the gut not in a state of constant inflammation? Though work has been done to characterize these interactions *in vivo*, many questions remain unanswered.

Conclusions

Within this dissertation, I have explored how intestinal bacteriophage respond to the development of obesity, as well as characterize a mechanism by which these alterations occur. In Chapter 2, I showed that intestinal phages change much more rapidly than bacterial populations during the development of obesity. To expand on this, in Chapter 3, I explored a potential mechanism by which these changes in bacteriophage populations occur. I found that immune products such as hydrogen peroxide and hypochlorous acid can influence the reproductive potential of both a temperate and virulent bacteriophage, but in different ways. Collectively, these studies point to bacteriophage as being important members of the gut microbiome and possess the potential to greatly alter the form and function of the gut. Moving forward, my work provides an exciting foundation for exploring relationships between phage, bacteria, and the host and the consequences of these relationships.

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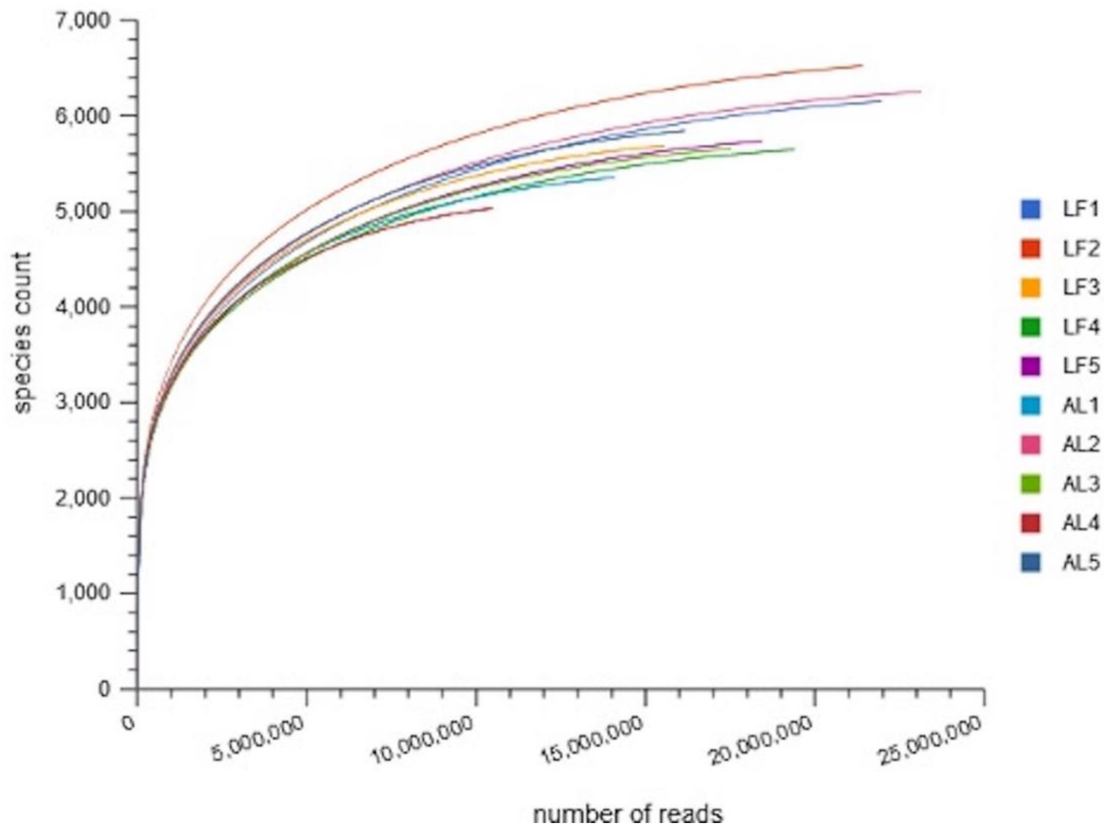
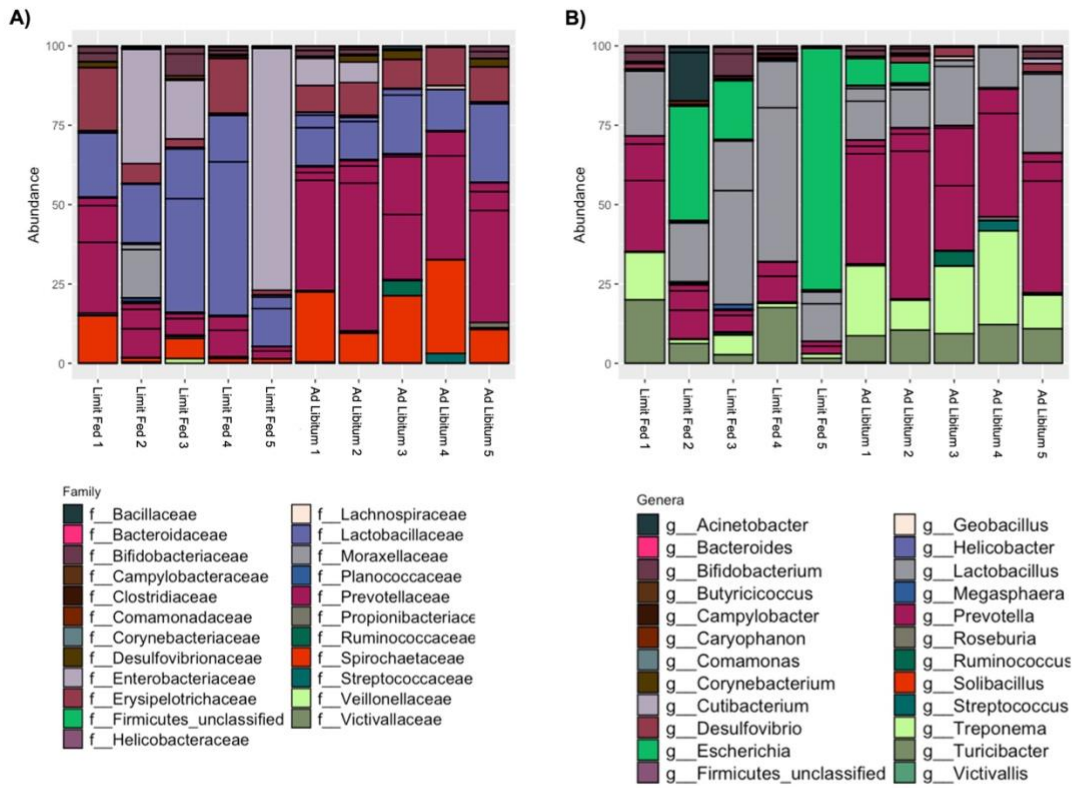
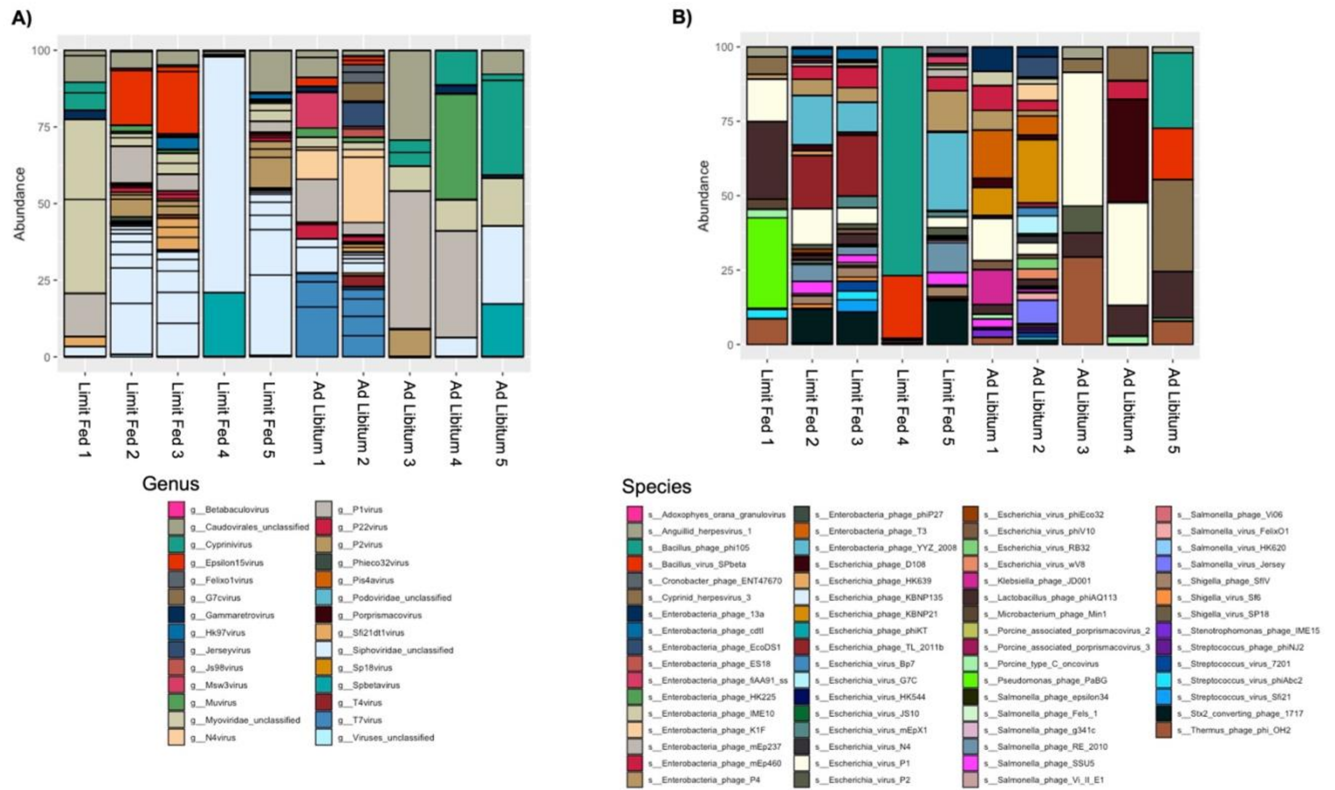
Appendix 1. Supplemental Figures for Chapter 2



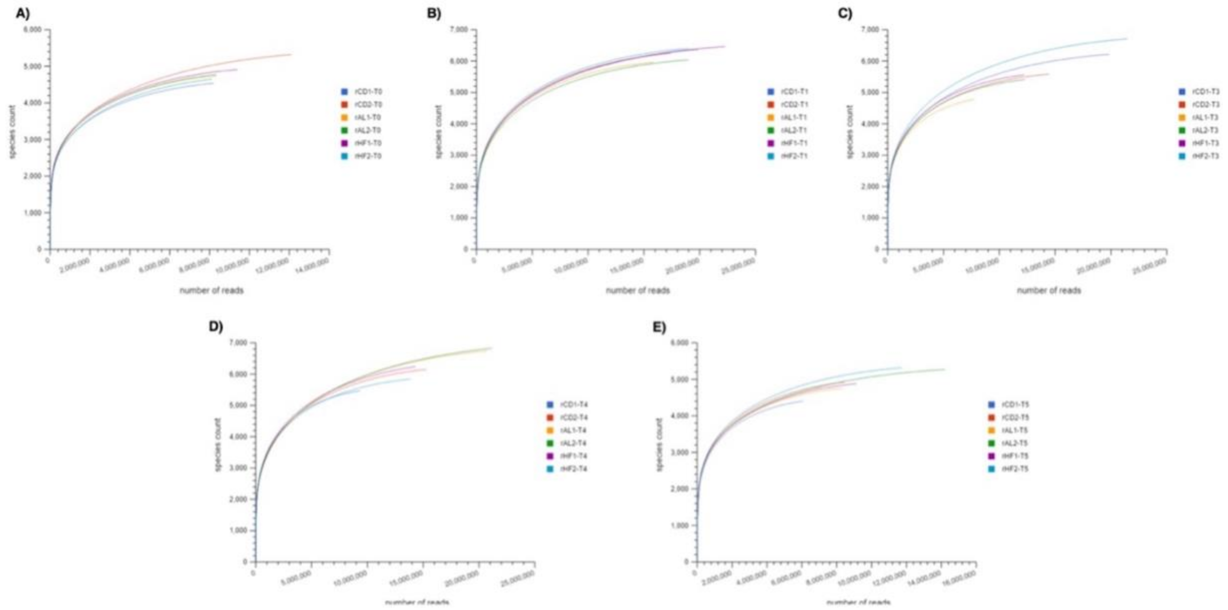
Supplemental Figure 1. Alpha Diversity of the intestinal microbiome of limit fed and *ad libitum* Mangalica Pigs. Rarefaction curves depicting the number of species compared to the number of reads in each sample. The total species count and total read count were used to generate rarefaction curves.



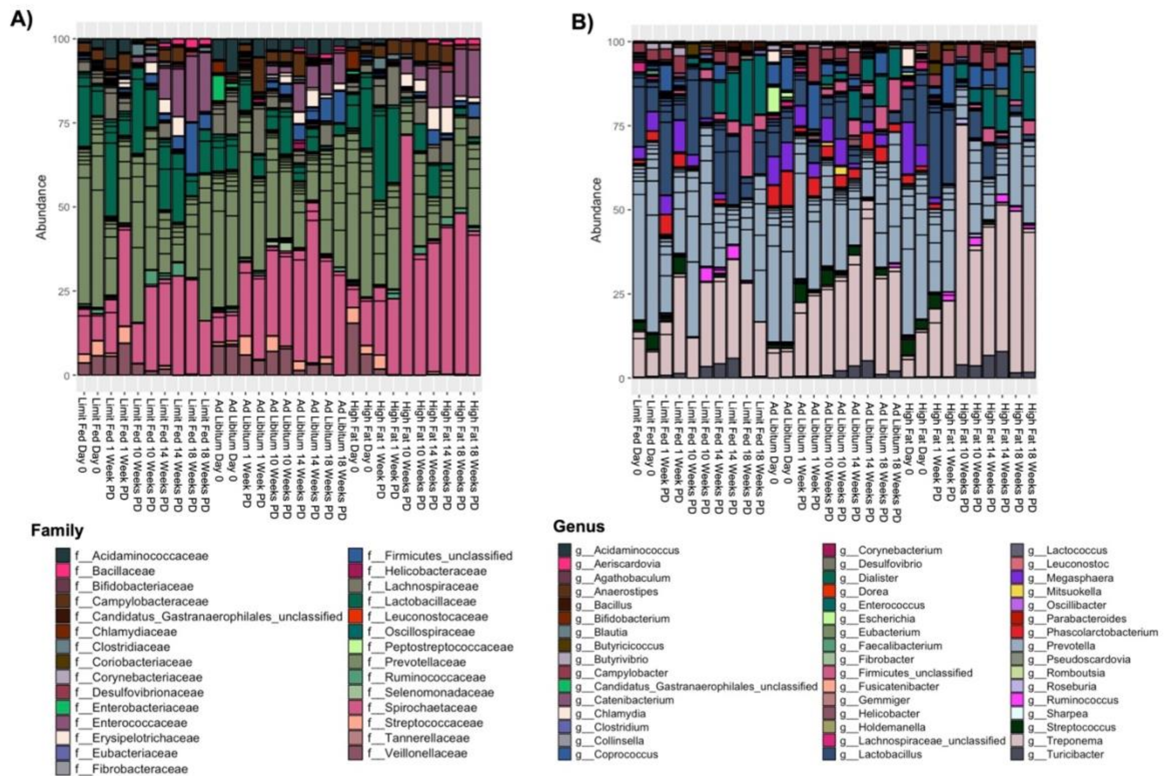
Supplemental Figure 2. Additional characterization of bacterial constituents of the intestinal microbiome in *ad libitum* and limit fed Mangalica pigs. Bar plots displaying relative abundance of bacteria at the **A) Family** and **B) Genus** level, in Mangalica pigs on either a limit fed or *ad libitum* feeding



Supplementary Figure 3. Characterization of viral constituents of the intestinal microbiome in *ad libitum* and limit fed Mangalica pigs. Bar plots displaying relative abundance of viruses at **A) Genus** and **B) Species** level, in Mangalica pigs on either a limit fed or *ad libitum* feeding.



Supplementary Figure 4. Estimating Alpha Diversity over time after Dietary exposure in weaned Mangalica Pigs. Rarefaction curves were generated to determine the overall alpha diversity of each sample by comparing the total number of reads and total number of species within each sample at **A)** Time 0, **B)** 1 Week, **C)** 10 Weeks, **D)** 14 Weeks, and **E)** 18 Weeks post-diet exposure.



Supplementary Figure 5. Additional characterization of the longitudinal impact on bacterial species in the intestinal microbiome following dietary exposure in weaned Mangalica pigs. A) Family, and **B) Genus** level bacterial composition in Mangalica pigs fed either a limit fed, *ad libitum*, or *ad libitum* + high fat diet prior to and after 1, 10, 14, and 18 weeks of dietary exposure.

Appendix 2. Collaborative Projects

Integrative Longitudinal Analysis of Metabolic Phenotype and Microbiota Changes During the Development of Obesity

Keah V Higgins[1]* and Lauren N. Woodie[2]*, Haley Hallowell[1], Michael W. Greene[2], and Elizabeth Hiltbold Schwartz[1]

*Co-authors

[1]Department of Biological Sciences, Auburn University, Auburn AL

[2]Department of Nutrition, Dietetics and Hospitality Management, Auburn University, Auburn, AL

Keywords: metabolic phenotype, microbiota, bacteriophage, obesity, bacteria-phage dynamics

I contributed to this project through the help of animal husbandry, data collection, and data analysis. During the collection of data, I aided in harvesting organs for analysis, collecting fecal samples and extracting DNA from said fecal samples . In terms of data analysis, I created multiple iterations of figures describing relationships between bacteria and bacteriophage. Finally, I edited the manuscript before submission.

Higgins, K. V., Woodie, L. N., Hallowell, H., Greene, M. W., & Schwartz, E. H. (2021). Integrative Longitudinal Analysis of Metabolic Phenotype and microbiota changes during the development of obesity. *Frontiers in Cellular and Infection Microbiology*, *11*. https://doi.org/10.3389/fcimb.2021.671926

Maternal Dietary Protein Intake Influences Milk and Offspring Gut Microbial Diversity in a Rat (*Rattus nervegicus*) Model

Matthew F. Warren[1,2], Haley A Hallowell[1], Keah V Higgins[1], Mark R. Liles[1], Wendy R. Hood[1]

[1]Department of Biological Sciences, Auburn University, Auburn, AL

[2]Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC

Keywords: milk microorganisms, bacteria diversity, phylogenetic distance, 16S rRNA gene sequencing

In this study, I provided analysis determining the dissimilarity in the microbiome between different dietary groups and generated a figure used in the manuscript.

Warren, M.F.; Hallowell, H.A.; Higgins, K.V.; Liles, M.R.; Hood, W.R. Maternal Dietary Protein Intake Influences Milk and Offspring Gut Microbial Diversity in a Rat (*Rattus norvegicus*) Model. *Nutrients* **2019**, *11*, 2257. https://doi.org/10.3390/nu11092257

In vivo Microbiome Profiling of the Luminal and Mucosal Surface of the Duodenum Using a Cannulated Yearling Bovine Model

Ricardo M. Stockler[1], Keah V Higgins[2], Haley Hallowell[2], Erin S. Groover[1], Elizabeth M. Hiltbold[2], Benjamin W. Newcomer[1], and Paul H. Walz[3]

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Keywords: duodenal cannulation, GIT microbiome, *in vivo* microbiome, bovine microbiome, metagenomic analysis

For this project, I collaboratively created the workflow and pipeline to annotate and analyze metagenomic sequences generated for this study. In addition, I aided in the generation of figures displaying differences in microbiomes between sampling sites.

Stockler RM, Higgins KV, Hallowell H, Groover ES, Hiltbold EM, Newcomer BW, Walz PH. *In vivo* Microbiome Profiling of the Luminal and Mucosal Surface of the Duodenum Using a Cannulated Yearling Bovine Model. Front Vet Sci. 2020 Nov 9;7:601874. doi: 10.3389/fvets.2020.601874. PMID: 33240966; PMCID: PMC7680733.