THE UNIVERSITY OF CHICAGO

# HOST-PATHOGEN-GUT MICROBIOTA INTERACTIONS: THE ROLE OF DIET, MICROBIOTA METABOLITES, AND THE ARYL HYDROCARBON RECEPTOR IN SURVIVAL FROM SEPSIS AND SURGICAL INJURY

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BY
ROBERT CHARLES KESKEY

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AhR - Aryl Hydrocarbon Receptor

SIRS – Systemic Inflammatory Response Syndrome

DAMPS – Danger Associated Moelcular Patterns

TLRS – Toll Like Receptors

NOD - Nucleostide-Binding Oligomerization Domain

DIC – Disseminated intravascular coagulation

NETs – Neutrophil extracellular traps

ARDs – Acute respiratory distress syndrome

SOFA – sequential organ failure assessment

GI – gastrointestinal

SCFA – Short chain fatty acids

RiPPs – ribosome synthesized and post-translationally modified peptides

T6SS – Type VI secretion systems

SFB – Segmented Filamenouts Bacteria

HDAC – Histone-deactylase inhibitor

GPCR – G-protein coupled receptors

X-assciated protein (XAP2)

c-Src – cellular sarcoma kinase

XRE – Xenobiotic response elements

TCCD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

TDO – tryptophan 2,3-dioxygenase

IDO1 – indoleamine 2,3-deoxygenase 1

IDO2 – indoleamine 2,3-deoxygenase 2

ILC3 – innate lymphoid cells 3

I3PA – indole-3-propionic acid

AC-FMT – autoclaved fecal microbiota transplant

NIH – National Institute of Health

HFb – high fiber

LFb – low fiber

HF-HFb – High fat/High fiber diet

HF-LFB – High fat/Low fiber diet

LF-HFB – Low fat/High fiber diet

LF-LFB – Low fat/Low fiber diet

SD – Standard rodent chow

IP – intraperitoneal

MVI – mouse virulent infection

MSS – Murine Sepsis Score

Sm – *Serratia marcescens*

OD – optical density

TSB – tryptic soy broth

BMDM – bone marrow derived macrophages

WD – western diet

7DP – 7 days of prehabilitation

3DP – 3 days of prehabilitation

ASH – antibiotics, starvation, sterile injury

DietPreHab – dietary prehabilitation

$Buk_{enz}$ – Butyrate kinase

$But_{enz}$- Butyrate:acetyl-coA transferase

MACs- microbiota accessible carbohydrates

AI-2 – Autoinducer-2

ARGs – antibiotic resistance genes

pMAC – peritoneal macrophage

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# ABSTRACT

Host-microbiota-pathogen interactions play a key role in determining survival from lethal bacterial infection leading to sepsis. Here we demonstrate that western diet, high in fat and low in fiber, increases the risk for pathogen colonization in the gut and results in gut-derived sepsis when mice are exposed to antibiotics, starvation, and surgical injury. The risk of postoperative sepsis in various surgical mouse models can be mitigated through dietary prehabilitation with a diet low in fat and high in fiber. Dietary prehab provides protection, in part, once the functional production of butyrate is restored. Furthermore, pathogen colonization and susceptibility can be mitigated with a diet high in fiber which increases microbiota resiliency by inducing production of the quorum sensing molecule AI-2 which stabilizes Firmicutes in the face of antibiotic exposure. The western diet alterations not only destabilize metabolite production of the gut microbiota, but also increase the presence of antibiotic resistance genes independent of antibiotic exposure conferring resistance to macrolides, fluoroquinolones, and cephalosporins. Gut-derived metabolites such as indoles were found to drive a recovery directed immune response in mice exposed to lethal bacterial peritonitis. During lethal infection, indole metabolites enhanced survival by activating AhR on macrophages which increased bacterial clearance and induce resolution of inflammation. Oral supplementation with tryptophan or direct injection of indole metabolites improved survival in this model. To further implicate the importance of indole activation of AhR, select bacterial pathogens actively inhibited indole activation of AhR via the secretion of small molecules such as enterobactin. Finally, indole metabolites in the gut of septic patients correlated with survival. In conclusion, the structure and function of the gut microbiota is influenced by diet and has major downstream implications on pathogen colonization, virulence expression and survival from a lethal bacterial infection such as murine peritonitis

# 1. INTRODUCTION

## *1.1 Host-pathogen interactions, infection in the modern era, and sepsis*

Our understanding of the microbiome has revolutionized our understanding of infection and sepsis[1]. Unfortunately, our current approach to treating and managing infection is built on a foundation of research that predates our recognition and understanding of the microbiota. The current approach based on decades of trial and error focuses on maintain sterility, administering antibiotics, and providing hygiene[2]. With antibiotic resistance on the rise and the persistent reliance on antibiotics to prophylaxes against infection, it is essential to began to improve our approach to treating and preventing infection[3–6]. In this dissertation, I will outline a body of work that demonstrates and begins to unlock the importance of the gut microbiota in improving our understanding of infection pathogenesis and the host-pathogen interactions that regulate it.

Host-pathogen interactions are the result of coevolution between organisms and microorganisms[7–9]. From a healthcare perspective, after the advent of germ theory, bacteria were typically synonymous with pathogens and disease. The idea of pathogenicity stems from the development of Koch's postulates: the microorganism must be found in abundance in those afflicted by the disease, the microorganism must be isolated in culture, the microorganism should cause disease when introduced into healthy organisms, and the microorganism can be reisolated from the diseased host[10,11]. Since Koch's postulate, our understanding of microbiology and infection has grown considerably. We have come to appreciate the large number of microorganisms that can colonize and exist in a symbiotic relationship. Furthermore, there are many microorganisms that have been labeled pathogens, but do not always cause disease despite

colonization of a host. Similarly, other microorganisms not typically considered as pathogens can cause disease under specific conditions. These varying interactions are important when considering both host-pathogen interactions and clinical infections [12]. Therefore virulence is a property of the microorganism that is only realized in a susceptible host [13]. Understanding the contextual relationship between microbes and the host are essential to understanding host-pathogen interactions and provide a nuanced understanding of infection that has been long overlooked.

Prior to the germ theory which led to the development of antibiotics, hygiene and vaccines, infection was one of the leading causes of death [14]. Since the advent of antibiotics, we have seen a significant downtrend in prevalence of bacterial infections and a significant improvement in survival [15]. However, in the post-antibiotic era, we are now observing that patients who die from infection are typically those with chronic life-limiting diseases (end stage renal disease, heart failure, chronic liver disease, cancer, etc.) and/or those who develope late onset infection-related complications as a result of treatment of acute life-threatening conditions [16]. The ease, and presumably safety, of antibiotics have resulted in their increased use. For example, many patients who may have symptoms of an underlying infection and/or concerns for a possible infection are immediately and often empirically treated with antibiotics. Such promiscuous use of antibiotics, although of survival benefit in terms of its immediate application the minute an infection is suspected, has also resulted in a significant rise of antibiotic resistant pathogens- an unintended consequence that has made the relationship between humans and microorganisms more complicated [6,17]. Recent studies have demonstrated that outcomes are worse when patients receive antibiotics that are too broad for the offending organism [18]. Furthermore, a rise in antibiotic resistant organisms is being observed that is complicating how

we treat infection and is limiting the effectiveness of our current antibiotic centric approach to infection. The estimated mortality from antibiotic resistant organisms in 2019 is believed to exceed 1 million people and a staggering 4 million deaths have been associated with antibiotic resistance [6]. The rise of antibiotic resistance alone has increased the pressure to alter our current approach to treating infection with antibiotics via programs such as antibiotic stewardship. However antibiotic resistance alone is only a small piece of the complex puzzle that is increasing the pressure to alter our approach.

## *1.2 Post-infectious and complications of sepsis*

Although our improvement in the treatment of infection has substantially improved over several decades, the impact of post infectious complications on long-term health is being increasingly realized, particularly patients who suffer from severe infections resulting in systemic manifestations such as sepsis. Sepsis is currently defined as a dysregulated host immune response resulting in end organ damage [19]. Our management of sepsis, described below in further detail, is primarily treating the underlying infection, with antibiotics, and providing supportive care until the collateral damage to end organs (kidneys, heart, lungs, liver, brain) recovers. Patients who survive sepsis develop significant co-morbidities and decreased quality of life [20–22]. Patients who survive the initial infection-related septic insult have increased functional impairments, increased cognitive impairment, and more importantly almost half of patients require readmission to the hospital [22]. These patients have deterioration in their overall health including worsening cardiovascular and renal disease [23,24] as well as worsening in their overall medical co-morbidities. For example, it has been observed that patients surviving a high acuity septic insult have an increased risk of myocardial infarction and stroke over the next 5

years [25]. Furthermore, such patients are at an increased risk of developing a secondary infection while hospitalized and at increased risk for re-hospitalization due to a new infection that occurs after discharge [26–29]. Even patients who do not develop sepsis, but are simply treated for an infection are at a significant increased risk for being admitted to the hospital with a new infection that leads to the systemic manifestations of sepsis [30]. Similarly, surgical patients are at increased risk for postoperative complications if they undergo surgery following being treated for infection and antibiotic exposure [31,32]. Despite the improved ability to treat infections with antibiotics and improvements in critical care management to allow patient to recover from the severe infectious insult, post infection sequalae can result in significant morbidity and mortality the etiology and mechanisms of which remain elusive. By redirecting our focus on understanding host-pathogen interactions and incorporating the role of the gut microbiota in regulating the host immune response to pathogens, a more informed approach to treating infection and its sequalae may be possible.

## *1.3 The pathophysiology of sepsis*

The current most feared outcome of an infection is the development of sepsis, a dysregulated host response resulting in end organ damage [19,33]. Sepsis is a leading cause of death and morbidity worldwide affecting over 1 million adults annually in the US associated with over 250,000 deaths [19,34,35]. Despite an intense amount of research focused on understanding the role of the innate immune system on sepsis pathogenesis, outcomes have only marginally improved with an observed shift of sepsis related deaths to older adults [36]. Traditionally, sepsis was defined by the manifestation of a systemic inflammatory response syndrome (SIRS –

tachycardia, tachypnea, hypotension, leukocytosis/leukopenia, fever/hypothermia) in the background of a known or suspected infection [19]. However with the recognition of sepsis as a medical entity, understanding of the immune dysregulation that accompanies the response after ostensibly containg the initiating infection with antibiotics and surgery has advanced its pathophysiology. For example, the septic response has been described of having an initial hyperinflammatory response which in turn is followed by a compensatory anti-inflammatory response [19,37,38]. Several studies have shown that septic patients who have worse outcomes are those in whom an increased levels of anti-inflammatory genes and increased expression of inhibitory ligands such as PD-1 and expansion of T-regulatory cells [39,40] are observed. Yet it remains unclear what specifically characterizes the driving force behind the expression of these anti-inflammatory properties which, in the aggregate has been referred to as "immune exhaustion" [41,42]. The timing of the anti-inflammatory response during sepsis remains unclear as it may be occurring simultaneously at the beginning of the initial insult. For example, some studies have demonstrated early expression of anti-inflammatory genes occurring as an overcompensated response from the initial pro-inflammatory phase [43]. This maladaptive overcompensated and immunosuppressive response may explain why a large number of septic patients develop a secondary infection that can result in failure to recover leading to death [43].

Although the timing of an anti-inflammatory phase in sepsis remains unclear, the proinflammatory response following the initial infectious insult is well characterized. The early hyperinflammatory response is believed to be driven by activation of pattern-recognition receptors on innate immune cells by pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs)[19,44,45]. Several studies have demonstrated the importance of toll like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)

receptors among others[44,46]. PRRs invariable lead to overlapping activation of similar pathways resulting in induction of the transcription factors NF-kB and STAT [44,46,47]. NF-kB and STAT both lead to the activation of well-known pro-inflammatory genes (including TNF, IL-1, IL-12, and type 1 interferons) [48–50]. The production of these early pro-inflammatory cytokines further exacerbates the initial pro-inflammatory response through the production of chemokines resulting in an influx of additional inflammatory cells [51]. The production of inflammatory cytokines and chemokines greatly impacts the vascular and lymphatic endothelium which in turn contributes to the vasodilation and end organ damage across various organs (i.e. liver, lung, kidney, etc) associated with sepsis [19,52]. The activation of these inflammatory cytokines results in downstream cascading events such as complement, particularly C5a, thrombomodulin-mediated disseminated intravascular coagulation (DIC), microvascular thrombosis and diffuse hemorrhage- all of which can contribute to the patient's continued manifestation of multiple organ failure leading todeath [19,53,54].

Several innate immune cells have been identified that contribute to the dysregulated immune response seen in sepsis. Monocytes/macrophages are prototypical innate cells that play an essential role in both clearance of pathogens and coordinating the appropriate immune response to prevent pathogen dissemination and resolve the initial inflammatory response [43,55]. Additionally, neutrophils and particularly neutrophil extracellular traps (NETs) have been demonstrated to play an essential role in the perpetuation of the inflammatory response seen in sepsis [43,56,57]. Although the molecular details of pathways associated with individual cells and individual cytokines has been demonstrated, there is still a lack of understanding of the precise elements that amplify or dampen the initial event or events leading to the dysregulated inflammatory response seen in sepsis. If virulence (harmfulness, clinical infection, organ failure)

is viewed as neither a property of the pathogen, nor that of the host, but rather a property of their interaction, accounting for the "interactome" involving both the host immune system and the pathogen during the progression of the septic immune response may be needed.

A major question in the field however remains: to what extent does the sepsis response itself drive outcome (organ failure, mortality) after the initial insult has been ostensibly treated? Stated another way, is sepsis simply "runaway inflammation?" Multiple systems are in play. For example, the hyperinflammatory response results in systemic vasodilation which in turn results in end organ damage due to hypoperfusion. Traditionally, end organ damage associated with sepsis is thought to result from hypoperfusion leading to respiratory failure/acute respiratory distress syndrome (ARDs), renal failure, liver failure, encephalopathy, circulatory collapse, and ultimately heart failure as every end organ attempts to combat the lack of perfusion [19]. However, the precise etiology of end organ dysfunction from sepsis is not clear. For example, renal failure in sepsis is associated with increased renal blood flow and does not appear to result from hypoxia and decreased oxygen delivery [58,59]. Another hypothesis regarding the pathogenesis of organ dysfunction in sepsis is that it is a direct result of structural damage and cell death. However, organ dysfunction can occur in the absence of significant cell death bringing into question of the underlying mechanisms and whether early dysfunction is a protective response [58–60]. Finally, there is a thought that end organ damage results from change in cellular energy utilization and expenditure in response to the overwhelming inflammatory response [61]. Given the complex and dynamic response that is occurring when the immune system is triggered to response to the inciting pathogen, the organ failure seen in sepsis may indeed be a reflection of hypoperfusion, hypoxia leading to cell death, and alterations in cell energy expenditure all contributing to organ failure to varying degrees. The end organ damage associated with sepsis has led to a change in

definition of sepsis stemming from measurement of end organ damage with the sequential organ failure assessment (SOFA) plus known or suspected infection[33]. All of this confusion has led to changes in the terminology to describe the elusive syndrome of sepsis. End organ failure affecting multiple vital organs (i.e. multiple organ failure) is linked to the infection itself and has replaced the generalized SIRS criteria which can occur in several hyperinflammatory states unrelated to an underlying infection. In contrast, the progression of the systemic inflammatory response and end organ damage associated with sepsis typically requires supportive care which includes fluid resuscitation and the use of vasopressor medication to improve end organ blood flow [61,62]. If patients can survive the initial systemic vasodilation and the organ damage that ensues, they often become susceptible to the immunosuppressive nature of the compensatory and somewhat maladaptive anti-inflammatory response [39,43]. This immunosuppressive state has been associated with the development of "late-onset sepsis" from healthcare associated pathogens that have been selected out by the use of antibiotics. It is important to recognize that often such patients undergo multiple insults including several takebacks to the operating room, invasive instrumentation, exposure to polypharmacy including multiple antibiotics, etc. In an attempt to treat sepsis in this context, early goal directed therapies have been proposed to reverse the end organ damage through goal-directed resuscitation and initiation of vasopressors [63,64]. Although initially felt to improve survival, further evaluation has demonstrated that the improved survival associated with many of these programs is the result of early recognition of sepsis and treatment of the underlying infection [65–67]. Yet, while various approaches targeting the host immune system have been proposed, most to date have resulted in abject failure. These include targeting TLR4, IVIG, removal of cytokines and endotoxin through hemoperfusion, delivery of GM-CSF, interferon-gamma, steroids, and vitamin C [68]. All of the targeted therapies are largely based on

translational studies that have led to an advanced understanding of the immune dysregulation that can occur, yet have failed to advance an understanding of underlying "interactome" that characterizes the host-pathogen continuum during all phases of sepsis.

A major area of improvement in this field is to depart from the notion that pathogen elimination and immune modulation together, but separately is the path forward to improving outcome. Rather considering the outcome of serious life-threatening infection to be neither a property of the pathogen nor that of the host alone, but rather a property of their interaction, may allow for more expansive interrogation of the host-pathogen-microbiome interaction. By not accounting for the life history of the patient and its influence on their microbiome, may impose limits on how an invasive pathogen and its hosts pathoadaptive response to eliminate it goes awry. Providing personalized care may require a deeper and more systems-level interaction approach than is currently employed. For example, the antibiotic first approach, although largely effective in eliminating and suppressing the virulence of pathogens, while clearly beneficial, may also be detrimental in terms of antibiotic resistance emergence and elimination of the probiotic effect of the microbiome.

### *1.4 The impact of antibiotic exposure on clinical outcomes*

The mainstay treatment of sepsis largely consists of supportive care, source control and rapid initiation of broad-spectrum antibiotic therapy. It is patently clear that patients suffering from septic shock are significantly benefited when antibiotic therapy is initiated within an hour of the onset of infection. A small delay (3-6 hours) can still benefit septic patients if they do not suffer from septic shock[65,67,69]. This latter distinction may be important when decisions

regarding who needs immediate antibiotics coverage and when a more appropriate delay is possible with the goal of preventing unnecessary exposure to broad-spectrum antibiotics. There is some evidence that waiting until an infection can be confirmed may improve survival when compared to empirically initiating broad spectrum antibiotics to avoid delays and the concern that infection will rapidly progress [70]. In fact, targeted antimicrobial therapy has been demonstrated to more effective and less harmful in such circumstances. A prospective study looking at compliance rates of broad spectrum antibiotic treatment for drug resistant pneumonia found that compliance with empiric broad spectrum antibiotics was associated with increased mortality [71]. Similarly, a recent study comparing inappropriate broad spectrum antibiotics versus targeted therapy found that patients who were overtreated (empiric antibiotics given when the anti-microbial coverage could be narrowed) versus those who were under treated (not given broad spectrum antibiotics to a multi-drug resistant or polymicrobial infection) were both associated with worse outcomes [18]. Given the general perception that antibiotics are safe and otherwise innocuous, the reflex of "if some is good, more must be better" prevails. Thus the overuse of broad-spectrum antibiotics based on the for treating sepsis at its earliest timepoint is concerning. However even more concerning is the promiscuous use of antibiotics in industrial farming [6,72]. Globally almost three quarters of all antimicrobials sold are utilized in industrial farming [72]. The rapid and robust use of antibiotics for livestock has been associated with an increase in the antimicrobial resistance present amongst bacteria across the world [72]. Inappropriate use of broad-spectrum antibiotics demonstrates the significant downside to their overuse and its harmful effect on mankind. Similar to the overprescribing of antibotics is the overpresciribn of their dose and dose schedule. Several studies have demonstrated that prolonged duration of antibiotics is associated with significant harm. In fact, shorter courses of antibiotics

in intra-abdominal infection and community acquired pneumonia have shown to be as effective as longer courses of antibiotics [73–75]. In contradistinction, longer courses of antibiotics have been associated with worse outcomes icluding readmission [73], increased risk of perioperative acute kidney injury and *C. dificile* infection [76]. While prolonged antibiotic use may decrease the efficacy of antibiotics as resistance mechanisms come into play, their collateral damage on the microbiota may also explain their association with poor outcome.

Antibiotics, especially those with anaerobic activity, can dramatically and durably alter the microbial ecology resulting in the acquisition and domination by normally low abundance but highly pathogenic species, such as *Enterococcus faecium* or *Klebsiella pneumoniae* [77]. In one study, intestinal prevalence of *Enterococcus* increased the risk of subsequent VRE bacteremia by 9-fold whereas a predominance of *Proteobacteria* in the gut microbiome increased the risk of gram-negative bacteremia by 5-fold. Bacteremia occurred a median of 7 days *after* intestinal domination by these pathogens [78]. The association between microbiota disruption and the risk of sepsis has been further supported by two large retrospective studies [79,80]. In both studies, admission to the hospital for an infectious related complication and antibiotic exposure significantly increased the risk of subsequent sepsis-related hospitalization within 90 days of the index hospitalization. Taken together, these studies indicate that disruption of the normal microbiota, our first line of defense against pathogens, can increase the risk of serious life-threatening infection leading to sepsis. Finaly, broad usage of anti-anaerobic antibiotics has been associated with increased mortality in the intensive care unit[77]. Exposure to anti-anaerobic antibiotics was associated with a decreased ventilator associated pneumonia free interval, decreased infection-free survival, and reduced overall survival [77]. Therefore the consequences of anti-anaerobic antibiotics is significant given its was reduction in gut bacteria density and an

increase in colonization with Enterobacteriaceae not only in the gut but also the respiratory tract [77].

Converging lines of evidence now demonstrated, in both murine and human studies, that critical illness itself is associated with a rapid reduction in gut microbiota diversity and increased colonization by Proteobacteria [81]. In a study out of our lab, we demonstrated that in critically ill patients there was not only an increase in hospital acquired pathogen colonization within the gut, but a substantial increase in colonization with antibiotic resistant and virulent pathogens [82]. Such alterations in the gut microbiota can not only have a major downstream impact on the host immune system (vida infra) but can also predispose individuals to gut-derived systemic infections. As sequencing technology has advanced, we have begun to understand that gut-derived sepsis goes beyond simple translocation of bacteria to the blood stream [83]. There is clearly an association between the gut microbiota and infection in distant organs where typical gut commensals move from one environment to the next, often by silently hijacking an immune cell, to cause a remote infection. One interesting finding is the predominance of gut-origin pathogens in the lung microbiota in both patients and and mice[84]. For example, following cecal ligation and puncture, the mouse lung microbiota become rapidly dominated by *Klebsiella pneumonia* and *Enterobacter cloacae*. As a human correlate, bronchial-alveolae lavage obtained from septic patients demonstrates an increase in the presence of *Bacteroides* within the lungs [84]. Finally, a major finding connecting how the treatment of critical illness can have a profound impact on the gut microbiota, which can in turn influence the lung microbiota can be observed during hyperoxia, an often-unavoidable circumstance needed to treat patients with lung injury. Hyperoxia delivered to mice by exposing them to 90% oxygen, can markedly reduce the obligate anaerobes in the gut resulting in the lungs acquiring multiple pathogens of gut origin thus

rendering the lung to pathogen invasion. [85]. Elucidation of a gut-lung, gut-brain and gut-liver axis is emerging and involves not only relocation of gut microbes to these remote organs, but also depletion of key metabolites from the gut microbiota that drive a recovery directed immune response [86–88].

As a results of this growing body of evidence, the gut microbiota are playing a more central role in the setting of gut-derived infection. The gut microbiota plays an important role in preventing pathogen colonization resistant against many common enteric pathogens [89,90]. The loss of normal composition of the gut microbiota due to antibiotic exposure and critical illness increases the risk for the development of enteric infections of all types [91]. A large portion of the early studies have demonstrated that enteric infections, especially *C. difficile* infections, are a result of the loss of the normal commensals that provide colonization resistance [92].

While in general, infection control measures outlined in clinical studies demonstrate various approaches to prevent the spread of pathogen, many are now considering a role for probiotics and prebiotics. Probiotics consist of communities of commensal cytoprotect bacteria, such as Lactobacillus and Bifidobacterium, that can promote colonization resistance and intestinal barrier function when administered as pills or food (i.e- yogurts) [93]. Prebiotics consist of nutrients, typically fermentable carbohydrates (i.e., fructo-oligosaccharides or galactosaccharides), that promote the colonization, growth and metabolite production of beneficial bacteria [94]. Unfortunately, probiotics in the forma of microbial consortia, have only shown minimal benefit in preventing infectious outcomes critically ill patients and may potentially result in delayed refaunation of the individual's native gut flora [95]. A recent study looking at time to refaunation after antibiotic exposure demonstrated that probiotics increased the time to restoration of the pre-antibiotic microbiota whereas autologous FMT resulted in rapid

restoration of the gut microbiota [95]. In contrast, synbiotics, the delivery of both prebiotics and probiotics, have shown some clinical benefit in reducing ventilator associated pneumonia, enteritis, and sepsis in neonates [96,97]. Currently, while there are many associations between the use of antibiotic and changes in the microbiota and clinical outcomes, much of it is context dependent and there is a reciprocal relationship between how the gut microbiota shapes both the host and pathogens and vice versa. In the following sections, the interaction between the gut microbiota and the host will be outlined in further detail.

## *1.5 Microbiota-host interactions and the importance of the gut microbiota in human health and disease*

Our growing understanding of the microbiota provides some degree of understanding of how antibiotic depletion of the gut microbiota can have a major impact on human health. Although bacteria occupy niches throughout the human body, the focus of this dissertation will be on the microbiota within the gut compartment, acknowledging that microbiota elsewhere also play an important role in maintaining health and promoting disease. The gut microbiota consists of complex, dynamic communities of bacteria that play an essential role throughout the entire gastrointestinal tract. The ability of the microbiota to impact the host can occur via three main methods – microbiota-microbiota interactions, direct microbiota-host interactions, and indirect microbiota-host interactions. To understand why alterations to the microbiota from antibiotics is being increasingly recognized to be associated with infectious outcomes, it is important to appreciate the role the gut microbiota plays in interacting with the host during homeostasis.

## *1.6 Microbiota-microbiota interactions and their impact on the host*

The gut microbiota consists of large communities of bacteria that not only interact with each other but are dependent on one another. Our knowledge of the bacterial communities within the gut are significantly limited as only ~20% of the bacteria found within the gut microbiota are cultivable and thus only a small percentage of the gut microbiota have been studies primarily in *in vitro* conditions. Therefore their potential interactions across multiple host compartments remains yet unknown. [98–100]. The gut microbiota has been found to encode greater than 100 times the number of genes as the human genome demonstrating the genetic complexity and potential of the organisms that make up the microbiota [100].

The human gastrointestinal (GI) tract offers a uniquely harsh environment that is optimal for only a limited community of bacteria to grow and thrive. The intestinal tract extends from the mouth to the anus. The bacterial density of the gut microbiota increases distally within the gastrointestinal tract with the largest microbial density occurring within the colon [101]. Both physico-chemical environmental "cues" and host derived "cues" shape the microbiota at specific niche sites. For example, the pH and oxygen content varies throughout the GI tract along with varying degrees of nutrients which in turn have a major impact on the community of bacteria that inhabit each portion of the gastrointestinal tract [101,102]. The host provides the environment the determines the niche suitable for colonization for communities of bacteria. The gut becomes colonized with bacteria that have been pre-adapted for a given environment and can alter their phenotype to increase their ability to colonize a given environment and occupy specific niches [101,103]. Individual and communities of the bacteria contribute to complex communities that interact to produce metabolites and nutrients necessary for their survival and that of the host. The

strong interaction and development of the communities of bacteria are stable over the course of a lifetime but are sensitive to extreme changes in the gut environment [104]. For example, one study demonstrated that 60% of bacterial strains remain stable over the course of five years [105]. Furthermore, longitudinal studies demonstrate that most individuals contain a 'core community of permanent colonizers' that can fluctuate their abundance over time [106]. In addition to adapting to the differing environment depending on the location of the GI tract, bacterial communities have adapted to either inhabit the intestinal mucosa or the intestinal lumen creating a network of communities occupying different niches within the intestinal tract that are dependent on each other for survival [107]. In the small intestine, the mucosa tends to be inhabited by bacteria from the phyla *Bacteroidetes and Clostridia* whereas bacteria from the phyla *Enterobacteriaceae* tend to inhabit the intestinal lumen [102]. The differences in microbiota within the mucosa and lumen of the intestinal tract are dependent on the intestinal microenvironment and are each other. For example, mucus associated *Clostridia* play an important role in preventing colonization with aerobic bacteria and maintaining health promoting anaerobes [108]. Work from our lab has demonstrated that there is a unique interaction between the crypt and luminal microbiota that is dependent on the microenvironment in which bacteria within the cecal crypts maintain a deoxygenated environment allowing for beneficial bacteria to reside while at the same time preventing colonization with potential pathogens. This colonization pattern alters the regenerative capacity of intestinal stem cells impacting the host health [109]. However, when dietary changes alter the oxygen gradient of the intestinal crypts, there is a loss of inhabitants of the cecal crypts with beneficial bacteria and colonization with potential pathogens from the phyla *Proteobacteria* [110]. The spatial arrangement and reciprocal interactions between bacteria that utilize digested products and metabolites from the host help shape an environment suitable for

other communities of bacteria to colonize. One example of the importance of the spatial organization of the gut microbiota occurs with the loss of the mucus layer either from a western diet or from deletion of the Muc2 gene, a gene necessary for mucus generation within the gut. When the mucus layer is depleted and the mucus associated microbiota are lost, there is increased colonization with enteric pathogen *C. rodentium* [111]. Thus, the complex relationship between the various bacterial communities on and within the mucosa and their dependency on local gut environmental conditions make the microbiota highly sensitive to environmental perturbations.

The interactions of the gut microbiota and stabilization of the gut microbiota is essential for preventing pathogen colonization. Early work from patients exposed to Streptomycin demonstrated increased colonization with *Salmonella* and the development of *Salmonella* enteritis[112]. A large component of pathogen colonization and resistance by the microbiota is dependent on nutrient availability[89,113]. When the intestinal microbiota is undisturbed, there is typically low availability of simple sugars within the large intestine and the colon is then dominated by communities of bacteria that metabolize complex carbohydrates and host secreted mucus [114,115]. The loss of these healthy promoting bacteria after antibiotic exposure results in the release of simple sugars by the host that are then utilized by potential pathogenic bacteria [116]. The communities of bacteria that inhabit the gastrointestinal tract are directly influenced by substrate availability. When viewing the colonic microbiota from an energy utilization and metabolism standpoint, the native microbiota can directly prevent pathogen colonization by limiting nutrient availability for potential pathogens. Exogenous species to the gut microbiota have difficulty competing with the communities of bacteria that have adapted to utilize the essential nutrients found within the gastrointestinal tract [114,117]. *Salmonella* and *C. difficile* have

been demonstrated to compete for simple sugars with native bacteria within the gastrointestinal tract [116]. Furthermore, pathogenic bacteria have been demonstrated to directly compete for important trace metals with the microbiota[113]. One study demonstrated the importance of iron utilization by observing that when mice were immunized against siderophores, there was reduced ability of *Salmonella* to colonize the gut [118]. Another finding of nutrient utilization by the microbiota to prevent pathogen colonization is the utilization of bile acids. Bile acids are produced from cholesterol in the liver and play an important role in fatty acid digestion and absorption within the gut [119]. The majority of bile acids are reabsorbed in the terminal ileum and are recycled to the liver in the enterohepatic circulation of bile acids [119]. However, the bile acids that are not absorbed in the small intestine can be conjugated to secondary bile acids by the colonic microbiota, particularly by *Clostridia* species [120]. The conjugation of bile acids by the native gut microbiota plays an important role in preventing *Clostridioides difficle* colonization and limiting the severity of the disease. Primary bile acids such as taurocholic acid can trigger the germination of *C. difficile* spores by binding the receptor CspC [121]. Secondary bile acids such as chenodoxycholic acid can inhibit bile acid induced germination, growth, and inhibit toxin TcdB of *C. difficile* [122,123]. Another nutrient produced by the microbiota that inhibits pathogen colonization is the production of SCFAs. Anaerobic bacteria *Bacteroides* and *Firmicutes* ferment complex carbohydrate and form short chain fatty acids (butyrate, acetate, and propionate). The majority of SCFAs are produced in the proximal colon and utilized by the host[124]; however, SCFAs can significantly alter the pH of the colon and can have a major impact on pathogen growth and colonization. Several studies have demonstrated that SCFAs significantly impact the growth of *E. coli* and *Salmonella* [125,126]. For example, butyrate has been shown to activate oxidative phosphorylation within colonic epithelial cells by activating PPARy, increasing

oxygen utilization and decreasing the oxygen and nitrogen concentration within the colonic lumen [127–129]. SCFAs have been demonstrated to decrease the virulence of *Enterobacteriaceae* species including *Salmonella* [130–132].

Outside of direct competition for nutrients and direct alterations in the microenvironment that play an essential role in determining the ability of pathogens to colonize the gut, bacteria have also developed the means to actively antagonize other bacteria [133]. Bacteria have the ability to produce antibacterial peptides that work to prevent expansion of other bacteria. These include the production of antibiotics such as polyketides, glycopeptides, aminoglycosides, and lincosamides [134–136]. Within the gut microbiota, the majority of antibacterial peptides are produced in the form of bacteriocins, ribosome synthesized and post-translationally modified peptides (RiPPs) [137]. One extensively researched group of bacteriocins are the lantibiotics produced by *Lactobacillales* [138]. Lantibiotics actively inhibit gram positive bacteria including *Listeria* and *Staphylococcus aureus* [139,140]. The majority of bacteriocins initially studied primarily target gram positive bacteria; however, recent studies are beginning to find bacteriocins with the ability to target gram negative bacteria. Bacteriocin NAI-107 was found to have the ability to fuse with polymyxin and significantly inhibit gram negative bacteria including *E. coli and Klebsiella pneumoniae* [141]. In a similar manner to bacteriocins, gut bacteria have the ability to produce larger antimicrobial proteins. The best example of production of antimicrobial proteins is production by *Bacteroidales* [142,143]. In particular, *Bacteroides fragilis* have been shown to produce antimicrobial proteins that target similar species and increase their ability to colonize the gut [142,143]. The main difference in antimicrobial proteins and bacteriocin peptides, is that antimicrobial proteins tend to require transportation into target bacteria to be effective as opposed to bacteriocins tend to target proteins in the cell wall of gram negative bacteria [114,133].

Finally, bacteria can utilize secretion systems, in particular type VI secretion systems (T6SS), to directly inhibit competing bacteria [144,145]. Interestingly, T6SS+ bacteria also encode immunity proteins that negate the secretion system and prevent self-targeting [145]. T6SS has been found to be widely expressed amongst *Bacteroidales* bacteria. The presence of T6SS+ *Bacteroidales* gives the bacteria to prevent colonization of the gut by other *Bacteroides* species [146]. The production of antibacterial proteins, peptides, and utilizations of T6SS demonstrate an essential role by which the bacteria that inhabit the gastrointestinal tract and actively work to maintain their environment to their own benefit and the benefit of the host. Furthermore, the groups of bacteria create environmental niches by competing for the availability of nutrients, developing symbiotic relationships with adjacent bacteria, and altering the microenvironment by altering oxygen content directly and indirectly.

The complex communities of bacteria present in each host are essential for maintaining homeostasis and preventing unwanted bacteria from colonizing. Certain pathogens, particularly *Enterobacteriacea*, elicit host inflammation with the goal of altering the intestinal environment such as by increasing oxygen levels [147]. Increased oxygen levels within the intestinal lumen decrease the ability of typical commensal bacteria to survive and allow for facultative anaerobic, commonly pathogenic bacteria to expand[147,148]. In addition, in the setting of increased inflammation, the respiratory burst by the host utilizes oxygen to produce reactive oxygen species including nitric oxide. Nitric oxide can be converted from thiosulfate to tetrathionate and pathogens such as *Salmonella* have the ability to utilize tetrathionate as a terminal electron acceptor [149,150]. These alterations further equip pathogens for survival in inflammatory conditions so they can outcompete commensal bacteria. In a similar manner, pathogenic *E. coli* species can utilize nitrate as a terminal electron acceptor which places them in a unique position

when nitric oxide concentrations increase within the intestinal lumen [151]. There is a dynamic interaction between the host and commensal bacteria in maintaining the normal intestinal microenvironment necessary for commensal bacteria to thrive and exert their colonization resistance against pathogenic bacteria. The impact of antibiotics and critical illness significantly disrupts this normal colonization resistance mechanisms by inadvertently eliminating many of the commensal bacteria resulting in increased pathogen colonization, niche specialization which together can contribute to the vicious cycle of repeat secondary infections in this patient population.

## *1.7 Indirect interactions between the microbiota and the host for prevention of pathogen colonization*

As our knowledge of the intestinal microbiota and the gut mucosal immune system grows, we have begun to understand the important interactions that can occur between the host and bacteria which is essential for maintaining a reciprocal and homeostatic relationship between the intestinal microbiota and the host. As discussed previously, colonization resistance against pathogens can occur directly through competition for resources, production of metabolites, alterations of the intestinal microenvironment, and production of antimicrobial peptides. One interesting finding is that host genetics can have a major influence on the composition of the intestinal microbiota. However, the influence of host genetics is diminished given the multiple environmental exposures that human progress has imposed including the use antibiotics and processed foods. Yet reports of associations between host SNPs and bacterial taxons [152–154] suggest that host genetics can alter the microbiota through variations within the host mucosal immune system which can directly impact the ability of bacteria to colonize the host. One

interesting cell line are Paneth cells which are secretory cells that exists in abundance within the small intestines [155,156]. Paneth cells have been shown to secrete antimicrobial peptides and are believed to play an important role in preventing bacterial colonization within the intestinal crypts. Paneth cells secrete antimicrobial peptides in response to bacterial products, both gram negative and gram positive, as well as host stimulation such as via cholinergic stimulation [155]. The primary antimicrobial peptides produced by Paneth cells fall under the category of alpha defensins which disrupt cell membrane integrity and inhibit cell wall synthesis of bacteria [155]. Alterations in Paneth cell function prevents *Segmented Filamentous Bacteria (SFB)* colonization [157]. *SFB* is essential for maintaining a Th17 immune response within the host and increases the inflammatory response to normal commensal bacteria [158]. To further implicate the role of the host immune system in dictating the composition of the intestinal microbiota, mice deficient in TLR or MyD88 demonstrate significant alterations in their intestinal microbiota composition – this is likely related to alterations in mucosal immune cells and the impact on Paneth cells [159].

Control of the intestinal microbiota by the host immune system goes beyond the innate immune system. The host immune system has been long noted to produce secretory IgA which coats the intestinal microbiota [160]. IgA coating of bacteria has been demonstrated to occur in both a bacteria specific and non-specific manner [161]. It is believed that IgA preferentially coats bacteria that are prone to mucosal colonization and have colitogenic potential [160]. The impact of IgA binding of bacteria remains unclear; loss of IgA results in a minimal change in phenotype and a minimal alteration in bacterial composition [160]. There is emerging knowledge in the field demonstrating a direct and reciprocal relationship between the intestinal microbiota and the host immune system. As such, alteration in one affects the other.

The composition of the microbiota plays an essential role in preventing pathogen colonization within the host. The composition of the microbiota is continually shaped by the host through the secretion of antimicrobial peptides and the response of the host immune system directly to bacteria and their metabolites. As knowledge in the field emerges, our understanding of how the gut microbiota play an essential role in response of the host to infection will be advanced and inform novel approaches to mitigate pathogen overgrowth.

## *1.8 Microbiota produced metabolites and their impact on the host*

Studies continue to demonstrate the important role that the gut microbiota plays in maintaining the host health and promotingdisease. It is becoming increasingly recognized that the interactions between the host and the microbiota extend beyond structural composition and membership of the gut microbiota and involve multiple intersecting elements on both the host and microbial side. Functionally, gut bacteria, their community structure and their membership play an important role in producing unique metabolites that can have a major impact on the host. In the following section, we will describe the production of unique metabolites exclusively produced by the gut microbiota and how they have been demonstrated to impact the host in both health and disease states.

## *1.9 Short chain fatty acids and the host immune system*

The production of health promoting metabolites by the gut microbiota requires that they be exposed to the proper dietary substrate. The production of short chain fatty acids (SCFA) is largely dependent on microbiota accessible carbohydrates; specifically carbohydrates that are

resistant to digestion within the upper GI tract and that are accessible to the gut bacteria for the production of metabolites [162–164]. Unfortunately, in many western societies, a diet that is poor in fiber and highly processed is the norm resulting in a significant alteration of SCFAs within their gut. In terms of sepsis risk, such SCFA depletion can put patients at risk. For example, previous studies have demonstrated that patients manifesting a sepsis phenotype can have a significant reduction in their stool concentrations of short chain fatty acids [165,166]. This is important as SCFAs are known to have multiple important roles in activating or downregulating the host immune system.

The typical SCFAs produced by the gut microbiota include acetate, butyrate, and propionate. Acetate and butyrate are the most abundant SCFAs produced by the microbiota [124,167]. Studies to date have focused on the importance of butyrate in altering the host immune system and therefore will be focused on during this section. Butyrate is an essential energy source for colonocytes, but also can play a major role in altering the host immune system. Butyrate can function as a histone-deacetylase inhibitor (HDAC) or an agonist for G-protein coupled receptors (GPCR) on host cells and significantly alter cell signaling [163,168,169]. HDAC inhibition has been demonstrated to globally reduce NFkB activation in mononuclear cells and downregulate the production of TNFa [170]. Butyrate has also been shown to play an important role in regulating Foxp3 expression within CD4+ T regulatory cells. This was first demonstrated by the seminal work showing that gut butyrate production by the microbiota was important in extrathymic Treg cell production and this occurred in an HDAC dependent manner [171]. When it comes to the importance of butyrate on the innate immune system, a more recent study demonstrated that butyrate played an important role in shaping macrophage bactericidal capabilities [172]. Schulthess et al. found that human monocyte derived macrophages derived in

the presence of butyrate had a significant increase in their killing of specific pathogens through HDAC inhibition which altered the metabolic and functional profile of macrophages increasing pathogen clearance [172]. In a similar manner, macrophages exposed to butyrate have been demonstrated to take on an M2, or anti-inflammatory, phenotype and reduce the severity of DSS colitis [173]. Butyrate has also been implicated for altering tumor associated immune cells including dendritic cells and in turn change the response of tumors to radiation [174]. Butyrate production by the gut microbiota also suppresses type I interferons and decreases the responsiveness of tumors to radiation [174]. The evidence of how SCFAs, particularly butyrate, shape the host immune system continues to grow. However, we are beginning to appreciate how the loss of butyrate seen in septic and critically ill patients is likely to contribute to a maladaptive immune response to pathogens.

## 1.10 Indole metabolites and the aryl hydrocarbon receptor (AhR)

Beyond SCFAs, other gut microbiota produced metabolites including bile acids have been shown to play an essential role in shaping the host response to a major perturbation. In this thesis, we will focus on **Tryptophan** metabolites and their role in altering the host immune system. Tryptophan is an essential amino acid and as such, its exogenous intake is required by animals, including humans [175,176] to maintain health. There are three major pathways by which tryptophan can be metabolized: it can be metabolized to produce indoles which occurs exclusively by the gut microbiota, to kynurenine by host cells, and to serotonin by the host [177].

In the context of immune activation within the host-pathogen-microbiota interaction, a main pathway of interest is the direct conversion of Tryptophan into indole metabolites such as indole-3-acetic acid, indole-3-propionic acid, tryptophol, and indole-3-lactic acid. These metabolites have been extensively studied due to their ability to activate an intracellular receptor known as the aryl hydrocarbon receptor (AhR) [178–180]. The aryl hydrocarbon is a cytoplasmic receptor that exists in an inactive state in the cytoplasm bound in a complex with proteins Hsp90, X-associated protein (XAP2), cellular sarcoma kinase (c-Src), and cochaperone p23 [180]. When an aryl hydrocarbon ligand binds to AhR, Hsp90 is released revealing the nuclear translocation signal allowing AhR to translocate into the nucleus [180]. In the nucleus, AhR dimerizes with ARNT which allows binding to xenobiotic response elements (XRE) and act directly as a transcription factor[180,181]. The canonical AhR signaling pathway consists of binding to XRE and ultimately leads to the production of CYP1A1,CYP1A2, CYP1B1, and AhR repressor [181,182]. CYP1A1 in turn has the ability to degrade AhR ligands. AhR/ARNT also has the ability to interact with other transcription factors including (KFL6, Rb, CUL4B) altering their transcription profile [183–185]. AhR can also impact cellular signaling following the dissociation of AhR from Src which can activate other signaling pathways that have a broader impact on downstream pathways of interest [186]. Furthermore, CUL4B can induce a ubiquitin ligase which can alter protein expression within cells [184]. The majority of AhR functions have been determined from knockout studies. AhR has been shown to play a role in organogenesis and has been found in the neuroepithelium and heart of mice during gestation [187,188]. In adults, AhR is highly expressed within the placenta, lungs, heart, pancreas, and liver [189]. Global AhR knockout mice can result in growth deficiencies, infertility, and vascular/cardiac hypertrophies, and accelerated aging [190–192]. AhR plays a wide role in normal physiology and is promiscuously expressed on a wide array of

different cells. Furthermore, the most experimentally employed endogenous AhR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCCD], has been observed to produce AhR-mediated toxicity and can result in significant alterations in the host immune response, in reproductive health, and can increase susceptibility to tumor development [178].

Potential ligands for AhR include exogenous, endogenous, and natural occurring molecules. Exogenous molecules include dietary molecules such as polyphenols and reservatol. More interesting are the tryptophan molecules that have been demonstrated to be AhR ligands. Host cells metabolizes tryptophan to serotonin or kynurenine [193,194]. Kynurenine is synthesized by tryptophan 2,3-dioxygenase (TDO) in the liver, but other cells can produce kynurenine through either indoleamine 2,3-deoxygenase 1 (IDO 1) and indoleamine 2,3-deoxygenase (IDO2)[194]. TDO is the predominant enzyme responsible for kynurenine production and is heavily regulated by glucocorticoid induction, tryptophan, heme, and the production of IFNy [195]. IDO is upregulated in the setting of inflammation and has been demonstrated to play an important role in regulating inflammation [196,197]. IDO is expressed by a variety of immune cells and is upregulated by IFNy and downregulated by anti-inflammatory cytokines (IL-10) [195]. Importantly, nitric oxide, often released during a physiologic perturbation, can also inhibit IDO [198]. These findings demonstrate that kynurenine production may play an important role in the immune response given its regulation by cytokines. Consistent with these findings, IDO has been shown to suppress effector T cells and increase Treg cells [199]. Interestingly, IDO has been shown to increase the tolerance of macrophages to apoptotic cells further demonstrating the importance of kynurenine production in regulating the pro and anti-inflammatory response [200]. Taken together, these findings demonstrate that tryptophan metabolism to kynurenine can have a broad impact on

the immune system. Expression of IDO and AhR by tumor cells demonstrates that Kynurenine activation by AhR results in immunosuppression and tumor progression [201]. Broadly, kynurenine has been demonstrated to have an anti-inflammatory impact on mice and can protect against LPS toxicity [202]. The extent of AhR activation by kynurenine and tryptophan metabolites extends further beyond interactions with the host immune system but has been demonstrated to play an important role in host-microbiota interactions.

AhR has been demonstrated to play an essential role in mucosal immunology and host-microbiota interactions [203,204(p3)]. AhR activation within ILC3 plays an essential role in IL-22 production which maintains mucosal barriers and increase production of antimicrobial peptides within the gut. Recent studies have demonstrated that indole production by *Lactobacillus* plays an important role in AhR activation within the gut mucosa and and can maintain a diverse microbial community [205]. The AhR has been demonstrated to play a role in the innate immune response outside of mucosal immunology. For example, the loss of AhR results in significant increase in mouse sensitivity to endotoxin[206]. When utilizing a global knockout of AhR, there is a significant increase in mortality in association with increased production of the pro-inflammatory cytokines IL-6 and TNFa, hypothesized to occur via interactions between AhR and STAT1[206]. AhR was further demonstrated to play an important role in response of macrophages to LPS and the subsequent development of endotoxin tolerance [207]. In this latter study, LPS sensitivity was dependent on both AhR and TDO. Furthermore, endotoxin tolerance can result from AhR activation and IDO1 expression. AhR activation on initial exposure interacted with Src kinase activity and phosphorylation of IDO1 which is important for the development of endotoxin tolerance [207]. As many as eighty-five (85) species of gram-positive and gram-negative gut commensals are capable of producing indoles from tryptophan via the enzyme tryptophanase

[208,209]. There are other bacterial species that have been noted to utilize aromatic amino transferases to convert tryptophan to indole-3 acetaldehyde and indole-3 pyruvate[210]. AhR activation is thought to potentially play a role in the pathophysiology of IBD with higher level of IDO1 activation, likely secondary to higher inflammatory levels, and a decreased production of AhR ligands by the gut microbiota in patients with IBD [211]. These findings are relevant as mouse models have demonstrated that AhR deficiency results in increased susceptibility to colitis likely secondary to the loss of IL-22 which is essential to maintaining the mucosal immune system [210,212,213]. In the setting of infection, the loss of AhR increases susceptibility to *C. rodentium* [214]. Furthermore, with some pathogens (i.e *Chlamydia spp)* subversion of the host to out compete other bacteria through induction of IDO1 has been observed causing increased tryptophan catabolism with suppression of the growth and colonization of other bacteria [215]. A recent study demonstrated that mice supplemented with oral indole-3-propionic acid (I3PA) had improved survival from cecal ligation puncture [216]. The mechanism by which I3PA improved survival in this model was not described, however it is hypothesized to occur via the relationship between AhR and the immune system or the impact of indole metabolites on bacteria as described above. Outside of the mechanism by which AhR ligates indole metabolites produced by commensal microbiota, recent studies also demonstrate that pathogens can interfere with ability of the AhR in these interactions. In a study by Moura-alves et al, it was demonstrated that various quorum sensing molecules produced by *Pseudomonas aeruginosa* can interact with the AhR in a manner that is growth phase-dependent [217,218]. Depending on concentration and time, various P. aeruginosa derived QS molecules had a differential impact on AhR signaling. These findings suggest that the aryl hydrocarbon receptor may have the ability to monitor the pathogen and coordinate the appropriate immune response based on the stage or severity of the infection.

The production of indole metabolites by the colonizing microbiota and their interaction with the aryl hydrocarbon receptor, when coupled with the ability of bacterial quorum sensing molecules to interdict in this process may suggest that host immune system is regulated by a complex interplay between the host-microbiota and the host-pathogen interactions.

## *1.11 Host-microbiota-pathogen interactions in the setting of surgery, sepsis, and critical illness*

As the scientific community has become to appreciate the important relationships that exists between the host and its microbiota, its role in human health and disease is being intensely investigated. My particular interests lie within the setting of surgery, sepsis, and critical illness in which there have been numerous studies demonstrating a compelling correlation between alterations in the gut microbiota and outcome [219–222].

Several studies to date have generated compelling evidence that the status of one's microbiome can influence outcome from a surgical stress or major injury. Our lab was the first to demonstrate a key and causative role for the gut microbiota on anastomotic leak, a common complication that occurs when a new connection is made between two ends of intestine leaks[223]. In a murine model of anastomotic leak, we demonstrated that collagenase producing bacteria inhibit anastomotic wound healing and contribute to anastomotic leak[223]. In a similar manner, mice that were colonized with pathogenic bacteria who then underwent an otherwise recoverable surgical stress (30% partial hepatectomy) with its attendant treatments were included such as short-term starvation and antibiotic exposure, the mice developed lethal sepsis from gut-derived organisms [224]. In this model, the typical surgical stressors of patients were recapitulated by having the mice fast overnight prior to the 30% partial hepatectomy, which, as mentioned, is an otherwise recoverable surgical injury, and then during the hepatectomy inject a consortia of

multidrug resistant pathogens obtained from the stool of a septic patient (referred to as the pathogen community (*S. marcescens*, *Klebsiella oxytoca*, *Enterococcus faecalis*, and *Candida albicans*) into the cecum . Mice that received a fecal microbiota transplant taken from a healthy littermate control following the surgery survive compared to mice that received an autoclaved fecal transplant (AC-FMT) who had a high mortality rate [224]. In order to determine the precise molecular mechanisms responsible for this finding, the model was shifted to a systemic infection model by injecting the polymicrobial community directly into the peritoneum. Mice in each group (FMT versus AC-FMT) received either an FMT or an AC-FMT at the time of infection. Similar to previous findings, mice that received an FMT survived whereas mice that received the autoclaved FMT (i.e. AC-FMT) died. Reiterative studies demonstrated that FMT had no impact on the composition of the gut microbiota, but significantly altered the levels of butyrate within the gut microbiota as judged by the metabolomic-based assay and by the increased presence of butyrate producers within the microbiome. Gene expression studies demonstrated that FMT maintained IRF3 within mice. *In vitro* studies of the effect of PC and butyrate on mouse embryonic fibroblasts demonstrated that PC suppressed IRF3 function while butyrate maintained its function suggesting that FMT maintained butyrate levels in the gut and prevented IRF3 suppression by the pathogen community and improved survival. However, direct interaction between the FMT, butyrate and IRF3 remained largely correlative in the mouse model and it remained to be determined how the gut microbiota and FMT were directly contributing to survival [224].

When mice were treated with antibiotics or exposed to a western diet, their gut microbiota became dominated with collagenase-producing bacteria and, in a model of colorectal

anastomotic surgery, the incidence of anastomotic leak increased [225]. Furthermore, anastomotic leak was decreased when western fed diet mice were prehabilitated with a low fat, high fiber diet [225]. Yet the contribution of the gut microbiota to surgical complications extends beyond anastomotic leak. For example, when mice are subjected to an otherwise recoverable surgery (i.e. a 30% partial hepatectomy), they develop lethal gut-derived sepsis that is most prominent when mice are treated with antibiotics and consume a western-type diet (high fat content, low fiber content). In this model, a western diet and antibiotic exposure results in the cecal contents being dominated by multi-drug resistant pathogens, such as *S. marcescens*, which the capacity to systemically disseminate and cause lethal sepsis. In contrast, mice that adhered to a low fat, high fiber diet survive without any signs of infection or bacterial dissemination despite undergoing surgery and being exposed to antibiotics [226].

The studies have clearly demonstrated that alterations to the microbiota whether through diet, antibiotics, or surgical stress, create an environment that impacts both the host, the pathogens and the gut microbiota such that there is increased susceptibility to lethal sepsis and infection-related complications such as anastomotic leak. The goal of these projects, in the context of developing actionable approaches to reduce infection-related surgical complications is to advance an understanding of the relationship between the host, the microbiota, and infecting pathogens. Taken together these results also indicate a direct interaction between the host environment and the microbiota that can be directly shaped by dietary modificatioin and lessening the stress of surgery. In this body of work we hypothesized that modifications of the gut microbiota can have a direct impact on infection-related surgical complications via their effects on the host immune system and pathogen virulence. To address these possibilities, I

further characterized the impact of the host gut microbiota on pathogen virulence following dietary manipulation and surgical stress and sought to define the relationship between the gut microbiota, the host and the infecting pathogen. This approach involved focusing on the impact of metabolites produced by the gut microbiota and to define their role on both the host immune system and pathogen virulence.

# 2. MATERIALS AND METHODS

### *2.1 Mouse Model of Surgical Stress:*

Six-week-old C57BL/6 mice (Charles River Laboratory) were housed in a temperature-controlled 12h light/dark cycled rooms at University of Chicago. Mice were fed on either standard rodent chow (Envigo) or a Western diet (Bio-Serv, catalog no. S3282) for 6 weeks. Following 6 weeks of WD consumption, mice subjected to dietary prehabilitation (DietPrehab) were transitioned to SD. All experiments were in accordance with National Institute of Health (NIH) guidelines and approval was obtained from University of Chicago Institutional Animal Care and Use Committee (IACUC protocol 71744).

Our previously described model of antibiotics, starvation, and hepatectomy was utilized [226]. After 6 weeks on their diets and/or completion of DietPreHab, mice were subjected to antibiotics twice per day for 5 days consisting of cefoxitin (30mg/kg i.p., Hikma Pharmaceuticals, Eatontown, NJ) and clindamycin (70mg/kg p.o.; Clindrops; Henry Schein, Dublin, OH). Prior to surgery, mice were subjected to 12 hours of starvation, but allowed water ad libitum. Mice were anesthetized using 80mg/kg ketamine and 5mg/kg xylazine administered i.p. A midline laparotomy was performed, the left lobe of the liver was exteriorized, and 30% of the liver was resected. Following surgery, the mice were resuscitated with 1cc of warmed NS subcutaneously (s.c) and for pain given 0.05mg/kg buprenorphine, and allowed to recover on a warming pad. Mice were assessed every 6 hours for signs of sepsis and scored using an established sepsis scores: score 1- ambulatory, active, normal coat, normal feces; 2- ambulatory, active, ruffled fur (mild sepsis); 3- ruffled fur, hunched poster, increased respirations (moderate sepsis); 4- ruffled fur, hunched posture, increased respirations, slow and staggering gait (severe

sepsis); and 5- animal on side, minimally responsive, rapid and shallow respirations, gasping (moribund). Mice were sacrificed when they reached a sepsis score of 4.

### *2.2 Mouse Diets and Antibiotic Treatment for high fiber diet studies and gut resistome experiments:*

Customized diets were developed in Tekland. They were varied in fat, microbiota accessible fiber (resistant starch, defined as a high fiber diet HFb), and microbiota non-accessible fiber (corn starch, defined as a low fiber diet LFb). High and low fiber diets contained the equal amount of carbohydrates. Mice were randomly assigned to *ad libitum* feeding on either: standard rodent chow (SD, Envigo), High-Fat, Low-Fiber Diet (HF-LFb, Teklad TD.08811), High-Fat, High-Fiber Diet(HF-HFb, Teklad TD.190230), Low-Fat, Low-Fiber(Teklad TD.120724), Low-Fat, High-Fiber(LF-HFb, Teklad TD.190229) for 6 weeks and weighed weekly. After 6 weeks of their respective diets, mice were subjected to the antibiotics twice per day for 5 days consisting of cefoxitin (30mg/kg i.p., Hikma Pharmaceuticals, Eatontown, NJ) and clindamycin (70 mg/kg p.o.; Clindrops; Hebry Schein, Dublin, OH). Following antibiotic administration, severity of diarrhea was scored from 0 – no diarrhea, 1 – slight perianal erythema and loose stool, 2 – severe perianal erythema and liquid stool. Intestinal length was measured by removing the entire GI tract en bloc from the distal esophagus to the anus and the small intestine, cecum, and colon were measured. All experiments were in accordance with National Institute of Health (NIH) guidelines and approval was obtained from University of Chicago Institutional Animal Care and Use Committee (IACUC protocol 71744).

*Gut resistome mouse experiments:*

Male 6-week old C56BL/6 (Charles River Laboratory) mice were housed within a temperature-controlled, 12- hour light/dark cycled room of the animal facility of the University of Chicago.

Mice were housed in barrier facility constructed to ensure prevention of adventitious infectious agents.

To avoid stress from social isolation, mice were housed at five mice per cage. The weights of all mice were monitored weekly. All experiments were performed in accordance with the National Institutes of Health guidelines, and approval was obtained from the University of Chicago Animal Care and Use Committee (Protocol 71744). Six cages of C56BL/6 mice were randomly assigned to two experimental groups: (1) Standard diet-fed mice (n=14); (2) Western diet-fed mice (n=25). The mice were assigned to ad libitum feeding with either a western diet (WD, 60% kcal fat, 0g fiber; Bio serve, mouse high-fat diet, cat#S3282) or standard diet (SD, 18% kcal fat, 18g fiber; standard mouse chow; Envigo) for 7.5 weeks. During the reversal period, the mice were weighed daily. Afterward, all mice were sacrificed by CO2 inhalation for 5 min. At sacrifice, a laparotomy was performed to remove the cecum within an anaerobic chamber. Approximately 25% of the fresh collected cecal content was analyzed by Biolog Phenotype MicroArray. A small portion of the freshly collected cecal content was put on a pH strip (Sigma-Aldrich - Hydrion® Brilliant pH dipsticks #Z264784). The remaining cecal contents were collected in 10% glycerol at the time of sacrifice and stored at –80°C until use for liquid culture experiments. For additional cecal microbiota and stool microbiota comparison, fresh stool samples were collected right before sacrifice of 5 WD- and 5 SD-fed mice.

***2.3 Mouse peritonitis model***

*This model was performed under the protocol ACUC71744.* Six to eight week old male mice were used in all experiments and co-housed in temperature controlled 12h light/dark cycled rooms at University of Chicago. For infection, mice were inoculated via intraperitoneal injection (IP) with a single dose of *S. marcescens MVI*. *S. marcescens* was grown on MacConkey agar for

32 hrs. and resuspended in 10% glycerol to an OD 0.1 and was further diluted 1:600 to achieve a final concentration of 5x $10^5$CFU/mL. Mice were injected intraperitoneally with 1ml of bacteria and their core body temperature measured hourly during the course of infection. The MSS scoring system (**Table 1**) was used to track the course of infection [227]. Mice were deemed to be "non-surviving" when a drop in their temperature was greater than or equal to 1 degree Celsius at 8 hours post injection. At 15 hours post injection, mice with a total sepsis score greater than 8 and a core body temperature less than 31 degrees Celsius were determined to be non-survivors. FMT treatment was conducted by sacrificing a healthy littermate and resuspending the cecal contents in sterile normal saline for a final concentration of approximately 50mg/mL. Approximately 1ml of FMT was delivered to each mouse via enema at the time of IP pathogen injection as previously described [224]. For macrophage depletion experiments, Clodronate liposomes or control liposomes (FormuMax Scientific Inc, California) were administered intraperitoneally 24 hrs prior to injection with *Serratia marcescens* (SM). Indole mixture i.p treatment was conducted by delivering 100μL of the indole mixture at the time of SM injection. For AhR (aryl hydrocarbon receptor) inhibition, 100μL of 1 mM StemRegennin (Selleck, Houston TX) was administered to each mouse i.p at the time of infection. In order to enrich the gut with indoles, the major ligands for AhR activation, its primary substrate, tryptophan was administered in the drinking water a concentration of 1mM for 14 days prior to injection with bacterial pathogens.

| Score | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| Appearance | Smooth coat | Slightly ruffled | Majority of fur on back is ruffled | Piloerection, puffy appearance |
| Level of consciousness | Active | Active, avoids standing upright | Active only when provoked | Non-responsive |
| Activity | Normal | Suppressed eating, drinking, or running | Stationary, except when provoked | Stationary even when provoked |
| Response to stimulus | Normal | Slowed response to touch | Very delayed response to touch | No response to touch |
| Eyes | Open | Not fully open, potential secretions | Half closed, secretions | Mostly or completely closed |
| Respiration quality | Normal | Periods of labored breathing | Consistently labored | Labored breathing with gasps |

***Table 1:*** *Murine Sepsis Score*

### *2.4 Bacteria used for bacterial peritonitis*

*Serratia marcescens* MVI, a known virulent strain, was isolated from the spleen of septic western diet-fed mouse who was moribund following a surgical stressv[226]. Aliquots of this stored and characterized strain was used in all experiments. For additional experiments to test the role of AhR and indoles on survival from human pathogens, previously characterized and sequenced pathogens obtained from the stool of critically ill patients in the ICU were injected intraperitoneally to induce lethal peritonitis, as previously describedv[82,224]. The human pathogens were a polymicrobial community (referred to as the pathogen community consisting of four microbes: *Serratia marcescens*, *Klebsiella oxytoca*, *Enterocccus faecalis*, and *Candida albicans*).

### 2.5 Biolog Assays to assess antimicrobial resistance:

The Phenotype MicroArray™ panels 11C and 12B (BIOLOG, Hayward, 398 CA, USA) were used to determine the antibiotic-resistance potential by measuring the metabolic activity as a degree of redox reduction due to mitochondrial respiration in the presence of a 4 graded increasing concentration of 48 different antibiotics. Freshly collected cecal content or stool

samples were homogenized in normal saline and afterward filtered using 70 µm DB Falcon Cell\ Strainers. An aliquot of the filtered sample was introduced to 16 ml IF-0a GN/GP base inoculating fluid (BIOLOG, Hayward, 398 CA, USA). The optical density (OD) was adjusted to an OD of 0.025 using the OD 600. Afterward, 7.5 mL of IF-0a bacterial solution was introduced to 100 mL of IF-10b GN/GP base inoculating fluid (BIOLOG, Hayward, 398 CA, USA), 1.2 mL Dye mix D (anaerobic bacteria) or 1.2 mL of Dye mix H (aerobic bacteria) (BIOLOG, Hayward, 398 CA, USA), 600 µL 1 M glucose solution and 10.7 mL sterile demineralized water. Each sample was plated on 96-well Phenotype Microarray Plate 11C and 12B that cover a range of common and clinically relevant antibiotics, as well as other antimicrobial compounds (Biolog Hayward, CA). Anaerobic plates were sealed with a PCR-film within the anaerobic chamber. Afterward, both aerobic and anaerobic plates were incubated for 24 hours at 33°C in the OmniLog incubator/reader (Biolog, Hayward, 398 CA).

***2.6 Shotgun metagenomic sequencing:***

Picogreen (Invitrogen) to quantitate genomic DNA samples. DNA was sheared using a Covaris and libraries were constructed with the Nugen Ovation Ultralow Library protocol. We aimed for an insert size of 400 bp to maximize data.  Amplified libraries were visualized on an Agilent Bioanalyzer DNA1000 chip, pooled at equimolar concentrations based on these results, and size selected using a Sage Blue Pippin 1.5% cassette. The library pool was quantified using a Kapa Biosystems qPCR protocol, then sequenced on the Illumina NextSeq in a 1x150 paired-end sequencing run using dedicated read indexing. The samples were demultiplexed with bcl2fastq.

### *2.7 Metagenomic analysis of cecal microbiota to assess the gut resistome and Shotgun metagenomic sequencing and bioinformatics analysis*:

Picogreen (Invitrogen) was used to quantitate DNA concentration in samples. DNA was sheared using a Covaris apparatus and libraries were constructed with the Nugen Ovation Ultralow Library protocol. We aimed for an insert size of 400 bp to maximize data. Amplified libraries were visualized on an Agilent Bioanalyzer DNA1000 chip, pooled at equimolar concentrations based on these results, and size selected using a Sage Blue Pippin 1.5% cassette. The library pool was quantified using a Kapa Biosystems qPCR protocol, then sequenced on the Illumina NextSeq in a 1x150 paired-end sequencing run using dedicated read indexing. The samples were demultiplexed with bcl2fastq. Post-sequencing quality control, trimming, and filtering were performed using Nesoni (https://github.com/Victorian-Bioinformatics-Consortium/nesoni). Next, we aligned the high-quality reads to the mouse genome using Gencode version GRCm38 using STAR aligner. Reads mapped to the human genome were excluded for further analysis. Non-human reads were processed for taxonomical and functional analysis using MetaPhlan3 [228] and HUMAnN3v[229], respectively. Quality-filtered reads were assembled into contigs using MetaBAT2v[230]. Metagenome contigs were screened for ARG genes using Abricate (https://github.com/tseemann/abricate) with the CARD database [231].

### *2.8 Liquid Culture Analysis of Antibiotic Resistance of Cecal Microbiota*:

The cecal content of 5 WD-fed and 5 SD fed mice was collected and stored in 10%-glycerol at –80 °C until use. The sample was homogenized in normal saline and afterward filtered using 70 µm DB Falcon Cell\ Strainers. The OD of the sample was adjusted to an OD of 0.01 in 5 ml tryptic soy broth (TSB) and 5 mL of Clostridial Broth. 180 µL of the sample was put on a 96 wells plate. 20 µL of the antibiotics was added in a three-fold increasing concentration rate:

Amikacin (10 mg/L), Ofloxacin (1/3/5 mg/L), Neomycin (1/5/10 mg/L), Cefoxitin (1/5/10 mg/L). Prior pilot results determined the concentration of 10 mg/L for amikacin. The anaerobic plates were tightly sealed with PCR-film and kept inside the anaerobic chamber at 37 ℃. Aerobic plates were kept outside the anaerobic chamber and placed in a 37 ℃ incubator. The OD was measured at 0h, 16h and 24h after inoculation. After 24h of liquid culture, the control samples were placed on Columbia Nalidixic Acid Agar (CNA agar) and MacConkey plates containing antibiotics at a concentration determined from the liquid culture results (Amikacin: 1 mg/L, Neomycin, 10 mg/L, Cefoxitin: 10 mg/L, Ofloxacin: 1 mg/L). Growing colonies were isolated and used for species and susceptibility identification.

***2.9 16S rRNA sequencing and sequence data analysis***

Microbial DNA extraction from cecal contents and stool was performed using a Power Fecal DNA isolation kit (Qiagen, Carlsbad, CA). For library preparation, DNA was amplified using the barcoded 12-bp Golay primer set designed for the Earth Microbiome Project (EMP). PCR was performed according to the manufacturer's protocol using the EMP primers, mPNA, AccuStart II PCR ToughMix, and the extracted DNA (Quntabio). After amplification, the PCR products were quantified by using a PicoGreen dsDNA quantitation assay (Invitrogen). The results of the quantification were used to normalize the amount of DNA from the PCR product used for sequencing and ensure that each amplicon was represented evenly during sequencing. Finally, an aliquot of the final pool was taken, and the DNA was purified by using an Agencourt AMPure XP PCR* purification system (Beckman-Coulter). The samples were then run on an Illumina MiSeq at Argonne National Laboratory (150 bp × 2).

Qiime2[232] was utilized for16S rRNA gene sequence analysis and demux emp-paired-end command was used to demultiplex and join the paired-end reads. Quality filtering was performed

using Deblur. Taxonomy and OTUs were assigned using a Greengenes classifier. Sequences were further analyzed utilizing the Phyloseq[233] package within R. Samples were rarefied to a depth of 10,000 reads per sample. For the alpha diversity, the Shannon index was used, and the beta diversity was analyzed using nonmetric multidimensional scaling (NMDS) plots that were generated based on a weighted UniFrac dissimilarity matrix. To determine significantly different OTUs between groups of interest, the DESeq2 [234] package within R was utilized and OTUs were compared between days of dietary pre-habilitation. Significant OTUs were determined by a *P* value (false discovery rate [FDR]) cutoff of <0.05).

PICRUSt [235] analysis was performed to predict microbiota function. The picrust2_out_pipeline function was utilized using a nearest sequence taxon index cutoff of 2. Contributions of samples and individual taxa to enzymes, designated by enzyme commission numbers, and pathways were determined utilizing KEGG designations. Taxon relative functional abundance was determined by the relative abundance of a given taxa multiplied by the number of gene copies present within the given taxa. The taxon relative functional abundance was compared across all significant taxa. In order to focus on butyrate producing pathways, enzymes and pathways specific to butyrate production were selected. STAMP [236] pipeline was used to compare the microbiota function between diets and dietary prehab days using Benjamin Hochberg correction. Taxa and functional shift scores were calculated within FishTaco [237] as previously described to determine taxa contribution to functional pathways. Briefly, the shift scores represent Wilcoxon rank sum statistics. Contribution between taxa attenuating and enhancing individual pathways were displayed for both case and control.

***2.10 Stool qPCR analysis for bacterial load and gut microbiota antibiotic resistance genes:***

Real-time PCRs were carried out in duplicate on an ABI StepOne plus real-time PCR system sequence detector with 2x FastStart SYBR green mix. All qPCR mixtures contained 10ul of 2xFast-Start SYBR green with dye1, 0.5ul of each forward and reverse primer (final concentration, 0.4mM), and 9ul of the DNA (equilibrated 25ng DNA). PCR primers(Integrative DNA Technologies) were utilized for Firmicutes, Bacteroidetes, and universal 16S (Table S1)[238]. Relative abundance as determined by PCR was estimated as:

$2^{CT}_{\text{Universal}} / 2^{CT}_{\text{Species Specific Primer}}$

Bacterial load was determined by using a standard curve of 16S amplification from *E. coli* DNA to allow for estimating bacterial DNA/mg stool.

*Antibiotic resistance gene expression:*

DNA was isolated from approximately 100mg of stool and cecal samples using the DNeasy. Approximately 25ng of DNA was utilized for quantitative PCR using Biorad SYBR green following the manufacturer protocols. Primers were utilized for antibiotic resistance genes of interest CfxA2 (Fwd – GCAAGTGCAGTTTAAGATT, Rev – GCTTTAGTTTGCATTTTCATC) and ErmG (Fwd – GTGAGGTAACTCGTAATAAGCTG, Rev – CCTCTGCCATTAACAGCAATG). Values were normalized to 16S.

***2.11 Cecal microbiota AI-2 detection assay:***

AI-2 was measured as previously described utilizing the AI-2 reporter strain *Vibrio campbelli (*ATCC strain BB170) and *Vibrio campbelli (*ATCC strain BB152*).* This latter strain constitutively produces AI-2 and was used as a positive control[239,240]. Briefly, the cecal contents

were isolated from the mice on their respective diets and resuspended in 0.1M MOPS (pH 7.0). The samples were centrifuged and filtered through a 0.2 micron filter. The filtrates were then vacuum dried and resuspended in water at a concentration of 50% weight/volume. The concentrated filtrate was then added to the AI-2 reporter strain and luminescence was measured overnight. Relative AI-2 production was normalized to the nadir of the negative control.

### *2.12 SCFA and Indole Metabolomic analyses:*

*Gas Chromatography-mass spectrometry utilized for dietary prehabilitation*

SCFA were extracted from mouse feces, utilizing approximately 50mg of mouse feces, using diethyl ether (Fisher Scientific), derivatized using N-tert-Butyldimethylsilyl methyltrifluoroacetamide with 1% tert Butyldimethylchlorosilane (Sigma) and run on an Agilent Single Quad GC–MS(5977A Single Quad and 7890B GC). 4-methylvalonic acid was spiked into each sample as an internal control and used to measure extraction efficiencies. Standard curves were generated utilizing butyric acid, propionic acid, and acetic acids. All values are normalized to sample mass and the amounts of SCFA were calculated based off of generated standard curves for each individual SCFA.

*Metabolite analysis utilized for bacterial peritonitis:*

Upon sacrifice, cecal samples were flash frozen and stored at -80°C before the analysis. Metabolite extraction from cecal samples was performed with 80% methanol spiked with internal standards, at a ratio of 100 mg of material/ml of extraction solvent, in beadruptor tubes (Fisherbrand, cat# 15-340-154). Samples were homogenized at 4°C on a Bead Mill 24 Homogenizer (Fisher; 15-340-163), set at 1.6 m/s with 6 thirty-second cycles, 5 seconds off per

cycle. Samples were then centrifuged at -10°C, 20,000 x g for 15 min and the supernatant was used for subsequent metabolomic analysis.

Metabolite Analysis using GC-nCI-MS and PFBBr Derivatization. The metabolite extract (100 µL) was added to 100 µL of 100 mM borate buffer (pH 10) (Thermo Fisher, 28341), 400 µL of 100 mM pentafluorobenzyl bromide (Millipore Sigma; 90257) in Acetonitrile (Fisher; A955-4), and 400 µL of n-hexane (Acros Organics; 160780010) in a capped mass spec autosampler vial (Microliter; 09-1200). Samples were heated in a thermomixer C (Eppendorf) to 65°C for 1 hour while shaking at 1300 rpm. After cooling to RT, samples were centrifuged at 4°C, 2000 x g for 5 min, allowing phase separation. The hexanes phase (100 µL) (top layer) was transferred to an autosampler vial containing a glass insert and the vial was sealed. Another 100 µL of the hexanes phase was diluted with 900 µL of n-hexane in an autosampler vial. Concentrated and dilute samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in negative chemical ionization mode, using a HP-5MSUI column (30 m x 0.25 mm, 0.25 µm; Agilent Technologies 19091S-433UI), methane as the reagent gas (99.999% pure) and 1 µL split injection (1:10 split ratio). Oven ramp parameters: 1 min hold at 60°C, 25°C per min up to 300°C with a 2.5 min hold at 300°C. Inlet temperature was 280°C and transfer line was 310°C. A 10-point calibration curve was prepared with acetate (100 mM), propionate (25 mM), butyrate (12.5 mM), and succinate (50 mM), with 9 subsequent 2x serial dilutions. Data analysis was performed using MassHunter Quantitative Analysis software (version B.10, Agilent Technologies) and confirmed by comparison to authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

*Indole/Tryptophan analysis*. Tryptophan metabolites were analyzed by LC-MS/MS. The metabolite extract (400 µL) was added to pre-labeled microcentrifuge tubes. Samples were dried down completely using a Genevac EZ-2 Elite. Samples were resuspended in 100 µL of 50:50 Water:Methanol and added to an Eppendorf thermomixer® C at 4°C, 1000 rpm for 15 min to resuspend analytes. Samples were then centrifuged at 4°C, 20,000 x g for 15 min to remove insoluble debris. The supernatant (80 µL) was transferred to a fresh, prelabeled MS vial with inserts or 96 deep-well plate (Agilent 5065-4402). Samples were analyzed on an Agilent 1290 infinity II liquid chromatography system coupled to an Agilent 6470 triple quadrupole mass spectrometer, operating in positive mode, equipped with an Agilent Jet Stream Electrospray Ionization source. Each sample (2 µL) was injected into a Acquity UPLC HSS PFP column, 1.8 µm, 2.1 x 100 mm (Waters; 186005967) equipped with a Acquity UPLC HSS PFP VanGuard Precolumn, 100Å, 1.8 µm, 2.1 mm X 5 mm (Waters; 186005974) at 45 oC. Mobile phase A was 0.35% formic acid in Water and mobile phase B was 0.35% formic acid in 95:5 Acetonitrile:Water. The flow rate was set to 0.5 mL/min starting at 0% B held constant for 3 min, then linearly increased to 50% over 5 min, then linearly increased to 95% B over 1 min, and held at 100% B for the next 3 min. Mobile phase B was then brought back down to 0% over 0.5 min and held at 0% for reequilibration for 2.5 min. The QQQ electrospray conditions were set with capillary voltage at 4 kV, nozzle voltage at 500 V, and Dynamic MRM was used with cycle time of 500 ms. Transitions were monitored in positive mode for 46 analytes (table on next slide). An 11-point calibration curve (ranging from 0.88 nM to 909 µM) was prepared for tryptophan, tyrosine, phenylalanine, serotonin, 5-HIAA, melatonin, tryptamine, kynurenine, kynurenic acid, anthranilic acid, and niacin. Data analysis was performed using MassHunter Quant software (version B.10, Agilent Technologies) and confirmed by comparison with

authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards.

*TMS-MOX Analysis.* Metabolites were analyzed using GC-MS with electron impact ionization. The metabolite extract (100 µL) mass spec autosampler vials (Microliter; 09-1200) and dried down completely under nitrogen stream at 30 L/min (top) 1 L/min (bottom) at 30°C (Biotage SPE Dry 96 Dual; 3579M). To dried samples, 50 µL of freshly prepared 20 mg/mL methoxyamine (Sigma; 226904) in pyridine (Sigma; 270970) was added and incubated in a thermomixer C (Eppendorf) for 90 min at 30°C and 1400 rpm. After samples are cooled to room temperature, 80 µL of derivatizing reagent (BSTFA + 1% TMCS; Sigma; B-023) and 70 µL of ethyl acetate (Sigma; 439169) were added and samples were incubated in a thermomixer at 70°C for 1 hour and 1400 rpm. Samples were cooled to RT and 400 µL of Ethyl Acetate was added to dilute samples. Turbid samples were transferred to microcentrifuge tubes and centrifuged at 4°C, 20,000 x g for 15 min. Supernatants were then added to mass spectrometry vials for GCMS analysis. Samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in electron impact ionization mode, using a HP-5MSUI column (30 m x 0.25 mm, 0.25 µm; Agilent Technologies 19091S-433UI) and 1 µL injection. Oven ramp parameters: 1 min hold at 60°C, 16°C per min up to 300°C with a 7 min hold at 300°C. Inlet temperature was 280°C and transfer line was 300°C. Data analysis was performed using MassHunter Quantitative Analysis software (version B.10, Agilent Technologies) and confirmed by comparison to authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards. Heatmaps were developed utilizing ggplot in R [241].

*Collection of peritoneal samples.* At fifteen hours post injection with *S. marcescens*, mice were sacrificed and grouped as survivors and non-survivors as described above. A small incision was made over the inferior portion of the abdomen and the peritoneum was carefully opened. A sterile sponge was inserted into the peritoneal cavity to absorb peritoneal fluid. The sponge was then placed into a sterile 10cc syringe housed in a 15cc sterile tube which was centrifuged for 15 minutes at 15,000 x g to allow for removal of the concentrated peritoneal exudate from the sponge. The peritoneal exudate was collected in this manner to avoid dilution with peritoneal lavage and allow detection of metabolites. Samples were incubated at -80°C for at least one hour, or up to overnight. Extraction solvent (4 volumes of 100% methanol spiked with internal standards and stored at -80°C) was added to the liquid sample (1 volume) in a microcentrifuge tube. Tubes were then centrifuged at -10°C, 20,000 x g for 15 min and supernatant was used for subsequent metabolomic analysis. Extraction solvent (4 volumes of 100% methanol spiked with internal standards and stored at -80°C) was added to the liquid sample (1 volume) in a microcentrifuge tube. Tubes were then centrifuged at -10°C, 20,000 x g for 15 min and supernatant was used for subsequent metabolomic analysis. Peritoneal exudate metabolites were analyzed using GC-MS with electron impact ionization. The metabolite extract (100 µL) mass spec autosampler vials (Microliter; 09-1200) and dried down completely under nitrogen stream at 30 L/min (top) 1 L/min (bottom) at 30 °C (Biotage SPE Dry 96 Dual; 3579M). To dried samples, 50 µL of freshly prepared 20 mg/mL methoxyamine (Sigma; 226904) in pyridine (Sigma; 270970) was added and incubated in a thermomixer C (Eppendorf) for 90 min at 30°C and 1400 rpm. After samples are cooled to room temperature, 80 µL of derivatizing reagent (BSTFA + 1% TMCS; Sigma; B-023) and 70 µL of ethyl acetate (Sigma; 439169) were added and samples were incubated in a thermomixer at 70°C for 1 hour and 1400 rpm. Samples were

cooled to RT and 400 µL of Ethyl Acetate was added to dilute samples. Turbid samples were transferred to microcentrifuge tubes and centrifuged at 4°C, 20,000 x *g* for 15 min. Supernatants were then added to mass spec vials for GCMS analysis. Samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in electron impact ionization mode, using a HP-5MSUI column (30 m x 0.25 mm, 0.25 µm; Agilent Technologies 19091S-433UI) and 1 µL injection. Oven ramp parameters: 1 min hold at 60°C, 16°C per min up to 300°C with a 7 min hold at 300°C. Inlet temperature was 280°C and transfer line was 300°C. Data analysis was performed using MassHunter Quantitative Analysis software (version B.10, Agilent Technologies) and confirmed by comparison to authentic standards. Normalized peak areas were calculated by dividing raw peak areas of targeted analytes by averaged raw peak areas of internal standards. Given the lack of concentrated peritoneal exudate in uninfected mice, a relative amount of metabolites were calculated by dividing the survival values by the average of the non-survivors.

### *2.13 Serum metabolite analysis of septic patients*

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Federal Research and Clinical Center of Intensive Care Medicine and Rehabilitology, Moscow, RF (Protocol code № 5/21/2 from 23 December 2021). Healthy controls were taken from healthy donors, n = 48: these were 19 women and 29 men aged 20 to 67 years. The donors showed no signs of an acute cold or inflammation. Cases of chronic liver, kidney, etc. diseases were excluded.  Patient's samples. Blood serum samples (n=26) were taken from 11 patients admitted in January-February 2024 to the ICU specializing in the treatment of acute pancreatitis and other severe abdominal infections at risk of sepsis. All patients included in

the study had PCT levels above the reference PCT value (0-0.05 μg/L), which confirms the presence of bacterial inflammation. All blood samples were collected from a peripheral vein into anticoagulant-free test tubes. Serum samples were obtained via blood centrifugation at 1500× g for 10 min on the same day. Serum aliquots were poured into disposable Eppendorf tubes, frozen, and stored at −80 °C. UPLC-MS/MS analysis was carried out in the Labs Bioanalytical Laboratory, as previously described [242].

### *2.14 Peritoneal macrophage isolation and RNA sequencing*

Peritoneal macrophages were isolated utilizing the macrophage isolation kit (Miltenyi Biotec, 130-110-434). Briefly, mice peritoneal cavity was flushed with ice cold 5cc of macrophage buffer. Macrophages were then stained with antibodies from the peritoneal macrophage isolation kit following the manufacturer's protocol. Once macrophages were isolated via bead isolation, RNA was extracted using the Qiagen RNeasy extraction kit. RNA sequencing was conducted by igenbio. Library preparation was conducted using the NEBNext Ultra II with poly-A selection kit according to manufacturer's protocol. QC was performed on the libraries and they were in turn sequenced on the Illumina NovaSeq S4 machine yielding 38M to 138M 2x 150 bp paired end reads per sample. Quality analysis was performed with ERGOs Read QC workflow with mean base sequences with a PHRED score > 35. Transcript abundances were obtained using Kallisto (v 0.46.1) which utilizes pseudo-alignment to determine the compatibility of read with target mRNA. The reference assembly and annotation of Mus musculus version GRCm39 was used for quantification. The abundances were imported into R using tximport (v 1.14.2) and counts were obtained using DESeqDAtaSetFromTxImport from DESeq2 (v1.26.0). PCA analysis was conducted using plot PCA function of DESeq2. Significantly differentially

expressed genes, log2 > 1.5 or < -1.5 and FDR < 0.05, were obtained utilized DeSeq2 comparing experimental groups to uninfected mice. Gene set enrichment analysis was performed utilizing DEGs using g:Profiler. DEGs were then uploaded into Ingenuity Pathway Analysis for further comparisons of the group. Raw RNA sequences uploaded to NCBI SRA (Reference number: SUB11571682)

### *2.15 AhR activation assay using hepatoma luciferase reporter cell lines*

Mouse hepatoma AhR luciferase cells (H1L1.1c2 cells) were obtained from Michael Denison PhD from UC-Davis [243]. As previously described, Mouse hepatoma cells were grown in alpha MEM media. For Luciferase assay, the cells were split into 96 well plates and seeded at 75,000 cells per well and allowed to grow overnight. The cells were then exposed to the metabolite of interest. After four hours of exposure to the metabolite, the hepatoma cells were washed and lysed utilizing the Promega luciferase assay kit. Luminescence was measured using SpectraMax i3x (Molecular devices), and AhR activity was determined based on relative luminescence to DMSO treated cells. For experiments studying the suppressive effects of *S. marcescens*, *S. marcesens* was grown in aMEM media at 37ºC on a rotary shaker, and the optical density was measured over the course of 32 hours to determine the growth curve. Supernatant from early and late log phase of the growth was isolated by centrifuging to pellet bacterial cells and the supernatant was filtered using a 0.2 micron filter. Further fractionation of the filtered supernatant was performed using 3kD molecular weight filters (Centrifugal filter units, Millipore) that allowed for separation of a <3kD and > 3kD fractions. Both fractions were restored to original volume using sterile aMEM cell culture media. The filtered with 0.22 um

filter fractions was then placed on the AhR cell line with or without the indole mixture to determine their impact on AhR signaling.

### *2.16 Bone marrow derived macrophages (BMDM)*

Bone marrow was isolated from WT mice as previously described [244]. Macrophages were derived utilizing L-conditioned media over 7 days of incubation. Following conditioning, macrophages were exposed to the experimental conditions at the same time as *Serratia marcescens* lysate. Briefly, *S. marcescens* was grown over night on MacConkey agar and diluted in liquid RPMI media to an MOI of 5. *S. marcescens* was then lysed utilizing the mBio tissue homogenizer. Supernatant was added to the BMDM cultures with or without experimental conditions including an indole mixture at a concentration of 0.01 mM and/or AhR inhibitor. Following incubation for six hours, supernatant was collected for protein expression and cells were lysed. RNA was extracted utilizing RNeasy extraction kits following the manufacturer protocol. Furthermore, cDNA was reverse transcribed from RNA utilizing the Biorad reverse transcription kit. The cDNA was then utilized for RT-PCR utilizing the primers of interests and fold change was calculated utilizing the delta-delta CT method.

*Phagocytic activity (Gentamicin protection assay*). Gentamicin protection assays were performed after deriving macrophages as previously described [245]. The BMDMs were exposed to *Serratia marcescens* at an MOI of 5. After exposure to *Serratia marcescens*, the cell plates were spun for 30 minutes at 900xG and underwent an additional 30 minutes of incubation at 37°C. Following completion of the incubation, the cells were incubated for an hour with 100uG/mL of gentamicin. The macrophage supernatant was replaced with 25uG/mL gentamicin

and incubated for a total of 4 hours to eliminate extracellular bacteria. Macrophages were lysed at 0, 2, and 4 hours and lysates were plated on MacConkey agar to assess for intracellular *Serratia marcescens.*

### *2.17 Bacterial* inhibition of Aryl hydrocarbon receptor

*S. marcescens* was grown in liquid MacConkey media at 37°C and the optical density was monitored over 24 hours to determine the growth curve (Supplemental figure XA). Once the growth curve was determined, supernatant was collected at the early log, mid log, and late log phase. The supernatant was filtered with a 0.2 micron filter. The supernatant was combined with the indole mix and added to the AhR reporter cell line and the AhR signaling was measured as described above.

### *2.18 Molecular docking analysis*

A molecular docking analysis was conducted to determine the degree of AhR binding between the AhR and small molecules produced by the microorganisms used in this model (*S. marcescens*, *K. oxytoca*, *E. faecalis*, *C. albicans*) as previously described [246–248]. The crystal structure of AhR was downloaded from PDB database. Then the organics, solvents, and ions were removed by PyMOL software, and hydrogens were removed by AutoDockTools-1.5.7 software. Finally, the AhR crystal structure was saved in 'pdbqt' by AutoDockTools-1.5.7 software. The 2D structures of enterobactin, prodigiosin, and melanin were obtained from PubChem (https://pubchem.ncbi.nlm.nih. gov/), which were then imported into ChemBio3D 14.0 software to minimize free energy and obtain 3D structures. These structures were further entered into AutoDockTools-1.5.7 and transferred into "pdbqt" format. Finally, AutoDock Vina software was used for molecular docking. The 3D and 2D results with the highest negative binding of free energy (kcal/mol) were visualized by PyMOL and LigPlot+ software. Potential candidates for

AhR suppression were chosen based on the docking score in the range of AhR antagonist Stemmreginin.

# 3. RESULTS

## *3.1 Dietary prehabilitation decreases postoperative sepsis in mice fed a western diet*[*]

### *3.1.1 Abstract:*

Here we determined whether preoperative dietary pre-habilitation with a low-fat, high-fiber diet reverses the impact of chronically consumed western diet (WD) examining two endpoints: the composition and function of the intestinal microbiota and postoperative survival.

The majority of surgical patients consume a WD and most (>50% are overweight- i.e. BMI >30). We have previously demonstrated that WD fed mice subjected to an otherwise recoverable surgical injury (30% hepatectomy), administered antibiotics, and undergoing a short period of starvation similar to surgical patients, develop reduced survival (29%) compared to mice fed a low-fat, high-fiber standard chow (SD) (100%). In this study, mice were subjected to 6 weeks of a WD and underwent dietary pre-habilitation with SD for 3-7 days prior to exposure to antibiotics, starvation, and surgery. 16S rRNA gene sequencing was utilized to determine microbiota composition. Mass spectrometry measured short chain fatty acids and functional prediction from 16S gene amplicons were utilized to determine microbiota function. As soon as at 24 hours, dietary pre-habilitation of WD mice resulted in restoration of bacterial composition of the stool microbiota, transitioning from Firmicutes dominant to Bacteroidetes dominant. However, during this early pre-habilitation, stool butyrate per microbial biomass remained low and postoperative mortality remained unchanged from WD. Microbiota function demonstrated

[*] This section has been reproduced and adapted from: Keskey, R., Papazian, E., Lam, A., Toni, T., Hyoju, S., Thewissen, R., ... & Alverdy, J. C. (2022). Defining microbiome readiness for surgery: dietary prehabilitation and stool biomarkers as predictive tools to improve outcome. *Annals of surgery*, *276*(5), e361-e369.

reduced butyrate contributing taxa as potentially responsible for failed recovery. However, after 7 days of prehabilitation (7DP), there was greater restoration of butyrate producing taxa and survival improved (29% vs 79% vs 100%: 7DP vs WD vs SD, p <0.001). The deleterious effects of WD on the gut microbiota can be restored after 7 days of dietary pre-habilitation. Moreover, stool markers may define the "readiness" of the microbiome to withstand the physiologic perturbations inherent in the process of surgery and may be predictive of enhanced survival.

### *3.1.2 Introduction:*

Despite bundled care and meticulous attention to asepsis, postoperative infection rates remain at unacceptable levels and are among the most costly complications [249,250]. Following elective surgery, evidence suggest that the pathogens causing postoperative infection originate from the gastrointestinal tract microbiota [114,226,251]. When operating within the abdomen, although current regimens of antibiotics that empirically target the gut microbiota have reduced postoperative infection rates to an all-time low[252,253], this approach carries the unwanted consequences of also eliminating many of the health-promoting microbiota that may aid in postoperative recovery[254]. The response of the microbiota to antibiotics is in part determined by diet [255] as the variability of the gut microbiome at the individual level is closely correlated to diet [256]. However, diet and its consequences on the gut microbiota over the course of surgical intervention has received little attention [257]. Although preoperative dietary interventions such as the use of immunonutrition and synbiotics have been proposed, their benefit in elective surgery remains unclear [258–260]. The extent to which diet can be manipulated to maintain the resilience of the microbiota to antibiotic exposure, starvation, and surgical injury is unknown.

The consumption of highly processed low fiber, high fat foods [261], has been demonstrated to have major implications on the health-promoting effects of the normal microbiota. Processed

diets result in a decrease in microbial populations involved in the production of short chain fatty acids (SCFAs)[163,262,263], key metabolites that drive a recovery-directed immune response following surgical injury [264]. As a result of adherence to a western diet, as many as 30% of surgical patients undergoing major surgery can be considered obese [265] which in turn is a major risk factor for the development of septic complications [266]. Therefore, a more complete understanding of how diet alters the microbiome in the context of outcome from surgical injury may inform novel approaches to reduce morbidity and mortality. We recently demonstrated that WD fed mice become sensitive to the overall process of surgery that includes **a**ntibiotics, pre-operative **s**tarvation, and a sterile injury (30% **h**epatectomy) (ASH) [226]. WD- fed mice subjected to ASH displayed high mortality rates associated with the presence of virulent and resistant pathogens originating from the gut microbiota [226]. Importantly, when mice were fed a plant-based, low-fat, high-fiber standard chow diet (SD) under the conditions of ASH, they recovered uneventfully and did not develop infection-related mortality[267]. Similarly, we previously demonstrated that WD-induced alterations in the gut microbiota adversely affected anastomotic healing allowing collagenase producing microbiota *to bloom* [268]. Remarkably, this effect could be reversed by short term dietary pre-habilitation that involved the feeding with SD to mice just prior to surgery[12]. Taken together, these studies suggest that the effects of a WD can be reversed by short-term dietary pre-habilitation and may mitigate the collateral damage on the microbiota that invariably occurs when surgical patients are subjected to short term starvation, antibiotics and surgical injury.

In the present study, we hypothesized that short-term **d**ietary **p**re**h**abilitation (DietPreHab) prior to a major surgical stress (i.e 30% hepatectomy) can improve post-operative outcome in mice chronically fed a WD. The aims of this study were to determine the time-

dependence of the DietPreHab to alter surgical outcomes in mice, its associated changes in the microbiome and to identify potential stool-based microbiota-related markers that can predict readiness for surgery when mice are subjected to antibiotics, short-term starvation, and a sterile surgical injury.

### *3.1.3 Results*

*DietPreHab attenuates WD associated weight changes and improves post-surgical outcome*

Prior studies from our lab have demonstrated that mice fed a WD subjected to antibiotic exposure and surgical injury (hepatectomy) developed gut derived sepsis; whereas, mice on a standard chow diet (SD) recovered under the same conditions [226]. In attempts to reverse the postoperative mortality seen after WD feeding, WD fed mice were transitioned to 3 or 7 days of DietPreHab (3DP versus 7DP) (**Figure 1A**). Following DietPreHab, the mice were administered 5 days of antibiotics, underwent 12 hours of starvation, and a 30% hepatectomy (ASH). DietPreHab led to a reduction in weight gain in WD-fed mice with the greatest attenuation of weight gain noted in the 7DP group (49.17 vs 46.7 vs 44.3 vs 16.0%: WD vs 3DR vs 7DR vs SD, $p < 0.01$) (**Figure 1B,C**). The exposure to 5 days of antibiotics led to 15% weight loss with a non-significant attenuation of antibiotic induced weight loss in the SD and 7DP mice (**Figure 1D).** Survival after exposure to the ASH was dependent on diet, and dramatically improved with 7DP (29% vs 38% vs 79% vs 100%: WD vs 3DR vs 7DR vs SD, **Figure 1E, $P < 0.05$**). Standard culture techniques of the blood, liver, and spleen demonstrated no growth among moribund mice. However, *Serratia marcescens*, a pathogen previously identified to be selected for by ASH treatment in this model, was detected in the spleen using DNA sequencing and varied among treatment groups. Levels of *S. marcescens* DNA were decreased in DietPreHab survivors, but not

in moribund DietPreHab and WD mice (**Figure 1F),** although these comparisons were not statistically significant.

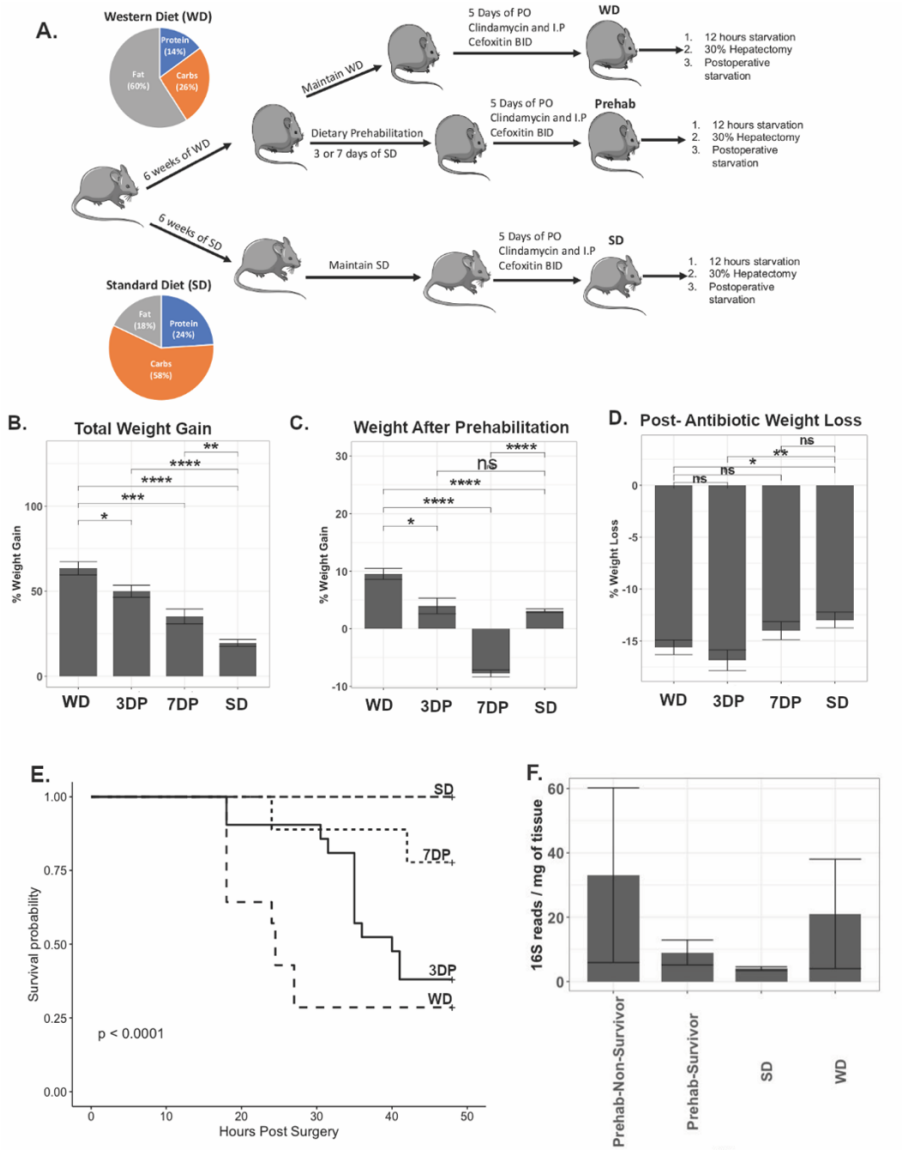



**Figure 1:Short course of preoperative dietary prehabilitation (DietPreHab) results in improved response to antibiotic exposure, starvation, and surgical injury.** The experimental design **(A)** demonstrates how the mice are subjected to their dietary intervention along with the diet composition of SD and WD represented within the pie chart. The total percent weight gain at the end of the dietary interventions **(B),**after prehabilitation **(C)**,and the amount of weight loss following 5 days of antibiotic exposure (**D)** are displayed. The survival curves **(E)** demonstrate the survival differences. The amount *S. marcescens* bacteria DNA detected by PCR in the spleen **(F).** WD (n = 14), SD (n = 14), 3DP (n = 21), and 7DP (n = 9) * p< 0.05, ** p< 0.01, *** p< 0.001, **** p <0.0001

*DietPreHab causes significant compositional alterations in the gut microbiota.*

The preoperative stool microbiota was assessed to determine whether a non-invasive biomarker could be used to define the adequacy of DietPreHab to reverse the impact of WD. After DietPreHab, there was a significant reduction in alpha diversity in both 3DP and 7DP (**Figure 2A**). Beta diversity based on weighted unifrac demonstrated a distinct clustering of SD separate from WD, 7DP, and 3DP (**Figure 2B**). When assessed at the phyla level, the stool microbiota of both 3DP and 7DP was similar to that of SD (**Figure 2C**). There was a significant increase in the abundance of Bacteroidetes and a significant reduction in Proteobacteria and Firmicutes after both 3 and 7 days of pre-habilitation (i.e 3DP, 7DP) (**Figure 2D-F**).
The functional sequelae of DietPreHab on the microbiota were investigated by measuring the fecal levels of SCFAs (butyrate, propionate, and acetate) using GC-MS. SCFA levels were measured in the cecum of WD and SD fed mice as the cecum harbors the densest biomass of bacteria in the gut. WD resulted in a significant reduction in butyrate and acetate within the cecum (**Figure 2G-I**).

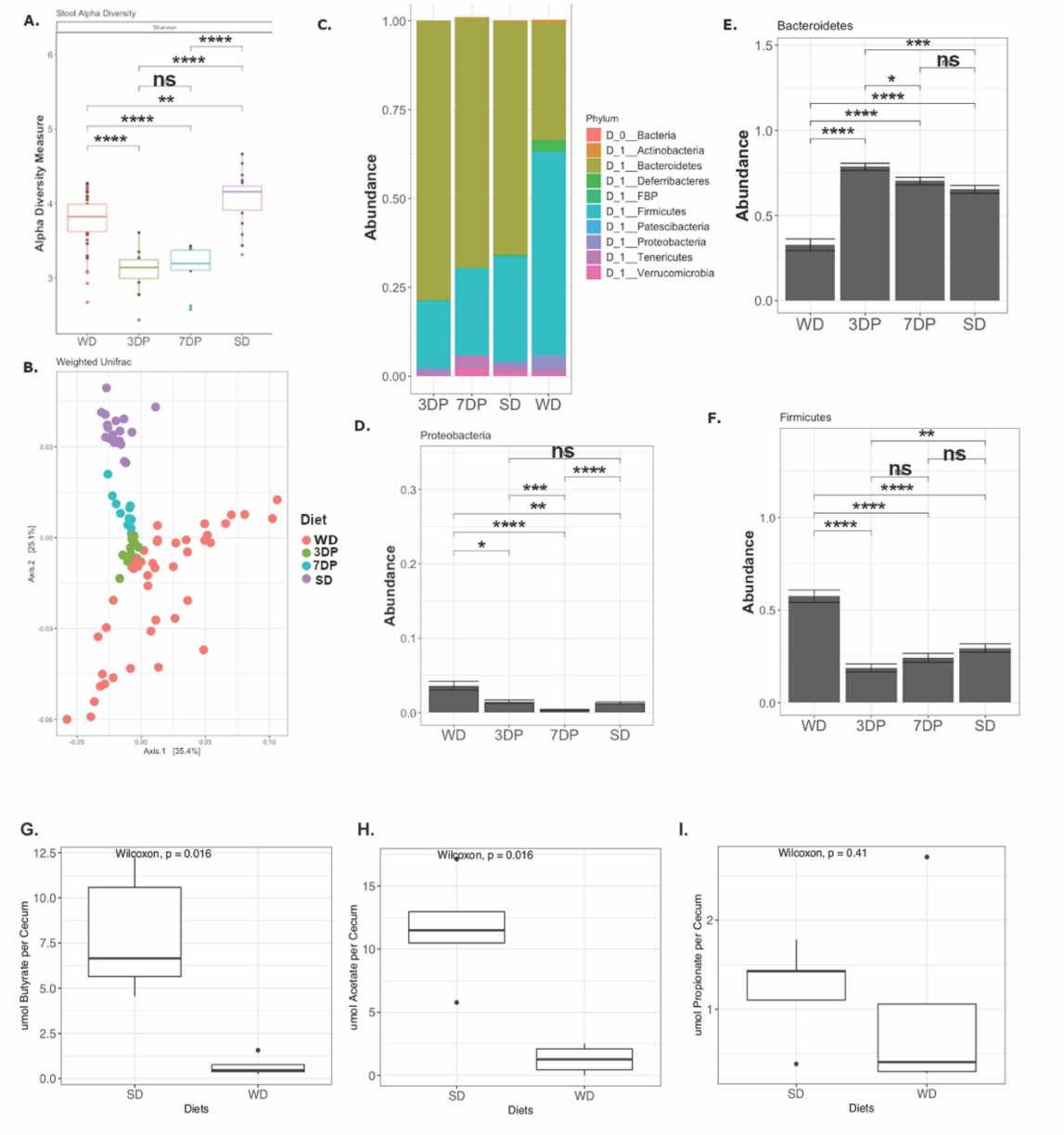



**Figure 2: Dietary prehabilitation results in restoration of SD stool microbiota composition after 3 days of prehabilitation** 16S rRNA sequencing was performed on stool microbiota for WD (n = 44), SD (n = 20), 3DP (n = 11), and 7DP (n = 10) mice prior to antibiotic exposure. (**A.)**The Shannon alpha diversity is reduced after dietary prehabilitation, and (**B.)** the beta diversity as measured by weighted unifrac demonstrates distinct clustering of the WD, SD, and prehabilitation (3DP and 7DP) stool microbiota**.** The composition of the stool microbiota at the phyla level demonstrates similarities between SD, 7DP, and 3DP (C). (D) The abundance of Proteobacteria, Bacteroidetes (E), and Firmicutes (F) were compared across SD, WD, 3DP, and 7DP. GC-MS was performed on total cecal content for SD (n=5) and WD (n =5 ) to measure total amount of butyrate (G), acetate (H), and propionate (I). in the cecum * p< 0.05, ** p< 0.01, *** p< 0.001, **** p <0.0001

When stool SCFAs were assessed as nmol per mg of expelled stool, there was a similar trend as seen in the cecum with SD fed mice having higher levels of butyrate and acetate as compared to WD fed mice (**Figure 3A-C**). Interestingly, butyrate levels dropped and remained low in 3DP mice. In contrast, butyrate levels after 7 days of pre-habilitation trended toward levels observed with a SD, not statistically significant.

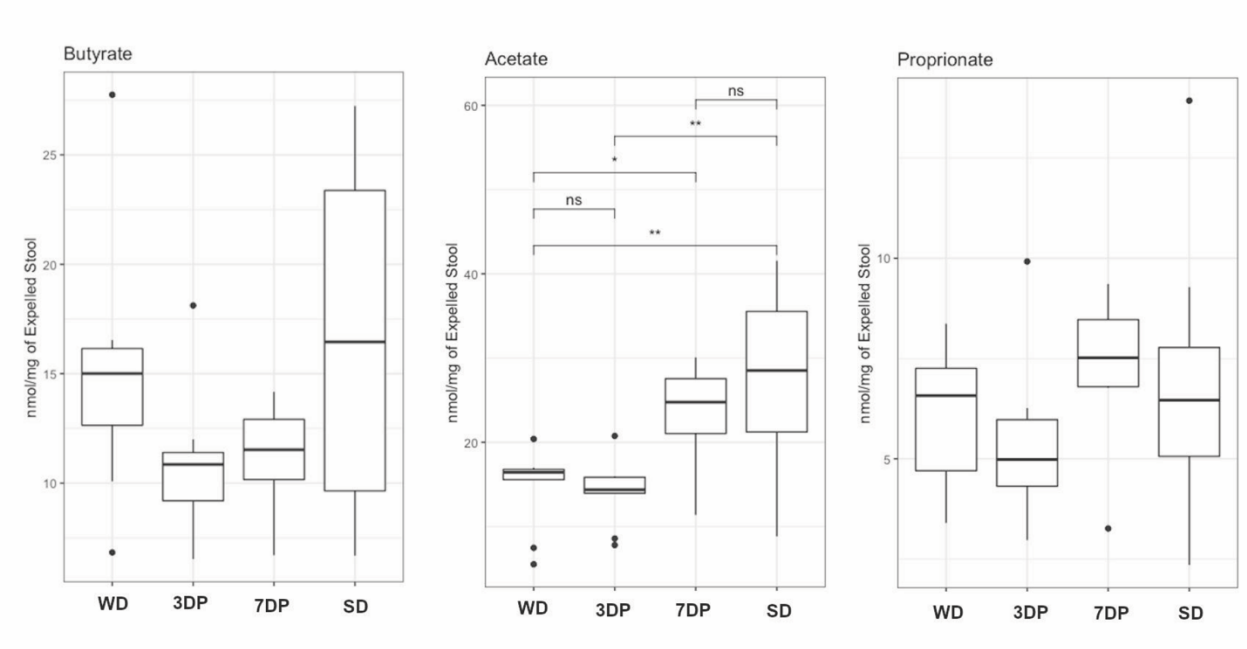



***Figure 3: Stool SCFAs are altered during DietPreHab.*** Stool butyrate levels are decreased in 3DP (n = 5) and 7DP (n = 5) compared to both SD (n = 5) and WD (n=5). Stool acetate levels are reduced in WD and 3DP mice compared to 7DP and SD mice. Propionate levels are similar across all groups.

*Rapid bloom of Bacteroidetes by DietPreHab fails to improve postoperative survival*

We have seen a transition from a Firmicutes dominance in WD to a Bacteroidetes dominance seen in SD, 3DP, and 7DP. To determine if DietPreHab induced alterations in the

abundance of Bacteroidetes and Firmicutes was sufficient to alter outcome, mice began the regimen of DietPreHab and stool levels of Bacteroidetes and Firmicutes were measured daily by qPCR (**Figure 4A**). Once the stool microbiota composition switched from Firmicutes to Bacteroidetes dominance, mice received antibiotics, short-term (overnight) starvation and underwent a 30% hepatectomy (ASH), referred to as the prediction group (**Figure 4A**). Prior to starting DietPreHab, all mice displayed a Firmicutes dominance microbiome over Bacteroidetes with an average Firmicutes abundance of 65.2% and Bacteroidetes abundance of 31.9%. After 24 hours of pre-habilitation, 75% of the prediction group mice had a reversal to Bacteroidetes dominance and all mice had a reversal to Bacteroidetes dominance after 48 hours. However, the prediction group had outcomes similar to WD indicating that single time measurements of Bacteroidetes and Firmicutes was not sufficient to improved survival from ASH (**Figure 4B**). In order to clarify the changes in the microbiota over the course of DietPreHab, alterations in Bacteroidetes and Firmicutes were measured daily during DietPreHab. There was a significant spike in levels of Bacteroidetes (Kruskal-Wallis, $p < 0.001$) and a drop in Firmicutes (Kruskal-Wallis, $p < 0.001$) after 24-48 hours of DietPreHab, followed by a gradual decrease of Bacteroidetes and increase of Firmicutes reaching stable levels after 1 week (**Figure 4C**). In addition to the spike in abundance of Bacteroidetes at the early DietPreHab period, there was an increase in bacterial load (ng bacterial DNA/mg of stool, Kruskall-wallis $p = 0.07$) in the first two days that then decreased reaching a stable level between 4 and 7 days of DietPreHab (**Figure 4D**).

Given the failure of fecal composition alone to predict outcomes, we focused on fecal SCFAs levels during DietPreHab. Over the course of DietPreHab, there was an initial reduction in fecal butyrate and acetate followed by a steady increase; however, these levels were never

completely restored to the levels of SD fed mice. The initial reduction in stool SCFA may represent an increase in utilization by the host or other bacteria after a deprivation of SCFAs associated with WD (**Figure 4E**), suggesting that spot concentrations of SCFA in feces may not be an adequate indication of the amount of SCFA present. To better understand the relationship between bacterial alterations and their contribution to butyrate levels, a relative butyrate index was developed. The relative butyrate index (RBI) was calculated by normalizing the butyrate levels to the bacterial load. When the RBI was calculated, a significant decrease in RBI on day 2 and 3 ($p < 0.05$) was observed with DietPreHab followed by an increase at day 7. Taken together, these data indicate that neither stool microbiota compositional changes ***alone*** nor SCFA concentrations ***alone*** are sufficient to predict survival. Rather, the functional output of the microbiota relative to the bacterial biomass present may provide a more accurate index for tracking how the microbiota respond to dietary manipulation.

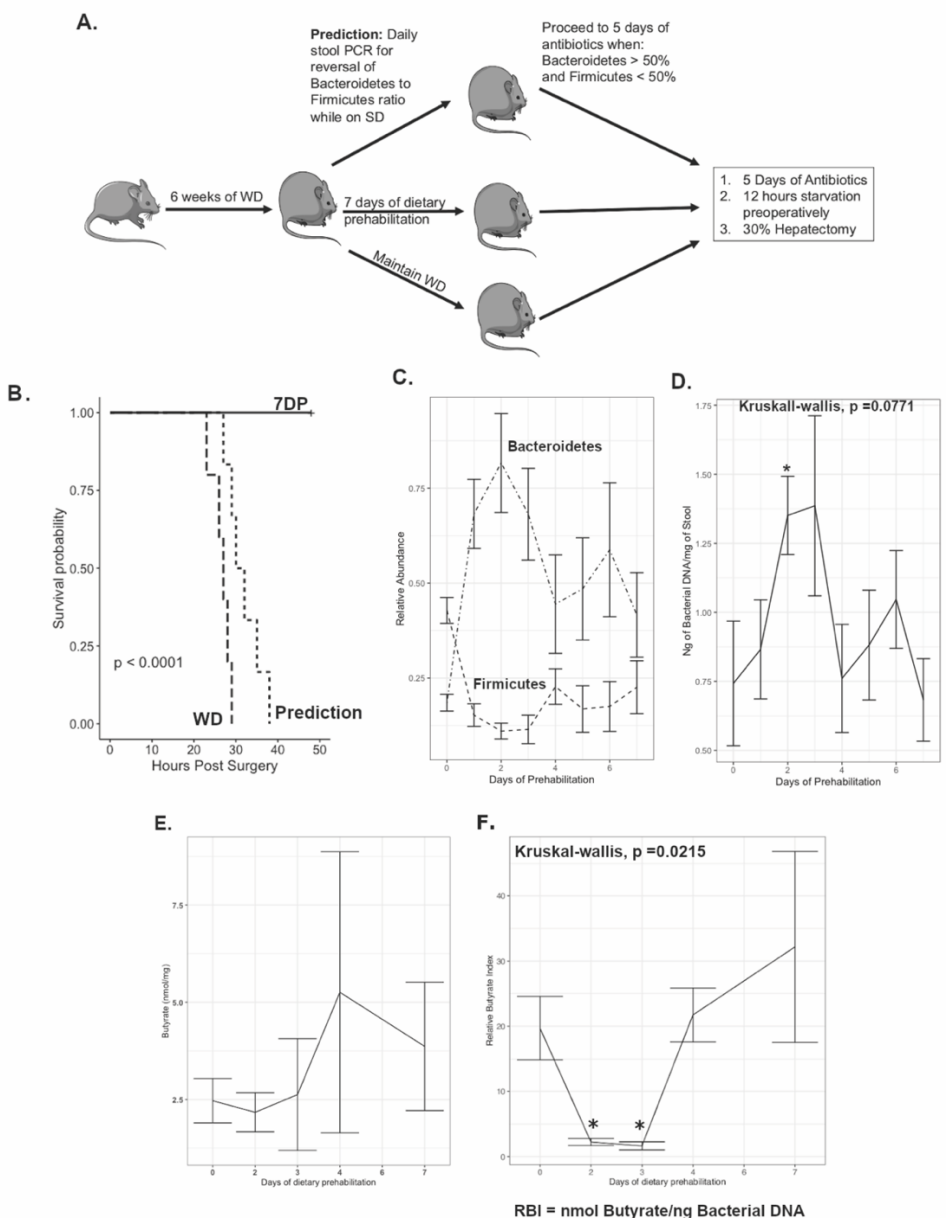



**Figure 4:Microbiota compositional changes are not sufficient to predict survival during dietary prehabilitation** (**A.**) The experimental design outlining the prediction group which underwent daily PCR of the stool to determine when the mice would proceed to antibiotics, starvation and surgery. The survival differences between 7DP (n = 5), Prediction (n = 8), and WD (n = 5) are outlined in **B**. The abundance of Bacteroidetes and Firmicutes as measured by PCR during the course of dietary pre-habilitation (**D**, $p < 0.001$ Kruskal-Wallis) and bacterial load within the stool (**E**, Kruskal-Wallis $p = 0.07$, wilcox pairwise comparison $p < 0.05$ on day 2 vs day 0 and day 1). The stool butyrate levels **(F)** and relative butyrate index(RBI) which is the butyrate normalized to the bacterial load **(G)** demonstrate the alteration during DietPreHab (Kruskal-wallis $p < 0.05$, pairwise day 0 compared to day 2 ($p = 0.06$) and day 3 ($p = 0.01$))
*WD and SD mice display distinct butyrate producing taxa*

To better understand the functional changes occurring within the stool microbiota, a in silico profile of WD and SD fecal microbiota were predicted from 16S rRNA gene amplicon sequencing using PICRUSt[235]. This allows for the determination of baseline functional changes prior to DietPreHab. WD and SD stool microbiota had distinct functional profiles (**Figure 5A**) with over 200 significantly altered pathways. When assessed from a compositional standpoint, there were approximately 600 unique taxa that were significantly, differentially abundant between SD and WD fed mice. These taxa were predominantly of the order *Bacteroidales* in SD fed mice and *Clostridiales* in WD fed mice. Using FishTaco [237], individual taxa contributions to the microbiota functional profile were determined (**Figure 5C**). When specifically focusing on butyrate metabolism, there was a significant difference in the taxa associated with butyrate production in WD and SD stool microbiota (**Figure 5B**). When comparing the taxa contributing to butyrate production, SD mice tended to have more taxa from the order *Bacteroidales* compared to WD fed mice having taxa from the order *Clostridiales*. However, when the abundance of butyrate producing enzymes were assessed across the entire stool microbiota, there were no major differences in abundance of butyrate pathways between WD and SD stool microbiota suggesting that the reduced butyrate production measured in the cecum was likely a consequence of elimination of dietary fiber rather than complete elimination of butyrate producers (**Figure 6).**

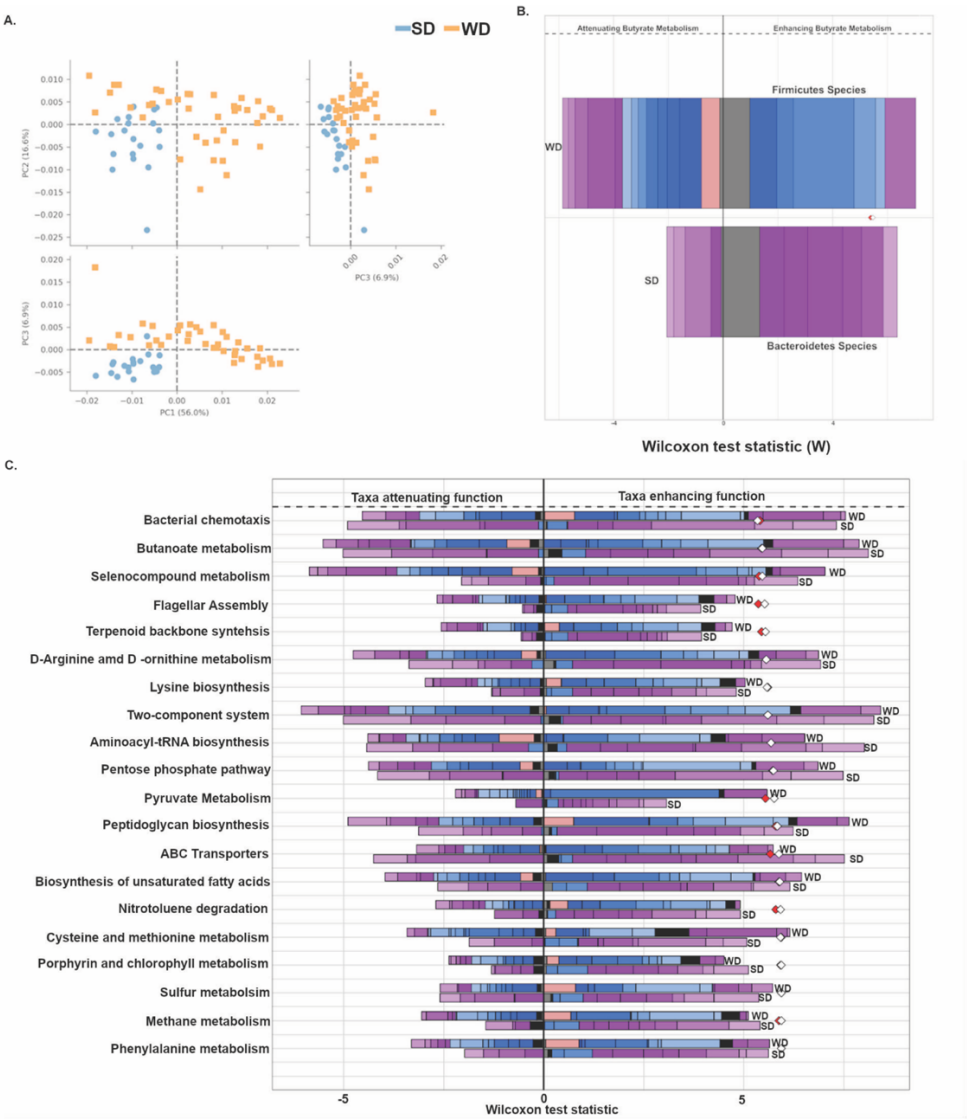



**Figure 5: WD and SD stool microbiota have distinct functional profiles** The WD and SD microbiota profiles demonstrate clustering by diet after analysis with STAMP **(A)**. FishTaco was used to assess the contributions of differentially abundant taxa to significantly altered pathways between SD and WD . The Wilcoxon rank sum score represents the degree of alterations contributed by the taxa for a given pathway. The top bar represents WD taxa contributing to the pathway and the bottom bar represents SD taxa contributing to the pathway. The bars to the left of center represent taxa that attenuate the pathway and bars to the right of center represent taxa that enhance the pathway. The different colors on the bar represent different taxa. Comparison of butyrate metabolism between WD and SD (B) demonstrates significantly different taxa contributing to butyrate metabolism between SD (Bacteroidetes – Pink/Purple) and WD (Firmicutes - Blue). FishTaco analysis of 16S rRNA amplicons for SD and WD mice to determine functional profile of the microbiota. The top 20 most significant pathways are displayed on the y axis. Each color represents different taxa that either attenuate or enhance the referenced pathway. Top bar represents WD and bottom bar represents SD (C).

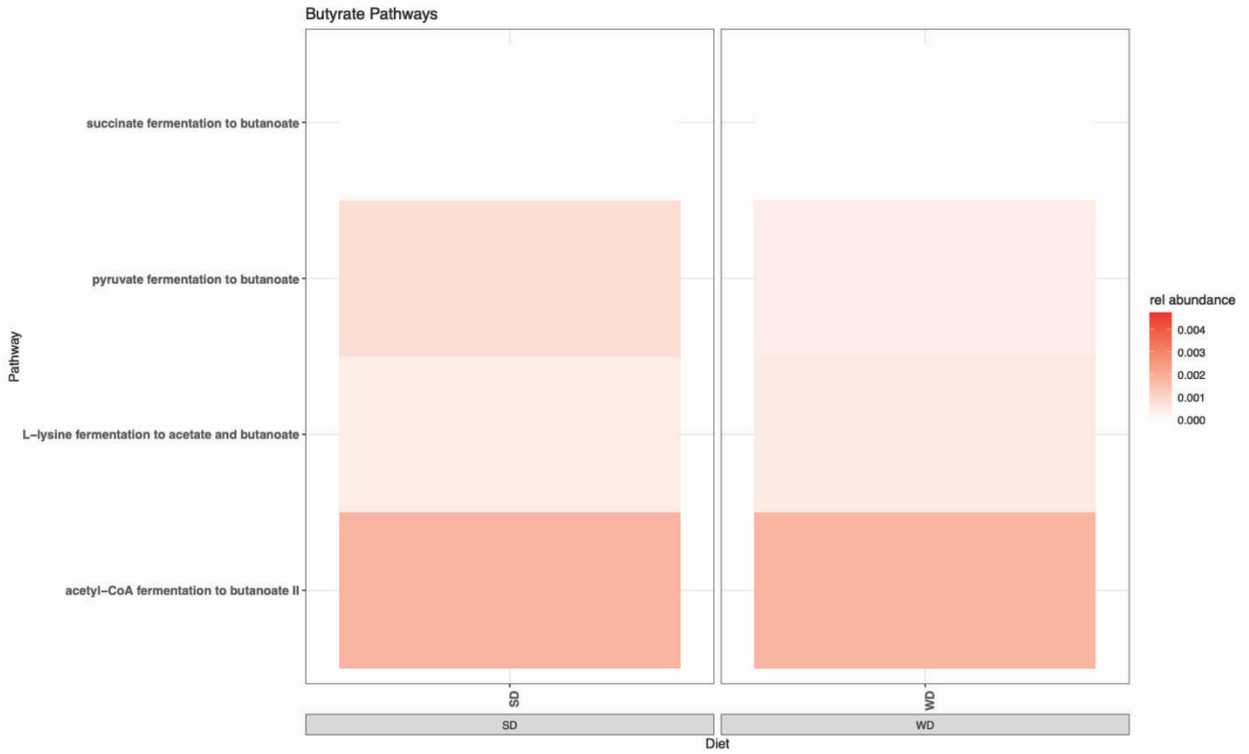



***Figure 6: Relative abundance of butyrate pathway between SD and WD mice*** PICRUSt analysis was performed on 16S rRNA amplicons collected from the stool of SD and WD fed mice and the relative abundance of pathways that contribute to butyrate synthesis was compared between the groups.

*DietPreHab results in early reduction of butyrate contributing taxa followed by its rapid restoration*

Given the significant alteration in the functional profile of WD and SD stool microbiota, the functional alterations during DietPreHab were determined using PICRUSt. The alteration seen in the RBI during DietPreHab and its correlation with survival led to a focus on butyrate production which can be produced by four general pathways from glucose, succinate, lactate, and acetate [269] (**Figure 7A**). Traditionally butyrate producing taxa are quantified by the terminal enzymes butyrate kinase ($buk_{enz}$) and butyrate:acetyl-coA transferase ($but_{enz}$) [270]. In order to determine how the alterations in the stool microbiota during DietPreHab contributed to butyrate production, the predicted presence of butyrate related enzymes within the significantly altered

taxa during DietPreHab were analyzed to determine their contribution to butyrate production over the course of DietPreHab.

When focusing on the terminal enzymes $buk_{enz}$ and $but_{enz}$, there is an initial reduction in $buk_{enz}$ and $but_{enz}$ containing taxa during early DietPreHab that is restored by day 7 (**Figure 7B,C**).

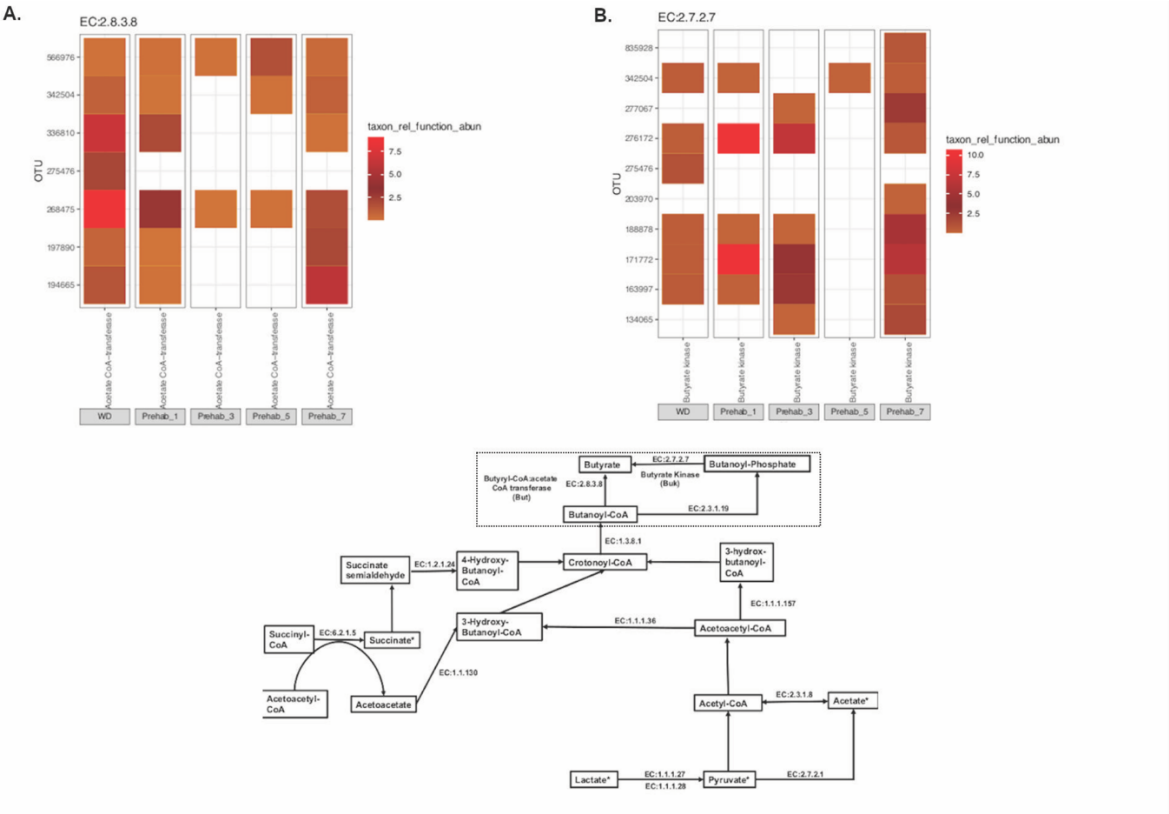



***Figure 7: Butyrate contributing taxa are initially reduced during DietPreHab and restored at day 7*** When assessing the contributions of butyrate to butyrate metabolism, individual enzymes that have been shown to contribute to butyrate metabolism were queried amongst all significantly differentially abundant taxa during DietPreHab as determined by DESeq ($p < 0.05$, FDR). Each individual enzyme corresponds to KEGG enzyme call number and can be related to butyrate prediction as indicated by the pathway in **A** (modified from KEGG pathway 00650). The heatmaps for each individual enzyme represent the relative taxon contribution abundance to a given enzyme. The end enzymes But (EC:2.8.2.8) and Buk (EC:2.7.2.7) are demonstrated in **B** and **C** respectively.

This pattern of initial reduction and then restoration at the end of DietPrehab was consistent for the majority of butyrate related taxa (**Figure 8A-F**). The new taxa present in 7DP containing the enzyme $buk_{enz}$ included 4 additional members of the order *Clostridiales*. Butyrate producing bacteria were initially decreased during DietPreHab and then were replaced by a new community of butyrate producing bacteria by day 7 of prehabilitation corresponding with the trend in RBI. This initial loss of butyrate producers may explain why DietPreHab was ineffective at improving surgical outcomes prior to 7 days. Additionally, when the functional profile of the stool microbiota on the early, ineffective days of DietPreHab (days 1 and 3) was assessed, the functional profile focused on cell wall synthesis and division corresponding with the rapid increase in bacterial load (**Figure 9)** which correlates with increased bacterial load. These findings suggest that altering WD induced functional changes to the microbiota is dependent on time and substrate availability. For the gut microbiota to alter its functional profile to increase butyrate production after dietary prehabilitation with SD, it must go through a period of disruption before beginning to restore production of butyrate.

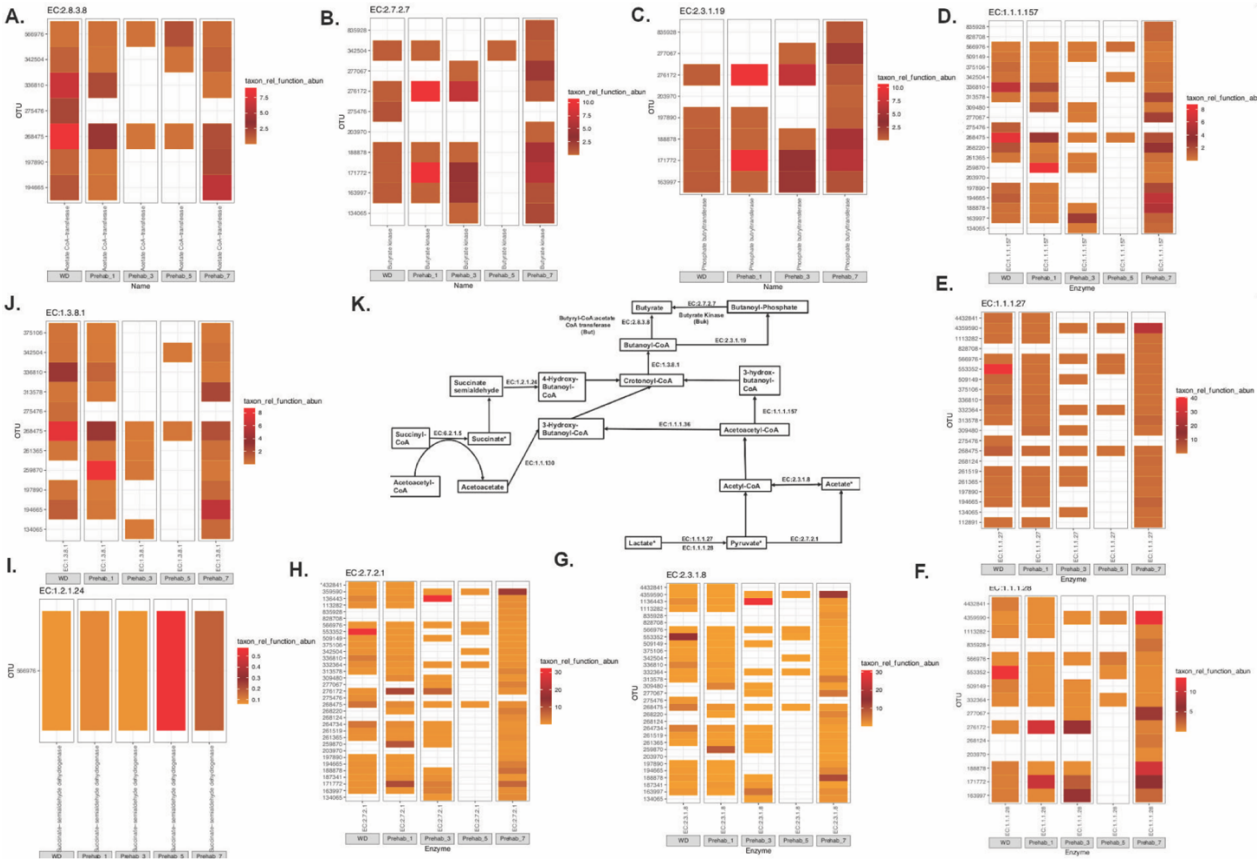



**Figure 8: Significant taxa contribution to butyrate synthesis pathways** Over the course of DietPreHab determined by DESeq2 were compared for their contribution to butyrate pathway (**K**). Each panel (**A-J**) represents an enzyme within the butyrate synthesis pathway (**K**). The taxon relative abundance takes into account the presence of the given gene within the genome of the taxa multiplied by its relative abundance.

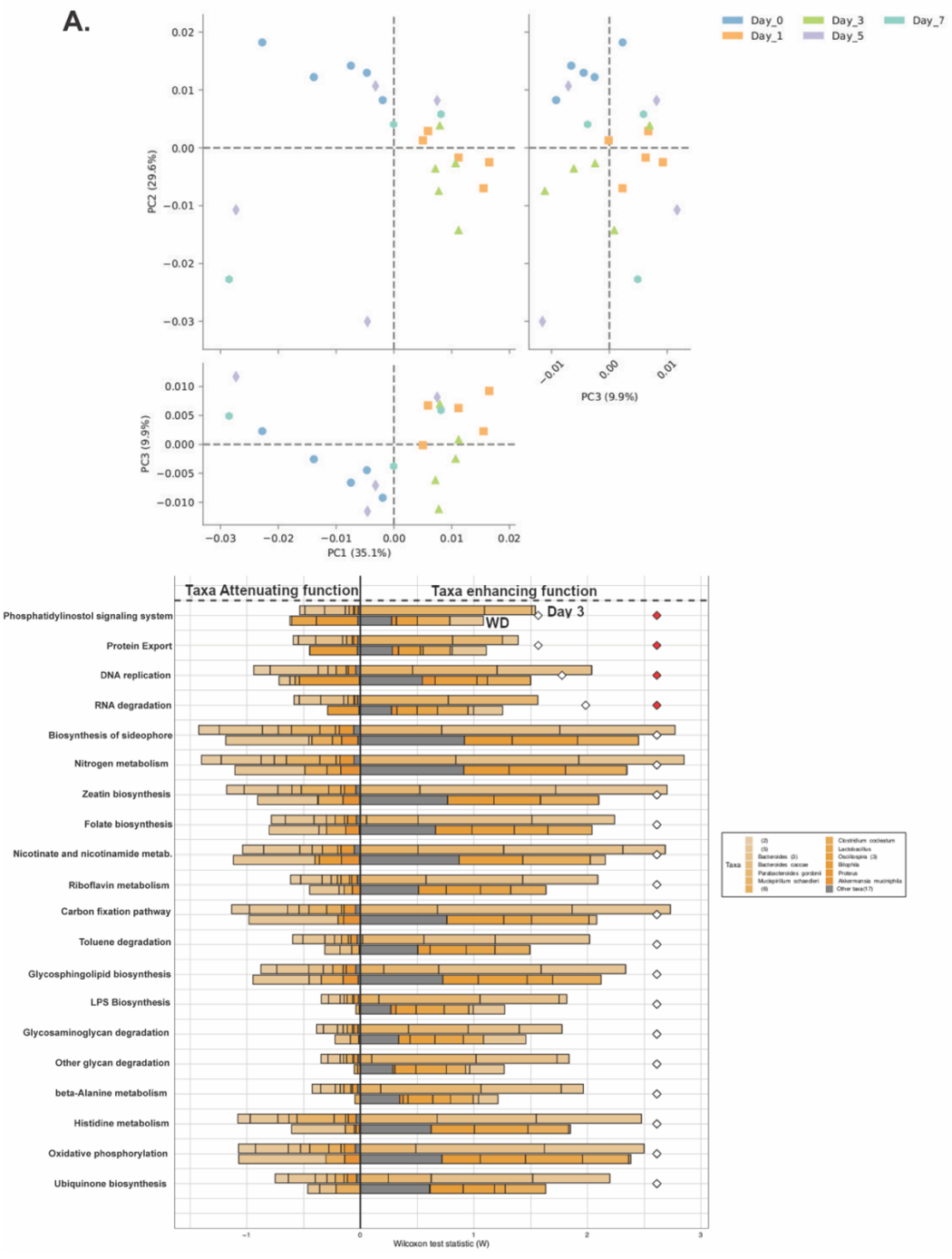



**Figure 9: Functional profile of the microbiota during dietary prehabilitation** Using PICRUSt and STAMP, the functional profile of the microbiota during dietary prehab are compared on PCoA plot (A). FishTaco analysis (B) demonstrates a comparison between Day 3 and WD for the top 20 most significantly different pathways. Top bar for each pathway represents 3 days of DietPreHab and the bottom bar represents Day 0 (WD). Each color bar represents a unique taxa. The bars to the left represent taxa that attenuate function and those to the right represent taxa that enhance the given pathway.

### *3.1.4 Discussion:*

In the line with our previous findings [226], the current work confirmed the adverse influence of a high-fat, low-fiber WD on the intestinal microbiome and its association with a poor outcome when the host is exposed to antibiotics, short-term starvation and operative trauma. The outcome of surgically operated mice exposed to WD was able to be reversed with as little as 7 days of dietary pre-habilitation using SD. Results from this model also revealed that stool-based biomarkers may provide insight on how to assess "microbiota readiness" for surgery in terms of applying dietary pre-habilitation. We identified butyrate, a major secreted product of anaerobic bacteria that has known beneficial effects on the immune system, as a potential candidate biomarker [173,264,271,272].

An interesting finding in the present study was that when mice were fed a WD alone, it did not lead to complete loss of butyrate and butyrate-producing taxa. The attenuation of butyrate in this model, which is most striking in the cecum, may have been related to the absence of dietary fiber in WD as others have shown [163,273]. These findings, when combined with our previous data demonstrating that survival of mice following a 30% hepatectomy and exposure to healthcare associated pathogens can be improved when butyrate producing taxa are restored with a fecal transplant, suggest a central role for butyrate on mortality in this model [264], and are reaffirmed with the alterations in butyrate-taxa seen in DietPreHab. However a direct causal relationship between butyrate and surgical outcome remains to be demonstrated.

Data from this study also suggests that the utility of single time point microbiota measurements in the stool may not accurately reflect the dynamic changes to the intestinal microbiota that occur during dietary interventions. During dietary pre-habilitation, there is

clearly an immediate response of the microbiota to the dietary intervention (**Figure 4C**) followed by a steady progression toward homeostasis after 1 week. Failure to account for both functional (i.e SCFA) and compositional changes of the intestinal microbiota over time may limit the ability to accurately determine the readiness of the intestinal microbiota, and hence the host immune system, for a major surgical stress.

There are several limitation to this study. We performed our experiments utilizing a single strain of mice from a single vendor. This allowed for controlling the background genetics of the mice and allowed for maintaining consistency within the microbiome; however, the results of dietary pre-habilitation may be dependent on the baseline microbiota and genetic background of the mice. Additionally, a western diet has many negative metabolic alterations of the host that are known to increase the risk for surgical complications [266,274] which we did not account for or directly assay. It is known that a western diet associated microbiome has a major impact on the host immune system and response to infection [262,275]. No cytokines or serum markers of inflammation were assessed in this study. Lastly, the precise cause of mortality among mice fed a WD and subjected to the combined exposure of ASH remains unknown. Despite extensive culturing of organs, a distinct pattern of bacterial dissemination of pathobiota from among the gut microbiota was not established as causal to death in this model as we have observed in many similar studies with this model [226]. Capturing the microbes responsible for mortality may require higher resolution next generation sequencing technology applied across multiple organs beyond the spleen using qPCR as performed here.

## *3.2 A high fiber diet reduces mortality in a mouse model of lethal gut derived sepsis and attenuates antibiotic-induced alterations in the microbiome via production of AI-2*

### *3.2.1 Abstract:*

Antibiotics disrupt the intestinal microbiome and can increase the risk for infectious complications. In this series of experiments, we focus on the impact of dietary fiber and fat on the gut microbiota to determine how dietary induced alterations in the microbiota correlate with the host response to antibiotics. We hypothesize that dietary fiber plays a critical role independent of its fat content, in mitigating the negative impact of antibiotics on the host. Mice were fed refined diets varying in fat and fiber content for six weeks, which was compared to standard plant-based rodent chow diet (SD). After six weeks, mice were administered antibiotics (PO clindamycin and IP cefoxitin, as might occur prior to intestinal surgery, twice daily for five days. Severity of antibiotic induced weight loss, intestinal short chain fatty acids (SCFA) and microbiota composition using 16S rRNA gene amplicon sequencing were determined. Dietary fiber led to increases in both cecal and colonic length, reduction in antibiotic-induced diarrhea, and reduced antibiotic-induced weight loss. With regards to microbiota composition, high fiber (Hfb) led to an increase in Bacteroidetes, with a concomitant decrease in Firmicutes and Proteobacteria. These compositional changes were associated with distinct alterations in butyrate producing communities and an increase in SCFA production in Hfb groups. Fermentable fiber demonstrated an increase in the AI-2 quorum sensing pathway in the intestinal microbiota which has been shown to increase resiliency of the microbiota to antibiotic treatment. The addition of dietary fiber, independent of fat, resulted in increased resiliency of the microbiota with fewer species significantly altered following antibiotic exposure. Fermentable fiber had a positive impact on the intestinal microbiota

structure and function independent of fat content, resulting in increased resiliency of the microbiota and correlated with improved antibiotic tolerance of the host. Dietary supplementation with microbiota-accessible fiber offers a way of mitigating the negative effects of antibiotics.

### *3.2.2 Introduction:*

Recent antibiotic exposure can independently increase the risk of infection-related postoperative complications inclusive of sepsis [31,276]. Despite continued improvements in antibiotic stewardship, healthcare providers, particularly surgeons, continue to rely on antibiotics to prevent infection and use them promiscuously assuming they are both safe and otherwise innocuous. Here we hypothesized that the increased risks associated with antibiotic exposure may be related to antibiotic-induced alterations within the intestinal microbiota [277–279]. Antibiotic induced changes to the microbiota lead to compositional and functional changes to the microbiota which correlate with impaired host health and increased infectious complications [280,281].

Recently, diet has been shown to play a major role in the response to and recovery of the intestinal microbiota after exposure to antibiotics [255,280]. However, there is limited evidence determining how the state of the microbiota prior to antibiotic treatment affects the response of the host to antibiotic therapy. An improved understanding of how the composition and function of the intestinal microbiota may impact the response of the host to antibiotics may help mitigate risks associated with their use. Currently the vast majority of patients in the United States adheres to a western diet that is low in fiber and high in fat. A western diet can dramatically shape the composition and function of intestinal microbiota resulting in a loss of health promoting bacteria [282–286]. These western diet induced alterations can have a detrimental impact

on the host as the combination of western diet and antibiotic exposure has been shown to have an additive impact on the microbiota[280]. The combination of a high fat diet and ciprofloxacin exposure resulted in a significant impact on the composition and function of the microbiota when compared to a control low-fat diet [280]. Similarly, we have recently shown mice fed a western diet and subjected to antibiotics and surgical stress developed lethal, gut-derived sepsis [226]. These findings suggest that the resiliency of the intestinal microbiota is greatly reduced when mice are treated with a low-fiber diet.

Due to the variability in the source of fiber across high fat commercially available diets, it remains unclear whether or not the resiliency of the microbiota to antibiotics is a result of the reduction in fat or increased fiber. The majority of experiments attempting to determine the impact of dietary fat on the microbiota use refined low and high fat diets in which fermentable fibers, carbohydrates that are accessible by the microbiota for fermentation, are absent and therefore conclusions on the effect of dietary fat on microbiota composition have been made in the background of diets depleted in fermentable fiber [273]. Fermentable fiber can be referred to as microbiota accessible carbohydrates (MACs) which are essential for maintaining health-promoting bacteria [162]. MACs are resistant to digestion by a host, and therefore available for utilization by gut bacteria, and provide a metabolic substrate that results in significant alterations in the community structure and metabolite production of the microbiota [162]. Therefore, the absence of fiber or lack of access of the microbiota to fiber sources in experimental diets may result in a reduction associated short chain fatty acids (SCFAs), which play an essential role in host health [273]. Recent studies have demonstrated that the absence of fermentable dietary fiber, independent of dietary fat, results in a reduction in health promoting Bacteroidetes and SCFAs [273]. Compositional and functional changes to the microbiota induced by fiber depletion may

impact the host immune system and increase pathogen colonization both of which would be further exacerbated by antibiotic treatment [163,287]. To further implicate the importance of dietary fiber, a recent study demonstrated that the recovery of the intestinal microbiota after antibiotic exposure is significantly impaired when dietary fiber is removed [255].

In this paper, we aimed to delineate a role of microbiota accessible fiber and fat on the ability of host to resist the negative impacts of antibiotic treatment. We developed customized diets that varied in fat, microbiota accessible fiber (resistant starch, defined as a high fiber diet), and microbiota non-accessible fiber (cellulose and corn starch, defined as a low fiber diet). We hypothesized that alterations in microbiota accessible dietary fiber, independent of fat content, would have a significant impact on the intestinal microbiota and the host response to antibiotics.

### *3.2.3 Results:*

*Dietary fat content influences weight gain independent of fiber content and dietary fiber content impacts intestinal tract length independent of fat content*

It is clear from the previous chapter that a WD has a major impact on both the compositional and functional changes of the intestinal microbiota that in turn, may increase the response of the host to physiologic and injury-related stress (antibiotic exposure, starvation, surgical injury). However, it is unclear whether it is the dietary fat or microbiota accessible carbohydrate, fiber, that is driving the sensitivity to host stress. This chapter will focus on a series of experiments utilizing customized diets to determine the contribution of diet and its effect on the intestinal microbiota to explain the ensitivity to antibiotics.

Mice were placed on their respective diets for a total of 6 weeks. Custom diets containing low fat-low fiber (LF-LFb), low fat-high fiber (LF-HFb), high fat- high fiber (HF-HFb), and high fat-low fiber (HF-LFb) were specifically designed and produced by Tekland.

(**Figure 10A**). As expected, the dietary fat content had a significant impact on weight gain at the end of 6 weeks when compared to SD (**Figure 10B)**. In the high fat diets, fiber content did not result in significant changes in weight gain (42.0 vs 38.9%, HF-LFb vs HF-HFb). In the low-fat diets, there was similar weight gain to SD; however, the absence of microbiota accessible fiber resulted in a significant reduction in weight gain as compared to LF-HFb (10.4 vs 15.6%, LF-LFb vs LF-HFb $p \leq 0.01$) (**Figure 10B**).

As previously shown [273], fiber supplementation had a significant impact on the lower gastrointestinal tract, increasing the cecum length in both low fat (2.6 vs 3.7cm, LF-LFb vs LF-HFb, $p \leq 0.05$) and high fat (2.1 vs 3.6cm, HF-LFb vs HF-HFb, $p \leq 0.05$) diet fed mice (**Figure 10C**). The addition of dietary fiber also resulted in a significant increase in colon length in both low fat (6.1 vs 7.2cm, LF-LFb vs LF-HFb, $p \leq 0.05$) and high-fat (6.5 vs 8.0cm, HF-LFb vs HF-HFb, $p \leq 0.05$) diet fed mice (**Figure 10D**).

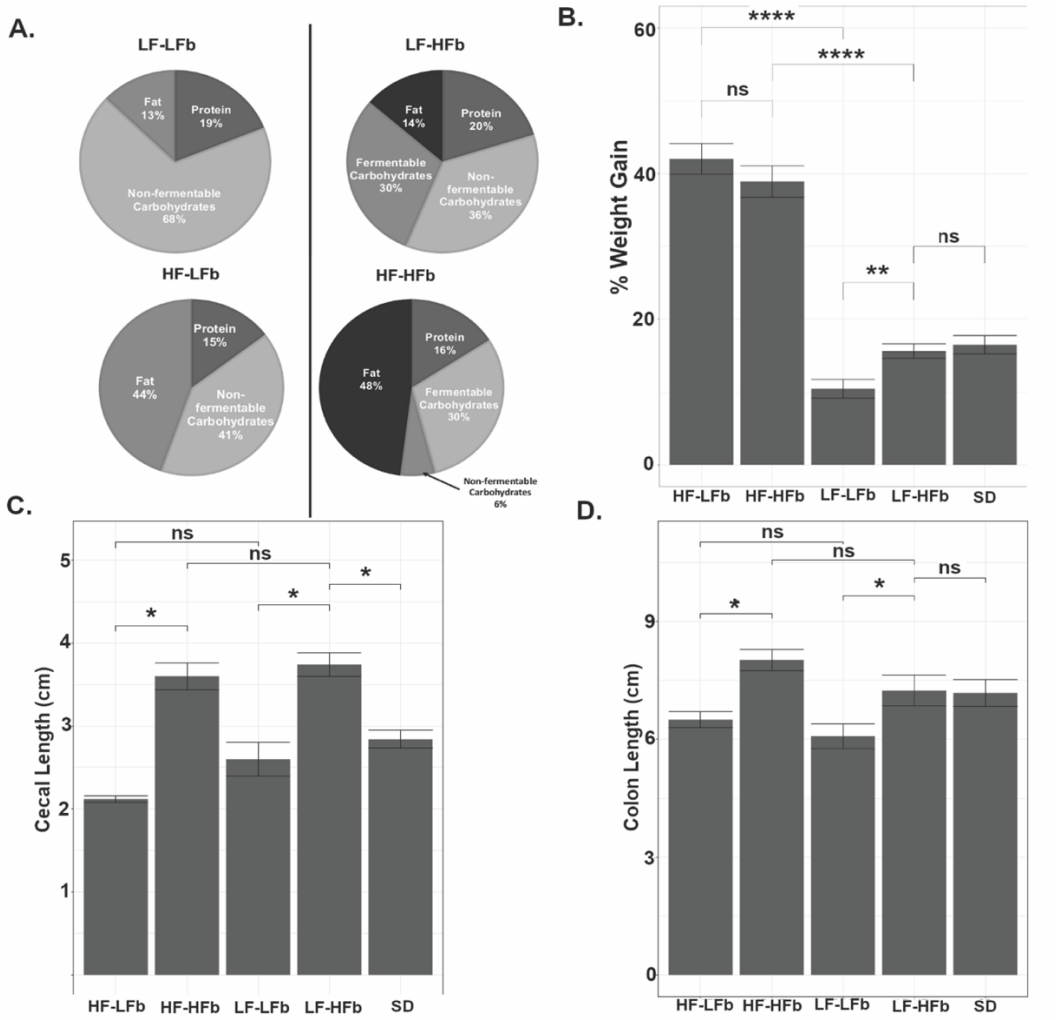



**Figure 10: Fiber supplementation has no impact on weight gain and impacts the intestinal tract independent of dietary fat** The compositional make up of the experimental diets are described in panel A where LF = low fat, HF = high fat, LFb = low fiber, and HFb = high fiber. The weight gain after 6 weeks on their respective diets is demonstrated in panel B. The impact of the experimental diets on cecum (C) and colon length (D) were measured at the end of 6 weeks of feeding on their diets. Weights (SD n = 20, LF-LFb n = 20, LF-HFb n = 20, HF-LFb n = 40, HF-HFb n = 20), Intestinal lengths (n = 5). * - p <0.05, **- p <0.01, *** - p <0.001, **** - p<0.0001

*Dietary fiber attenuates antibiotic induced diarrhea and antibiotic induced weight loss*

After 5 days of antibiotics, the severity of antibiotic induced diarrhea was compared between diets and rated on a 2-point scale, where 0=no diarrhea, 1=mild diarrhea, 2=severe diarrhea (**Figure 11A**). The addition of microbiota accessible fiber resulted in a significant

attenuation of diarrhea severity in both high-fat (1.85 vs 1.1, HF-LFb vs HF-HFb, $p \leq 0.01$) and low-fat (1.6 vs 0.3, LF-LFb vs LF-HFb, $p \leq 0.01$) diet fed mice (**Figure 11A**). No diarrhea was developed in LF-HFb diet fed mice, similar to that in SD fed mice (**Figure 11A**). These results demonstrate that both high microbiota accessible fiber and low dietary fat can mitigate antibiotic induced diarrhea. The combination of high fat with low fiber (HF-LFb) had the most drastic effect on antibiotic induced weight loss (**Figure 11B**) while there were no differences in all other diets as compared with SD.

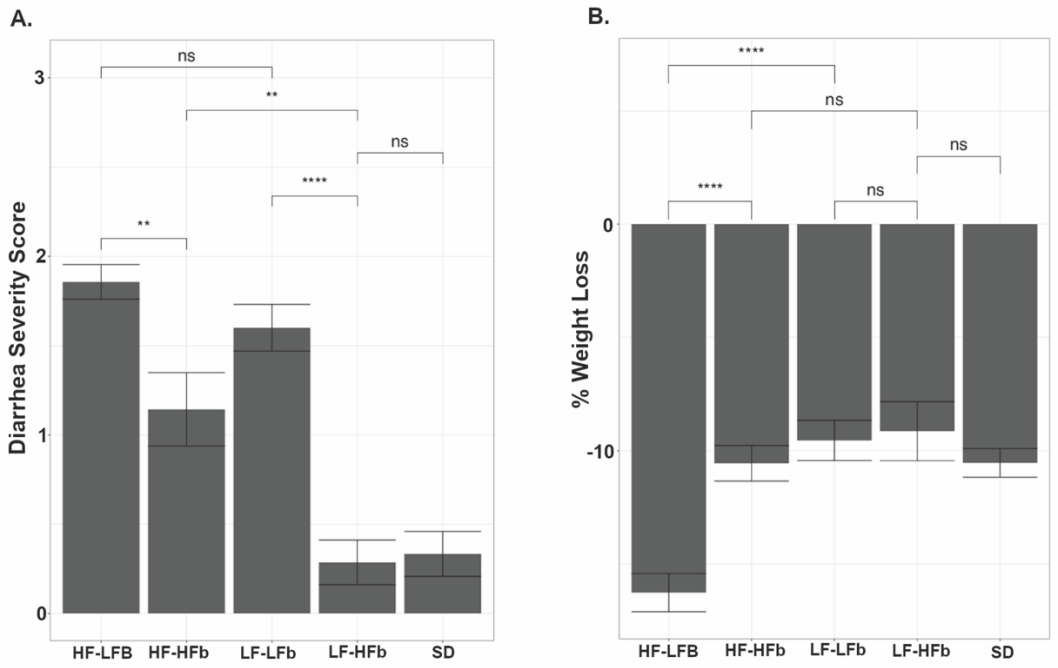



**Figure 11: Fiber supplementation mitigates antibiotic induced weight loss and antibiotic induced diarrhea** Mice were administered 5 days of antibiotics (PO Clindamycin, i.p cefoxitin) and diarrhea severity was scored from 0-2: 0 = no diarrhea, 1 = mild diarrhea, and 2 = severe diarrhea (A). Antibiotic induced weight loss was compared between respective diets after 5 days of antibiotics (B). n = 5 for all groups. * - p <0.05, **- p <0.01, *** - p <0.001, **** - p<0.0001

*Dietary fat and fiber both impact the intestinal microbiota composition*

The impact of diets on the intestinal microbiota was assessed using 16S rRNA gene amplicon sequencing analysis in expelled stool and within cecal contents. The amount of fiber

and dietary fat both resulted in significant alterations to the microbiota composition of the both stool and cecum (**Figure 12A,B**). Within the cecum, the addition of fiber resulted in a significant increase in Bacteroidetes and a reduction in Firmicutes and Proteobacteria (**Figure 12C,D)** ($p < 0.05$). In addition, the lower fat diets (LF-LFb and LF-HFb) had significantly lower abundances of Firmicutes and Proteobacteria and higher levels of Bacteroidetes compared to the high fat diets (HF-LFb and HF-HFb). When Beta diversity was assessed with weighted unifrac analysis, a distinct clustering of the microbiota based on the dietary fiber present within the diet in both the cecum and stool microbiota (**Figure 12E**, **F**) was observed. The microbial compositions in HF-HFb and LF-HFb diets were clustered closer to that in SD diet and were distinct from the HF-LFb and LF-LFb diets in both the cecum and stool ($p=0.001$). Alpha diversity did not appear to be impacted by fat content but was reduced in the high fiber diets (HF-HFb and LF-HFb) compared to the low fiber diets (HF-LFb and LF-LFb) (**Figure 12E,F**). The decrease of alpha diversity on HFb diets may be explained by the single source of fiber present within the diets (**Figure 12G,H)**.

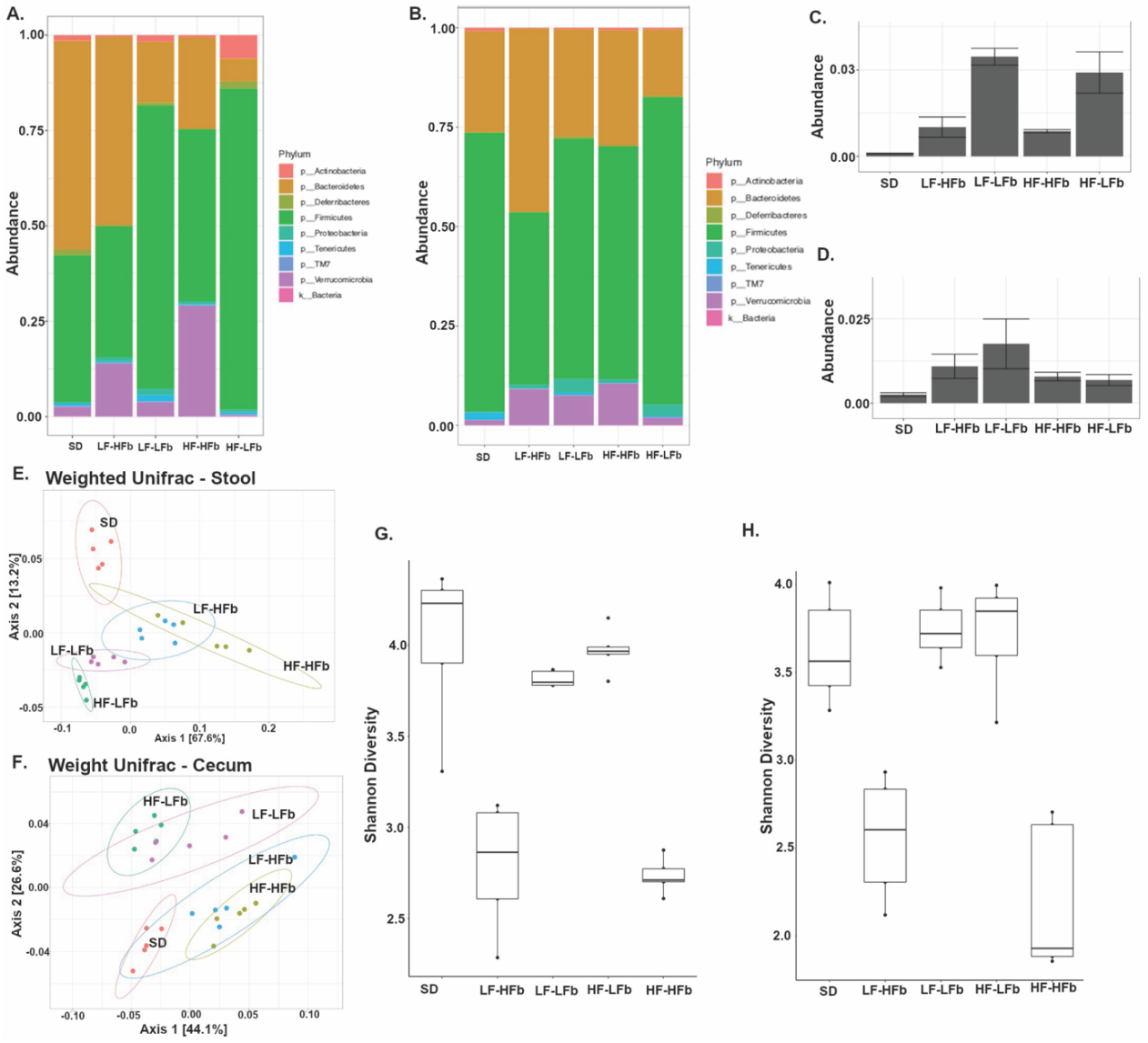



**Figure 12: Dietary fiber significantly impacts intestinal microbiota composition in both low and high fat diets** 16S rRNA sequencing was performed on both the cecal content and the stool prior to administration of antibiotics. Dietary fiber significantly altered the composition of the stool (A) and cecal (B) in a similar manner. Proteobacteria abundance was significantly different between the diets in both the cecal (C) and stool (D) microbiota. Beta diversity determined by weighted unifrac resulted in clustering of both the stool (E) and cecal (F) microbiota by presence of dietary fiber. Addition of dietary fiber resulted in a reduction in stool (G) and cecal (H) microbiota alpha diversity. n = 5 for all groups.

*Dietary fiber results in increased short chain fatty acid production and butyrate producers*

To determine the impact of the dietary interventions on intestinal microbiota function, GC-MS was utilized to measure SCFAs within the cecum prior to antibiotic treatment (**Figure 13A-C**). SD-fed mice had significantly higher SCFAs production compared to the custom diets. In high-fat diet fed mice, the addition of dietary fiber resulted in a significant increase in total cecal butyrate (81.5±8.9 nmol vs 276.2±1, HF-LFb vs HF-HFb, $p \leq 0.05$) and acetate (236.8±27.7 nmol vs 738.4 ± 485 nmol, $p \leq 0.05$). In low-fat diet fed mice, there was a non-significant increase in butyrate (201.4± 56.4 nmol vs 567.8 ± 404 nmol, LF-LFb vs LF-HFb) and acetate (809.1± 444.7 vs 1293.6± 509.6 nmol, LF-LFb vs LF-HFb). When high-fat and low-fat diets were compared, a reduction in fat content resulted in a significant increase in cecal butyrate (201.4± 56.4 vs 81.5±8.9 nmol, LF-LFb vs HF-LFb, $p \leq 0.05$) and acetate (809.1± 444.7 vs 236.8± 27.7 nmol, LF-LFb vs HF-LFb, $p \leq 0.05$). In the presence of high dietary fiber, cecal SCFAs levels were similarly observed to be independent of fat content (HF-HFb vs LF-HFb). Thus, the effect of fat on SCFAs production was more evident on the low fiber diets compared to the high fiber diets. Similarly, the effect of fiber was more significant in the high fat diets compared to the low-fat diets. Additionally, the variety of microbiota-accessible carbohydrates present within SD compared to the single source of fiber in the custom diets may explain the significant increase in butyrate in SD compared to the high-fiber diets (1267.9±140.7nmol vs 567.8±404.1nmol vs 276.2±179.7nmol, SD vs LF-HFb vs HF-HFb, $p \leq 0.05$).

To further investigate the bacterial communities contributing to butyrate production, ASVs that were significantly altered between diets (DESeq, $p < 0.05$, FDR) were assessed for the presence of butyrate kinase (Buk) and butyryl-CoA-transferase (But) using PICRUSt. It was

observed that the presence of fermentable fiber selected for a very specific set of butyrate producers mainly of the genus *Clostridiales,* again independent of fat content (**Figure 13D**). Additionally, it is important to note that there was a subset of butyrate producers whose abundance was reduced with the addition of the single source of fermentable fiber used in this study. Again, this finding may be related to the single type of fiber source used in our experiments in which selection for a particular subset of bacteria that more efficiently utilize the fermentable fibers was used in our custom diets.

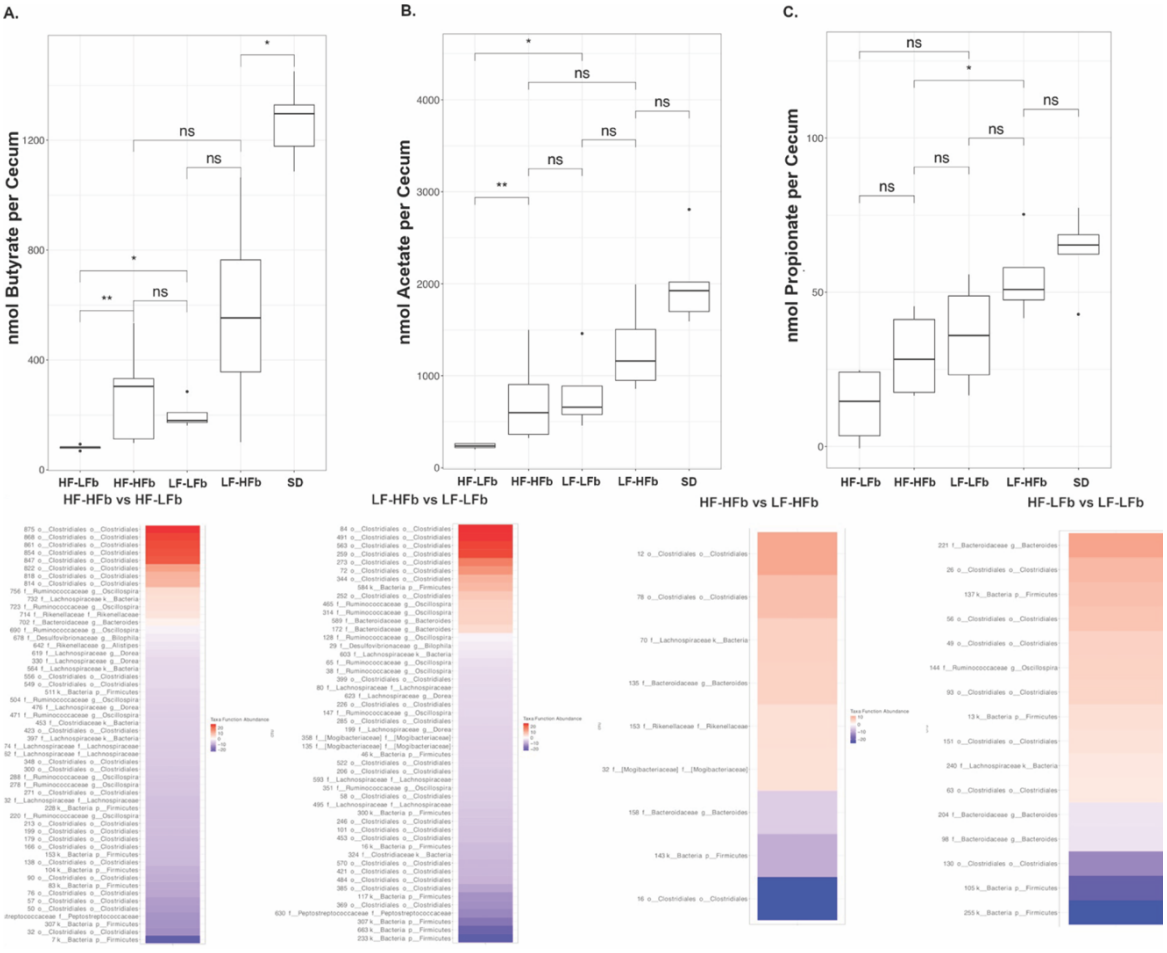



**Figure 13: Addition of dietary fiber resulted in an increase in cecal short chain fatty acids independent of fat content** GC-MS was used to measure levels of cecal SCFAs. The total amount of SCFA per cecum was compared between respective diets. Cecal butyrate (A), acetate (B), and propionate (C) were measured. n = 5 for all groups. The bottom panel demonstrates the

**Figure 13 (continued):** presence of significantly differentially abundant species between diets with butyrate kinase (DESeq, $p < 0.05$ FDR) and determined by PICRUSt.

*Dietary fiber significantly alters stool and cecal microbiota functional profiles*

To further evaluate the differences in the functional profile of the intestinal microbiota between the various diets, PICRUSt was utilized to perform functional prediction based on 16S rRNA amplicons of the stool and cecal microbiota. Similar to the findings evaluating microbiota composition, functional profiles were similar between LFb and HFb diets. On pairwise comparison between LFb diets (HF-LFb and LF-LFb) there was only one differentially identified pathway that reached significance in the stool whereas there were 64 significant pathways in the cecum (**Figure 14A,B**). When a pairwise comparisons was performed between HFb diets (HF-HFb and LF-HFb), there were no significance was identified in the cecum and only 20 significant pathways were observed in stool. When high fiber and low fiber diets were compared, there were a greater number of differentially identified functional pathways that were significantly different between the diets (1769 significant pathways HF-HFb vs HF-LFb and 703 significant pathways LF-HFb vs LF-LFb, $p < 0.05$ FDR). When the diets were compared to SD, the stool microbiota functional profile of LF-HFb appeared to be the most similar to SD with only 26 pathways identified to be significantly different (957 v 1305 v 938 v 26; HF-HFb v HF-LFb v LF-LFb v LF-HFb, $p < 0.05$ FDR).

When the post-antibiotics functional profiles were compared, the diets appeared to be more similar based on fat content than when the refined diets were compared. HF-HFb had only 64 distinct pathways from HF-LFb after antibiotic treatment compared to 529 distinct pathways when HF-HFb was compared to LF-HFb. Similarly, there were only 90 distinct pathways between LF-HFb and LF-LFb. In summary, the dietary fiber content appeared to dictate the

functional profile of the stool and cecal microbiota prior to antibiotic treatment and dietary fat content appeared to determine the microbiota profile post antibiotic treatment (**Figure 14C**).

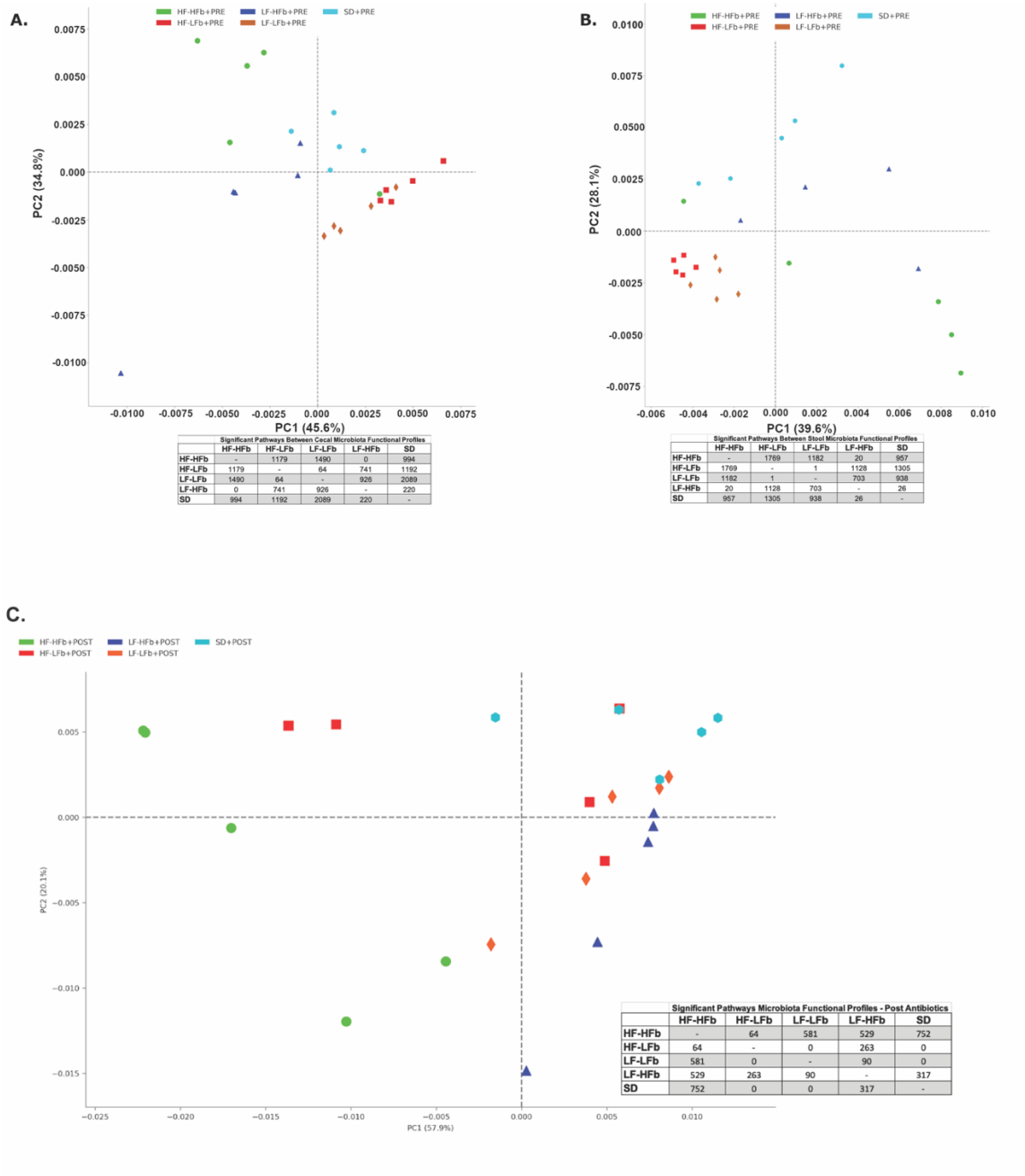



**Figure 14: Dietary fiber results in distinct intestinal microbiota profile independent of fat content** PICRUSt was used to determine the functional profiles of both the cecal (a) and stool microbiota of the experimental diets. Principle component analysis of the cecal (A) and stool (B)

**Figure 14 (Continued):**demonstrates distinct clustering by fiber content as opposed to fat content. Tables below the PCoA plot demonstrate the number of significantly different pathways determined by pairwise comparisons ($p \leq 0.05$ , FDR) . Post antibiotic treatment there were more shared functional profiles based on fat content than based on fiber content (**C**). n = 5 for all groups.

*Fermentable Fiber Independent of Fat Increases Acetyl-CoA production and Induces LuxS/Autoinducer-2 within the Microbiota*

The pathway differences between the diets were further assessed and the two of the most significantly altered pathways included acetylene degradation and S-adenosyl-L-methionine cycle I (**Figure 15A**). Both of these pathways were significantly increased in high fiber diets independent of fat content (**Figure 15B,C**). Acetylene degradation is essential for the production of acetyl-CoA which is important for the production of both butyrate and acetate and may offer an explanation for the increase in acetate and butyrate seen with the addition of fermentable fiber [288]. Finally, S-adenosyl-L-methionine is a pathway associated with the production of the quorum sensing molecules utilizing LuxS/Autoinducer-2 (AI-2). AI-2 has been identified to be produced by different species of bacteria and deemed a universal signaling molecule that allows for interspecies communication [289]. LuxS/AI-2 has also been found to play a role in biofilm formation, virulence, and antibiotic resistance [289]. Specifically, increased production of AI-2 has been observed to increase the resiliency of Firmicutes to antibiotic treatment [240]. Fermentable fibers may be able to increase metabolites that provide important host benefits in the setting of antibiotic exposure while simultaneously increasing the ability of the microbiota communities to cross-communicate and maintain their resiliency in the face of antibiotics.

When evaluating for resiliency of the microbiota to antibiotics, the impact of antibiotics on the cecal microbiota appeared similar based on their abundance at the phyla level (**Figure 15F**). However, when a closer evaluation of the post-antibiotic cecal microbiota were compared

at the species level, significant differences between diets was observed. The pre and post antibiotic cecal microbiota of each diet were compared and significant ASVs were determined using DESeq ($p < 0.05$). The high fiber diets were less impacted by antibiotics with less significantly altered ASVs between pre and post antibiotic microbiota when compared to low fiber diets (62 vs 45 vs 229 vs 139: HF-HFb vs LF-HFb vs HF-LFb vs LF-LFb). When the percent change in ASVs was compared between diets post antibiotics, there was a significant attenuation of antibiotic depletion when fiber was added to the high fat diet and a non-significant attenuation of ASV lost in the low-fat diet (**Figure 15H)**. Taken together, these data suggest that consumption of a high fiber diet is protective to health-promoting microbiota when exposed to antibiotics.

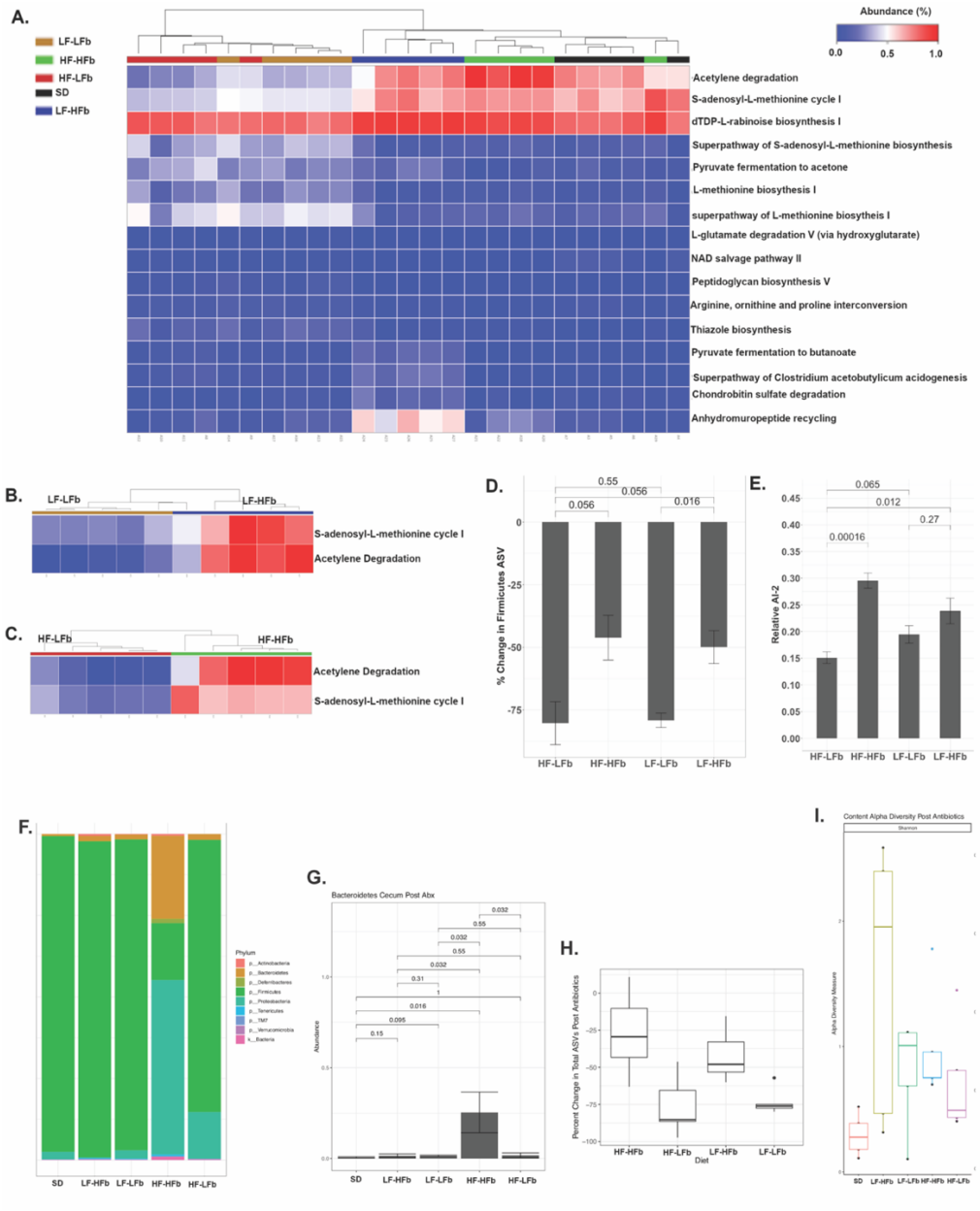



**Figure 15:Dietary fiber significantly increases acetyl-CoA production and synthesis of interspecies quorum sensing molecule AI-2 (S-adenosyl-L-methionine cycle I)** A. heat map comparing the most significantly abundant pathways between all diets ($p < 0.05$, FDR). Compare the abundance of acetylene degradation and AI-2 synthesis between low fat (LF-LFb and LF-HFb, **B**) and high fat (HF-LFb and HF-HFb, **C**) diets. Percent reduction of Firmicutes ASV following antibiotic treatments for each diet (Tukey HSD analysis of variance: HF-HFb vs HF-LFb, $p = 0.056$; LF-HFb vs LF-LFb, $p = 0.016$). Relative amount of AI-2 production (**E**) within the cecal microbiota of each respective diet (HF-LFb vs HF-HFb , $p = 0.00016$; LF-LFb vs LF-HFb , $p = 0.27$). The post-antibiotic cecal microbiota composition based on 16S is demonstrated at the phyla level (**F**). The overall abundance of *Bacteroidetes* was compared between diet groups with a significant increase in abundance seen in HF-HFb mice (**G**). Change

**Figure 15 (continued):** in total ASVs was compared between groups and demonstrated to be significantly lower in low finer diets (**H)**. There were no significant changes in alpha diversity in the cecal microbiota following antibiotic treatment between the groups (**I)**Given the findings of increased abundance of pathways resulting in AI-2 production within the high fiber diets and the relationship between AI-2 and resiliency of Firmicutes to antibiotic treatment, the impact of antibiotics on Firmicutes species within each diet was investigated further.

Overall, the addition of fiber resulted in an attenuation of antibiotic induced loss of Firmicutes ASVs in both low and high fat diets (**Figure 15D**). Using a *Vibrio* AI-2 reporter strain, there was a significant increase in relative AI-2 production in the HF-HFb cecal microbiota compared to the HF-LFb cecal microbiota ($p < 0.01$) and a non-significant increase in AI-2 production in the LF-HFb cecal microbiota compared to the LF-LFb cecal microbiota (**Figure 15E)**. These results indicate that supplementation of a diet with high fiber content improves the host response to antibiotics, irrespective of fat content. The resiliency in response to antibiotics correlated with increased AI-2 production by the cecal microbiota which may explain the increased resiliency of Firmicutes in the face of antibiotics. This further suggests the importance of environmental factors, including host diet, that may influence how the host responds to clinical stress via dietary consumption. As previously discussed, the microbiota plays an important role in preventing pathogen colonization and these findings suggest that we may be able to mitigate some of the antibiotic induced infectious complications by considering patient diet, particularly fat and fiber content, and how it may impact the host.

### *3.2.4 Discussion:*

Independent of dietary fat, fermentable fiber has a major impact on both the intestinal microbiota composition and function which correlate with microbiota resiliency and improved host response to antibiotics. These results suggest that dietary supplementation in the form of

plant-based fiber may offer a method for mitigating the infectious complications that often ensue following antibiotic exposure.

It is now well-established that the intestinal microbiota plays an essential role in shaping the host immune system and preventing pathogen colonization. Although antibiotics are generally regarded as safe (GRAS), exposure to antibiotics is can significantly impact the intestinal microbiota and thus negatively impact the host immune system leaving patients more vulnerable to pathogen colonization [290–293]. Numerous studies have now demonstrated that loss of health promoting Bacteroidetes and SCFAs result in a significant increase in pathogen colonization which, can in turn result in additional infection-related complications [133,294]. Here we have shown that the addition of dietary fiber supplementation in both low and high fat diets increased Bacteroidetes abundance, reduced Proteobacteria abundance, and increased intestinal SCFAs- all of which may aid in maintaining the host immune system and preventing pathogen colonization when antibiotic use is unavoidable.

The combination of a western diet and antibiotics are environmental exposures that leave the host susceptible to both infectious and inflammatory diseases. A recent study demonstrated that the combination of a high fat diet with antibiotics perturbs the intestinal microbiota in a manner that alters the mitochondria activity of colonocytes, resulting in increased intestinal inflammation in a manner similar to patients with pre-Inflammatory bowel disease [295]. Additionally, our recent study demonstrated that when mice are fed a WD, are exposed to antibiotics, and then are subjected to surgical injury, they develop lethal-gut derived sepsis- this is not observed when mice are fed their standard diet (SD) of chow (i.e. high fiber plant based, low fat). In fact no sepsis is observed in any mice consuming their standard chow diet which speaks to the importance of properly modeling these events given that most patient consume a

western type diet and are prescribed prophylactic antibiotics prior to surgery. Both of these findings highlight the need to develop interventions to mitigate the impact of antibiotics on the intestinal microbiota. The addition of plant-based, fermentable fiber may offer an opportunity to improve antibiotic resiliency of the microbiota and mitigate postoperative sequelae given that antibiotics are invariably used as prophylaxis against infection prior to surgery. The addition of fermentable fiber in our studies resulted in a microbiota capable of producing short chain fatty acids and increased quorum sensing signaling, as judged by the expression of AI-2, which has been shown to play a role in microbiota interspecies communication and resiliency to antibiotic treatment [240].

Although the addition of resistant starch provided improvement in the intestinal microbiota and the host response to antibiotics, these diets did not restore the microbiota at the same level of resiliency observed with the the plant-based standard rodent chow diet. The high-fiber diets used in these experiments represent a refined (i.e. processed) diet with the addition of a single, plant-based fiber. Different plant based fibers have been shown to result in species specific variations within the intestinal microbiota and therefore a food source such as standard rodent chow, which contains a wide variety of unprocessed plant based fibers, will have a broader impact on the intestinal microbiota [296]. Finally, microbiota diversity and resiliency has been found to be associated with increased dietary diversity[297]. Utilizing a single fiber type may explain the reduction in alpha diversity in our study and failure of fiber supplementation to result in a microbiota resiliency similar to that of mice feeding on a standard rodent chow. However, one could hypothesize that if supplementation with multiple fiber sources was used in this study, the results herein observed, may have been further strengthened.

Our study is not without limitations, we utilized a single strain of mice from a single vendor and the response of the intestinal microbiota to diet and antibiotics is likely influenced by the background genetics of the host. It is also important to understand that the microbiota response to dietary interventions is highly individualized [297] and not all patients may have the same response to dietary fiber as reported here. However, if further investigations are able to characterize how the microbiota responds to variation in diet across multiple fiber sources, then it may be feasible to design personalized diets for an individual's microbiota.

As healthcare providers continue to rely on antibiotics, it is important that we work to mitigate the negative impact of diet and antibiotics on the intestinal microbiota. Most hospitalized patients are placed on chemically defined, sterile diets that are completely lacking in plant-based fibers and thus will negatively impact the intestinal microbiota and metabolome [298]. As we attempt to mitigate the impact of antibiotics on the intestinal microbiota, it is essential that we begin to work to improve our current approach to hospital nutrition. The results of this study suggest utilizing plant-based fibers to fortify diets may strengthen the resiliency of the intestinal microbiota to antibiotic exposure and mitigate the infectious risk associated with antibiotic exposure.

## *3.3 Western diet increases the gut resistome without exposure to antibiotics*

### *3.3.1 Abstract:*

Approximately half of surgical site infections are caused by pathogens resistant to the antibiotics used for prophylaxis. We recently demonstrated that when mice are fed a western diet (WD) high in fat and low in fiber, are then exposed to antibiotics, and undergo an otherwise

recoverable surgery, they develop lethal sepsis associated with dissemination of multi-drug resistant pathogens. Here we hypothesized that a WD alone can drive the intestinal microbiome to become populated by antibiotic resistant bacteria in a manner independent of exposure to antibiotics. The cecal microbiota response to antibiotics was determined utilizing Biolog Phenotype Microarrays which measures metabolic activity under anaerobic and aerobic conditions in the presence of 48 different antibiotics. WD fed mice had a significant increase in antibiotic resistance within their microbiome compared to mice on standard low fat, high fiber diet (SD). In WD fed mice, there was statistically significant increase in metabolic activity in the presence of different classes of antibiotics including aminoglycosides, tetracyclines, cephalosporins, fluoroquinolones and sulfamethoxazole. On metagenomic sequence analysis, there was an increase in the abundance of antibiotic resistance genes (ARGs) within the cecal microbiota of WD fed mice including CfxA2, ErmG, TetQ, and LnuC. After just 7 days of WD, the ARGs ErmG and CfxA2 were detectable within the stool and significantly increased over time.

### *3.3.2 Introduction:*

The rise of antibiotic resistant organisms continues to plague the healthcare system resulting in over 3 million infections and almost 50,000 deaths per year [4,6]. Antibiotics have played an essential role in the advancement of medicine and improvement in treatment and prevention of infectious diseases; however, the efficacy of antibiotics in the era of antibiotic resistant organisms remains tenuous with an increasing number of antibiotic resistant organisms responsible for infections, resulting increased hospitalizations, morbidity, and death.

The intestinal microbiota is an important reservoir for pathogens and antibiotic resistance genes (ARGs) that can cause systemic infections when patients become immunocompromised by chemotherapy, major surgery and other significant physiologic perturbations [78,299–302]. The role of environmental exposures in increasing the presence of ARGs within the microbiota has been repeatedly demonstrated across multiple studies [303,304]. In human studies, it appears ARGs in gut microbiota are dependent on prior antibiotic exposures, diet, environment, and physiological stressors including surgical intervention [226,261,305]. These studies allude to the influence of a western diet, high in fat and low in fiber, in the development and accumulation of ARGs within the microbiota. Human studies have also demonstrated significant associations between dietary consumption and the gut resistome [305,306]. A recent twin study out of the UK demonstrated host genetics only accounted for 25% of the variation in the gut resistome, further supporting the dominant effect of environmental exposures on the gut resistome [307]. However, the mechanism and ability of diet alone to increase the presence of antibiotic resistance genes within the microbiota remains unexplored.

The gut resistome is directly influenced by environmental exposures which can induce changes to the resistome that are long-lasting. A human study looking at the gut resistome of swine farm workers demonstrated that the workers developed a gut resistome directly influenced by their exposure to the swine farm and persisted after the workers moved to a new environment [303]. Additionally, pre-term infants who are admitted to the NICU and require antibiotic treatment have been shown to have persistent colonization with antibiotic resistant organisms despite recovery and maturation of their microbiota after discharge[308]. To further implicate the role of diet and environment in the gut resistome, geographic and diet influences have been shown to shape the resistome of chimpanzees and humans alike. Captive apes tend to have a higher

abundance of antibiotic resistant genes within their gut resistome when compared to the resistome of wild apes[309]. Similarly, humans that have been westernized and engage in agriculture have a significant increase in ARGs compared to hunter and gatherer tribes[310]. It is clear environmental exposures including western diet shape the intestinal microbiota and lead to the accumulation and persistence of antibiotic resistance genes. To further support the importance of environmental influences on the gut microbiota, when the gut microbiota of wild mice were compared to lab mice, gut microbiota from wild field mice were more resilient to antibiotic therapy and less influenced by dietary interventions compared to the microbiota from lab mice [311].

A western diet (WD), high in fat and low in fiber, has long been shown to result in significant perturbations to the intestinal microbiota, changing their functionality [226,256,284]. The disturbance of the intestinal microbiota by a WD has been demonstrated to have a significant impact on how the intestinal microbiota respond to host stressors including antibiotic exposure, infection, and surgery [226,312]. Here we study the effect of WD on the development of antibiotic resistance within the gut microbiota. We demonstrate that WD alone results an increased antibiotic resistance of gut microbiota to several classes of antibiotics.

### *3.3.3 Results:*

*Western diet results in weight gain, acidification of the gut microenvironment, and significant alteration in the gut microbiota's response to antibiotics.*

As expected, mice feeding on a western diet significantly increased in overall weight (29.8 ± 3.1% vs 72.1 ± 11.3%, **Figure 16A**) and resulted in alterations in the intestinal

microenvironment demonstrated by a reduction in cecal pH (6.88±0.4 vs 6.33±0.23, **Figure 16B**).

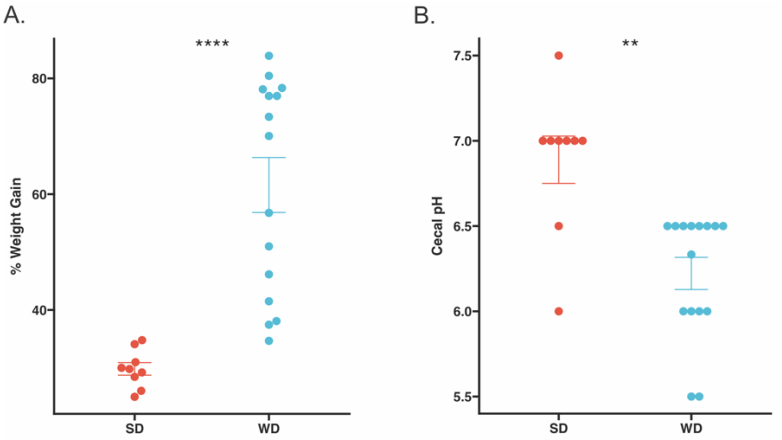



**Figure 16: Impact of WD on weight and cecal pH** The weight (A) and cecal pH (B) of WD fed mice (n = 15) and SD fed mice (n = 9) after 7.5 weeks of acclimation to their respective diets. ** - $p < 0.01$, **** - $p < 0.0001$

Biolog phenotype antimicrobial microarrays were used to characterize how diet impacted the cecal microbiota resistome. The cecal microbiota resistance profile was inferred from the level of metabolic activity in the presence of the highest concentrations of antibiotics on the Biolog microarrays. Additionally, the basal metabolic activity was determined at the lowest concentrations of antibiotics, and the antibiotic induced metabolic activity was normalized to basal metabolic activity. When the cecal microbiota was compared between standard chow diet (SD) and western diet (WD) fed mice under anaerobic (**Figure 17A**) and aerobic (**Figure 17B**) conditions, there were 23 individual antibiotics that had significantly increased metabolic activity in WD-fed microbiota under anaerobic conditions **(Figure 17A**), and 25 individual antibiotics under aerobic conditions (**Figure 17B**). The increased metabolic activity in the presence of antibiotics was similarly found to be present in the stool microbiota of WD fed mice (**Figure 18**). The antibiotic resistance profile based on Biolog was similar between the cecal and stool

microbiota with a significant increase in metabolic activity in the presence of 16 different antibiotics. PCA analysis of total antibiotic induced metabolic activity revealed a distinct clustering between groups of mice feeding on SD and WD in both the stool and cecal microbiota (**Figure 17C**). To validate the antibiotic resistance noted on Biolog, cecal microbiota from WD and SD mice were grown in liquid culture in the presence of individual antibiotics. The WD fed cecal microbiota demonstrated a significant increase in growth in the presence of amikacin, cefoxitin, neomycin, and ofloxacin (**Figure 17D**), suggesting that WD promotes a gut resistome to antibiotics.

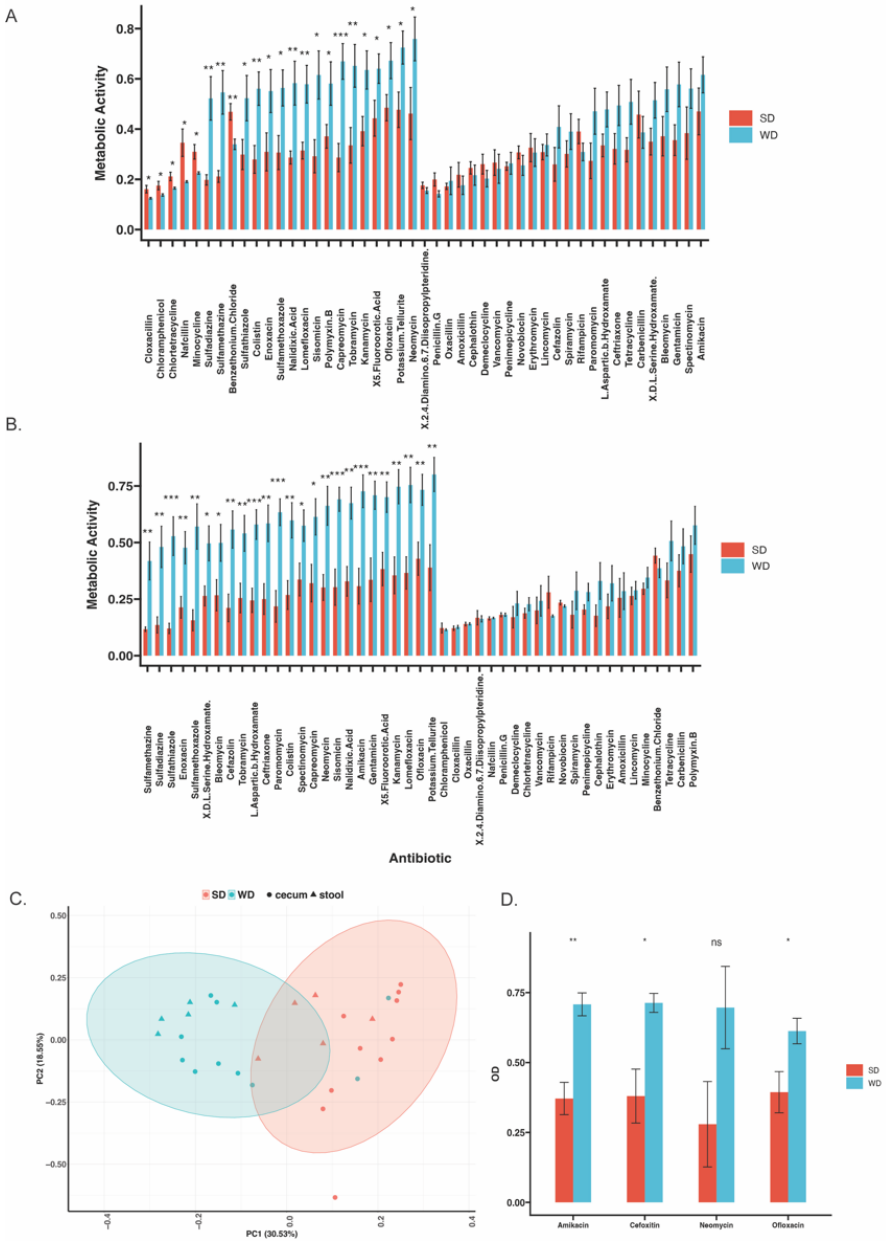



**Figure 17: WD results in a significant increase in the gut resistome** When the overall metabolic activity of the cecal microbiota was compared across all antibiotics on PM plates, there was a significant increase in metabolic activity across individual antibiotics in anaerobic (A) and aerobic (B) conditions (n = 10 WD, n = 9 SD). When comparing WD resistance profile across diets and between cecum and stool, there was clustering of samples by diet on principle component analysis (C). When cecal contents were cultured in the presence of antibiotics, there was a significant increase in growth amongst the microbiota isolated from western diet fed mice. The resistance of the cecal microbiota to individual antibiotics on PM plates under aerobic (A) and anaerobic conditions (B). Cecal microbiota from WD (n= 5 with 2 technical replicates) and SD (n = 5 with 2 technical replicates) were grown in TSB liquid culture for 16h in the presence of cefoxitin (10mg/L), amikacin (10mg/L), ofloxacin (1mg/L), and neomycin (10mg/L) (D). OD

**Figure 17 (continued):** was normalized to the starting OD. Consistent with the Biolog results, there was a significant ($p < 0.05$) increase in growth of the WD microbiota in the presence of all antibiotics except for Neomycin which approached significance ($p = 0.08$). * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

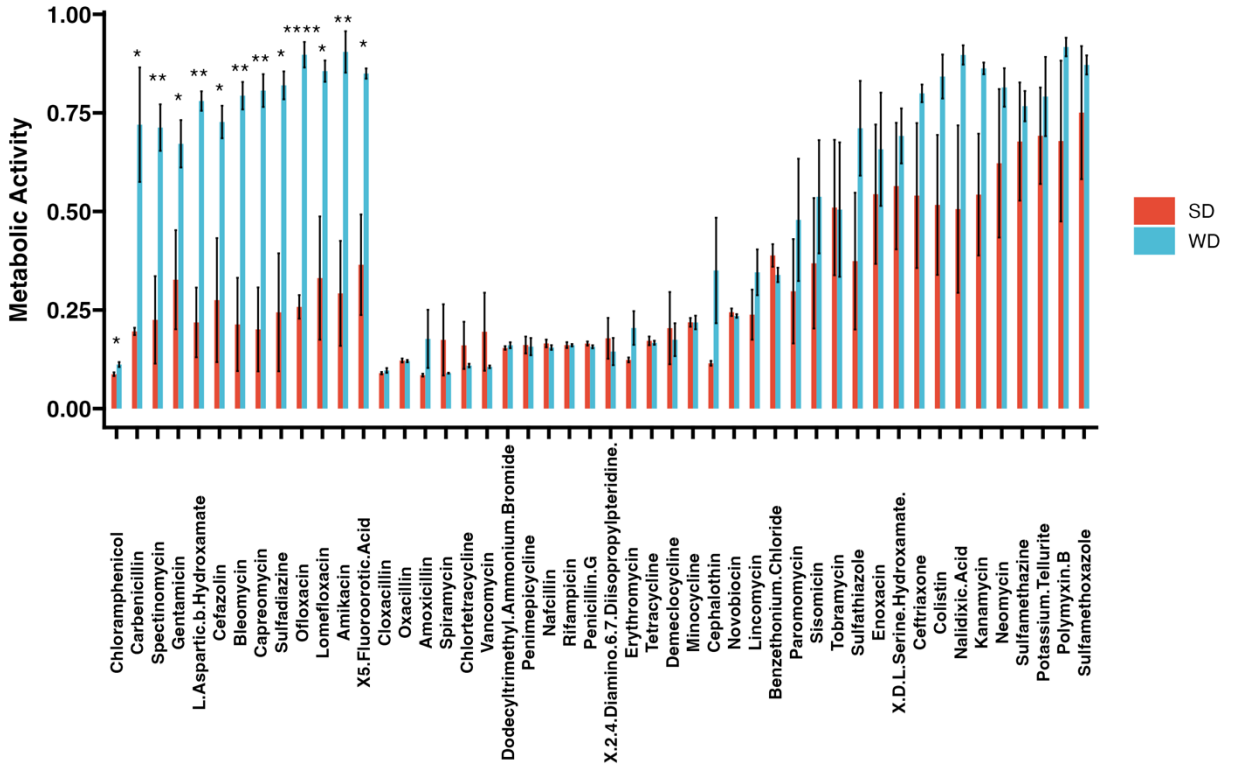



**Figure 18: Significant antibiotic resistance in WD stool microbiota** When the overall metabolic activity of the stool microbiota was compared across all antibiotics on PM plates, there was a significant increase in metabolic activity across individual antibiotics in anaerobic and aerobic (B) conditions (n = 10 WD, n = 9 SD). * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

The WD cecal microbiota Biolog activity was compared between different classes of antibiotics. The WD microbiota demonstrated significantly increased activity in the presence of a variety of classes of antibiotics under both aerobic (**Figure 19A**) and anaerobic (**Figure 19B**) conditions; including Aminoglycosides, Cephalosporins, Fluoroquinolones and Tetracyclines under aerobic conditions, and Sulfonamides, Aminoglycosides, and Fluroquinolones under anaerobic conditions.

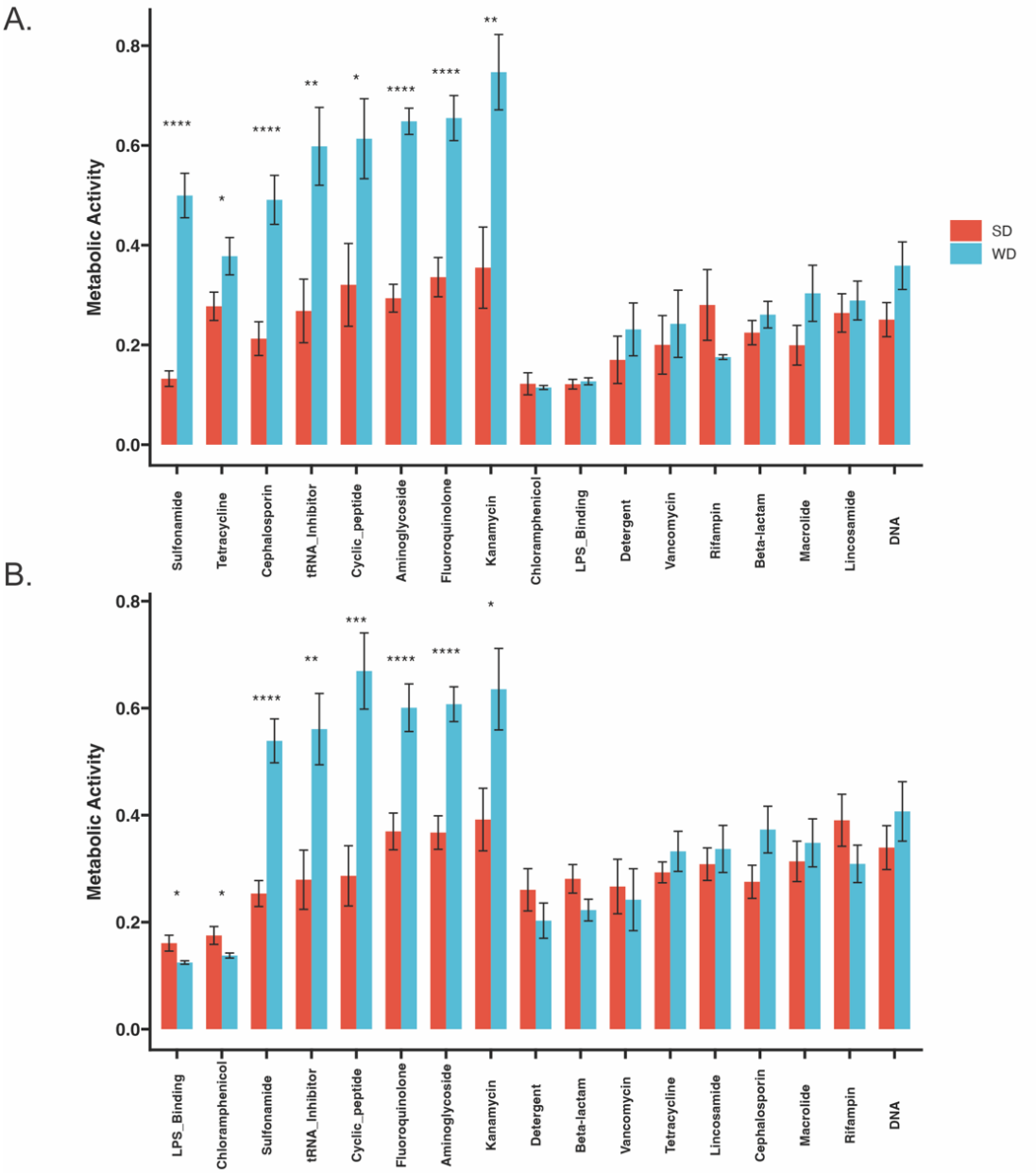



**Figure 19: WD microbiota maintain resistance across antibiotic classes** Biolog activity from WD and SD mice cecal microbiota under aerobic (A) and anaerobic (B) were compared grouping antibiotics by antibiotic class. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$

*Metagenomic sequencings reveals the accumulation of ARGs within the WD fed cecal microbiota*

Given the differences seen on Biolog between the cecal microbiota, shotgun metagenomic sequencing was performed to determine if the differences in metabolic activity seen on Biolog correlated with the antibiotic resistome of the cecal microbiota. On compositional analysis, WD feeding resulted in a reduction in both alpha (**Figure 20A)** and beta diversity (**Figure 20B**) in the stool and cecal microbiota when compared to SD fed mice. When the composition of the microbiota was compared between WD and SD fed mice, there was a significant increase in *Bacteroides vulgatus* within the cecum and stool of WD fed mice (**Figure 20C,D)** which is a normal gut commensal that has been shown to have pathogenic potential[313]. SD fed mice were found to have a large percentage of *Bacteroides ovatum* which is a normal gut commensal that plays an important role in stimulating fecal IgA levels.

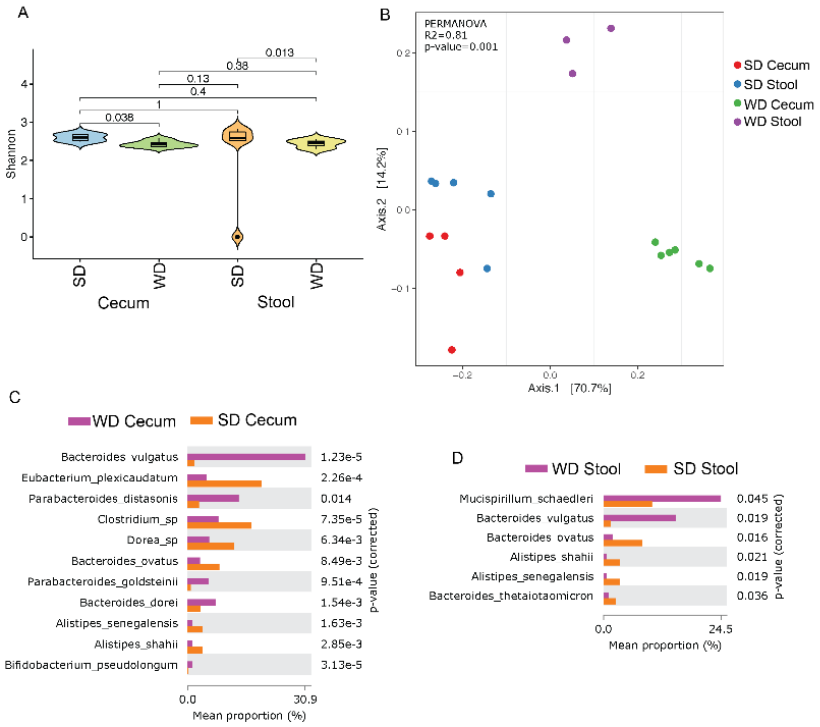



***Figure 20: WD affects composition of cecal and stool microbiota*** Analysis of metagenomics sequencing revealed significant difference in cecal and stool microbiota composition. WD

**Figure 20 (continued):** cecum and stool had significantly lower alpha diversity (A) when compared to SD cecum and stool microbiota. Furthermore, there was distinct clustering by diet and location (stool vs cecum) by beta diversity (B). The most significantly different abundant species between SD and WD cecal and stool microbiota are displayed in panel C and D respectively.

When determining the abundance of antibiotic resistance genes (ARGs), six different ARGs were determined to be significantly increased in WD fed mice among the cecal microbiota compared to SD cecal microbiota: *ErmG*, *CfxA2*, *mel*, and *mefA* (**Figure 21A**). These ARGs were also detected to be increased in the stool of WD fed mice with *CfxA2, mel*, and *tetQ* remaining undetectable in the SD stool and ErmG, tetX, and tetO at significantly lower levels (**Figure 21B**). To determine whether these ARGs were present prior to the beginning of the experiments, time 0 stool and cecal microbiota were analyzed. For WD fed mice, lnuC, mel, and tetQ were not present at time 0. CfxA2 and Ermg were present at time 0 and increased in frequency with WD feeding. Similarly for SD fed mice, CfxA2 and ErmG were detected at time 0 but decreased in abundance during the course of the experiment. To summarize, there are 6 ARGs that appear to nascently emerge in the WD microbiota despite having no prior exposure to antibiotics. There is a subset of ARGs that appear to be inherent to the mouse microbiota at the beginning of the experiment and increase with WD feeding. An additional subset was not detectable on sequencing at the beginning of the experiment but increased over time, indicating the ability of diet to alter the gut microbiota resistome. It is important to note that in the SD fed mice, the resistome appears to be isolated to the stool microbiota with complete absence of the WD-associated ARGs **(Figure 21A,B)**. To confirm the presence and abundance of these ARGs, PCR was performed on stool collected from WD and SD mice from day 1 and day 7 after initiation of their respective diets. There was a significant increase in *tetQ*, *ErmG*, and *tetW* expression levels in the stool of WD fed mice after 1 day of WD feeding (**Figure 21C**). All four

genes tested had a significant increase in expression in WD fed mice after 1 week of WD feeding (**Figure 21C**).

To further characterize how these ARGs were shaping the microbiota, the ARGs were binned to metagenomes and the species harboring the ARGs were determined by BLAST. Across the mice the WD-associated ARGs were detected in *Bacteroides dorei* (ErmG, mel), *Bacteroides fragilis* (ErmG), *Parabacteroides merdei (CfxA2), Mitsuokella sp. AF33-22 (lnuC), Bacteroides acidifaciens (tetQ), Lactobacillus reuteri (lnuC),* and Clostridium culturomicum (mefA). The species harboring these genes are typical Firmicute and Bacteroides species of the microbiota. These findings further suggest that simple environmental exposures such as diet can have major consequences on the antibiotic resistant profile of the gut resistome and should be considered when to individualize antibiotic regimen for gut derived pathogens in the clinical setting.

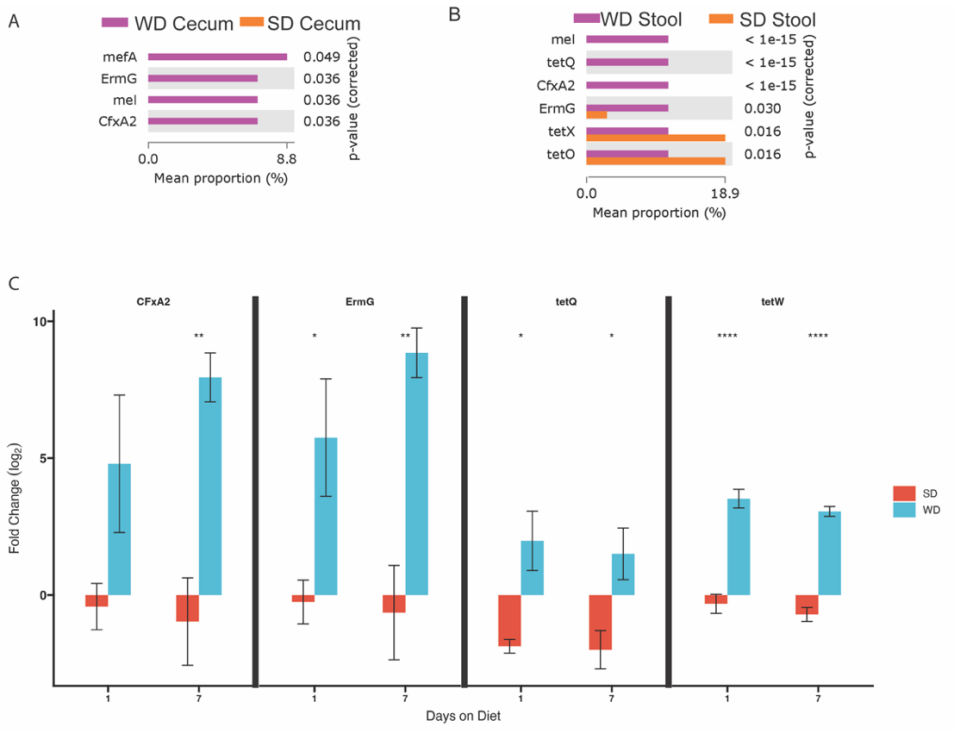



**Figure 21: Antibiotic resistance genes (ARGs) were identified within the metagenomes using the Comprehensive Antibiotic Resistance Database (CARD)** There was a significant increase in the abundance of antibiotic resistance genes in both the cecal (A) and stool (B) microbiota of WD fed mice. PCR was used to determine the presence and accumulation of antibiotic resistance genes tetQ, CfxA2, ErmG and tetW (C) within the stool of WD and SD fed mice after 7 days of being on their respective diet (n = 5 per group).

### *3.3.4 Discussion:*

Here we demonstrate that consumption of an experimental western diet, without antibiotic exposure, independently results in an increase in the prevalence of antibiotic resistance genes and development of the gut resistome. Prior studies, have demonstrated that western-type diets are associated with the development of antibiotic resistance within the gut microbiota, but have recognized the many confounding environmental factors [261,305,306]. This is the first study to demonstrate a diet-dependent alteration in the gut resistome when all other environmental and

genetic variables are held constant. Furthermore, the gut resistome profile appeared to be sensitive to dietary interventions with significant alterations of the resistance profile within days of dietary changes. These findings are important as we began to understand how diet and environmental exposure may explain the risk of colonization with antimicrobial resistant pathogens and the response of the gut microbiota to antibiotics.

The accumulation of ARGs seen within *Parabacteroidetes* is consistent with prior studies [314–316]. Bacteria from the phyla *Bacteroidetes* are common gut commensals, however they can behave as pathogens and in fact, are responsible for many anaerobic-related infections [315]. More importantly, many infections treated in the hospital are polymicrobial- both aerobic and anaerobic are often isolated and require appropriate antibiotics including those that target anaerobic bacteria. Extensive studies throughout Europe have demonstrated that treatment failure often occurs due to antimicrobial resistance amongst anaerobic species [317]. An observational study in humans demonstrated a significantly higher level of antimicrobial resistance within anaerobic commensal species including resistance to clindamycin and metronidazole, antimicrobials utilized to empirically treat anaerobes [318]. These findings in conjunction with this study emphasize the importance of understanding the dietary impact on the gut resistome and its future role in guiding antibiotic therapies. As we attempt to compensate and empirically target anaerobic bacteria, critically ill patients have worse outcomes when treated with anti-anaerobic antimicrobials [77].

It remains unclear how the presence of these antibiotic resistance genes impact both the host and gut microbiota in the setting of antibiotic exposure. Clinically, antibiotic resistance testing accounts for single species' response to antibiotics, but bacteria within the gut form complex communities of many different species that have co-evolved strategies to deal with

environmental pressures. Previous studies have demonstrated that when communities of bacteria are exposed to antibiotics there is a selection for genes that engage in cooperative drug resistance [319]. It is unlikely that horizontal gene transfer occurs across phyla of bacteria; however, it is plausible that accumulation of ARGs even within 'non-pathogenic' bacteria may have the ability to act as an antibiotic sink and shield potential pathogenic bacteria from antibiotic exposure [320]. A recent study demonstrated that mice colonized with beta-lactamase producing *E. coli* and treated with a beta-lactam antibiotics had higher colonization with beta-lactam sensitive pathogens, *Listeria monocytogenes* and *Clostridium difficile*, suggesting that antibiotic resistant pathogens within the gut can shield potential pathogens from antibiotics [320]. Additionally, these antibiotic resistant bacteria may have the ability to outcompete other native microbiota species commonly associated with promotion of health. Given the growing emphasis of diet in shaping both the composition of the intestinal microbiota and the antibiotic resistance profile, bthe major influence of diet on the gut resistome should be considered when providing patients with empiric antibiotic treatment against gut-derived pathogens. Currently, when antibiotic prophylaxis is used or treatment of a gut-derived pathogen is implemented, there is little attempt to individualize the antibiotic regimen relative to the individual's gut microbiota. Our data suggest that empiric antibiotics against gut pathogens may benefit from being personalized to the resistance profile of an individual's gut resistome, and dietary interventions may provide a means of changing the gut resistome to alter pathogen susceptibility and improve the success of antibiotic treatment.

Our study is not without limitations, it was conducted in a single strain of mice from a single vendor. It is clear that both genetics and environment contribute to the gut resistome and it is likely that both of these factors may result in different results than demonstrated here. Finally,

Biolog and liquid cultures are artificial growth environments which in turn select for organisms capable of growing in these culture conditions and may result in loss of certain species of bacteria that may also contribute to the gut resistome *in vivo*. To account for these limitations, metagenomic sequencing provided a snapshot of the presence of antibiotic resistance genes in uncultivable bacteria.

As we continue to understand the contributing factors to the gut resistome, we may be able to advance care to curtail the rise of antibiotic resistance. Through dietary intervention and the use of antibiotic regimens personalized to an individual's gut microbiota, we may more efficaciously treat gut-derived infections as well as eliminate the use of empiric antibiotic selection that may be ineffective depending on an individual's gut resistome.

## *3.4 Pathogen subversion of indole-mediated activation of the aryl hydrocarbon receptor and survival following lethal peritoneal infection*

### *3.4.1 Abstract:*

In order for pathogenic species to be successful at causing infection, subvertion of host clearance mechanisms is often necessary to avoid elimination. Here we demonstrate the role of gut microbiota-derived metabolites (i.e. indoles) to promote survival by activating macrophages via the aryl hydrocarbon receptor (AhR) and the ability of infecting pathogens to counter this pathway by releasing exoproducts leading to AhR inhibition and mortality. Using a model of intraperitoneal (IP) infection with *Serratia marcescens, Klebsiella oxytoca* and a human multi-pathogen community isolated from the stool of a patient dying of sepsis, we demonstrated that indole-mediated activation of the AhR has a major influence on macrophage function and

survival. However, as a countermeasure, we also found that secondary metabolites produced by pathogens in this model are capable of inhibiting indole-mediated AhR activation. Enterobactin was identified as one such secondary metabolite capable of inhibiting AhR and increasing mortality from bacterial peritonitis. Finally, we observed that the lethal effect of these pathogens can be overcome when indoles are delivered systemically or increased through dietary tryptophan supplementation. Taken together, these findings suggest that survival from a lethal bacterial infection is dependent on the ability of microbiota-derived indole metabolites to activate AhR as a countermeasure to the infecting pathogen's ability to inhibit it.

### *3.4.2 Introduction:*

Despite the many advances in our understanding of the role of the immune system on the pathophysiology of sepsis, the precise factors that govern survival remain poorly understood [68,321]. Given its definition as a "dysregulated host response to an infection," survival from sepsis has traditionally been focused on understanding and modulating the immune response following a standardized infectious insult. This immunology centered approach has been difficult to translate to the human condition, as the behavior of the infecting pathogen(s) is presumed to have remained constant during the course of infection and fades into the background as inflammatory-mediated damage takes over.

Yet the infecting pathogen (s) may not be the only microbial factors participating in the host response to infection. For example, recent studies have demonstrated the importance of the role of the gut microbiota and their metabolites on survival following injury and infection [9,124]. Here we hypothesized that survival following a standardized systemic infectious inoculum (i.e.,

an $LD_{50}$ IP dose of *Serratia marcescens* and others), can be differentially influenced by the action of metabolites from both the gut microbiota and those of the infecting pathogen. Here we show that a dual, yet distinct, action of both microbiota and pathogen derived metabolites on AhR activity in macrophages, may play a role in survival following intraperitoneal infection. AhR is a cytoplasmic receptor that can interact with microbial ligands and regulate the host immune system [197]. In particular loss of AhR sensitizes mice to LPS response through its regulation of NF-kB [322]. AhR is also important for microbial tolerance and limiting immunopathology through production of IL-10 following endotoxin stimulation [207]. The ability of AhR to interact with microbial ligands and regulate the innate immune response places it at a unique position to play an essential role in survival from a lethal bacterial infection.

We have previously demonstrated that when the composition and function of the gut microbiota are altered by a combination of dietary modulation, antibiotic exposure and surgical stress, mice display an increased susceptibility to life-threatening infection [224,226]. Specifically, there is an increase in mortality following an otherwise recoverable surgical injury in association with decreased gut butyrate levels[5]. Increasing gut butyrate levels via dietary modulation has been associated with improved survival [312] thus implicating a central role for butyrate in this model. Similarly, when a fecal microbiota transplant (FMT) is administered, gut butyrate concentrations are increased, and survival improves from a human polymicrobial community causing bacterial peritonitis [224]. Although we previously determined that the FMT response occurred in an IRF3 dependent manner [224], causality could not be established between the metabolites produced by the gut-microbiota and mortality in this model. Therefore, to further clarify this issue, in the current report we hypothesized that specific gut-microbiota derived metabolites can be identified that are deterministic of "within-group" survival among co-housed,

genetically similar, inbred mice subjected to a standardized (i.e., $LD_{50}$) dose of a monomicrobial or polymicrobial intraperitoneal (IP) inoculum. We further hypothesized that opposing forces from the infecting pathogen itself might overcome this effect and subvert a beneficial effect from gut microbiota-derived metabolites.

Therefore, in the current study, we tested the hypothesis that "within-group mortality" rates following an intraperitoneal $LD_{50}$ dose of a gram negative bacterium, *S. marcescens*, is influenced by identifiable metabolites from the gut microbiota. This species was chosen based on our prior experience with its lethal effect in mice following surgical injury. The aims of this study were to determine extent to which metabolites from the gut microbiota versus those of the infecting pathogen (s) converge on the AhR to influence survival.

### *3.4.3 Results:*

*Survival following IP S. marcescens is dependent on the presence, composition and function of the gut microbiota and correlates with peritoneal macrophage phenotype*

In order to eliminate potential bacteria-bacterial interactions inherent in our previous model in which a four-pathogen community was inoculated into the peritoneum of mice to produce lethality, we began experiments using a monomicrobial model of lethal infection with the intraperitoneal (IP) delivery of an $LD_{50}$ dose of *Serratia marcescens* strain MVI (mouse virulent isolate) (**Figure 22A**). In the present study, to dissociate the effect of systemic illness on mortality and other measures in this model, we used core body temperature to capture a time point at which mice appeared clinically well, but their survival/mortality could be accurately predicted prior to becoming systemically ill [323–326]. Both sepsis score (**Figure 22B**) and core body temperature (**Figure 22C**) demonstrated distinct alterations over the course infection

between surviving and non-surviving mice starting at 8 hours and delineating by 15 hours post-infection. As expected, surviving mice had improved clearance of *S. marcescens* from the peritoneal cavity at 8- and 15-hours post injection compared to the non-survival group (**Figure 22D**). To confirm the requirement of the gut microbiota on survival from *S. marcescens* peritonitis in this model, mice were decontaminated of their microbiota by administering both systemic cefoxitin intraperitoneally (IP) and oral clindamycin via gavage for a course of 5 days that was completed 24 hours prior to IP inoculation of *S. marcescens*. Mice treated with antibiotic decontamination demonstrated a significant increase in mortality (**Figure 23E**). To further support the importance of the microbiota on survival in our model, mice were treated with an FMT (fecal microbiota transplant) consisting of cecal contents from a healthy littermate control, delivered via enema at the time of infection as previously described [224], which enhanced survival (**Figure 22E**). In summary, depletion of the microbiota with antibiotics enhanced mortality in this model, whereas microbiota repletion with an FMT enhanced survival, supporting a central role of the microbiota on outcome in this model.

To map changes in the microbiota to the response of the immune system, we examined the role of macrophages on survival in this model given their central role in clearing peritoneal infection [327]. When macrophages were globally depleted with clodronate liposomes, mortality was significantly increased and FMT was no longer effective at enhancing survival. (**Figure 22F**). Furthermore, the clearance of *S. marcescens* was associated with significant differences in canonical M1 and M2 gene expression in peritoneal macrophages (pMACs). On average, pMACs from surviving mice demonstrated lower expression of *Nos2*, a marker of the M1 phenotype (**Figure 22G**) with elevated expression of *Arg1*, a marker of the M2 phenotype (**Figure 22H**)[328]. This pattern was not observed in the pMACs of non-surviving mice. Taken

together, these data indicate that macrophages are required for both survival and FMT rescue and express a specific phenotype associated with survival in this model.

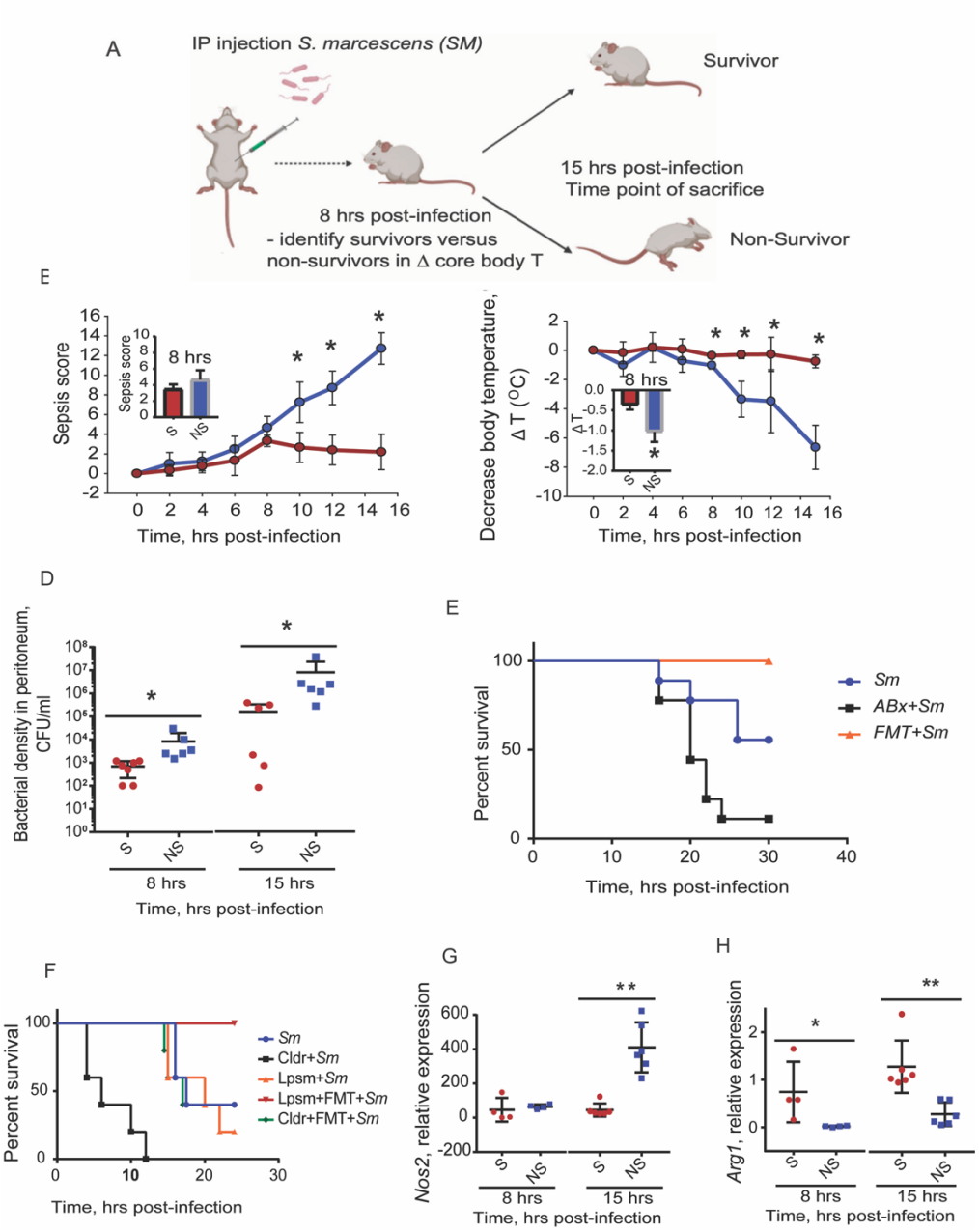



**Figure 22: Survival from S. marcescens peritonitis is dependent on the gut microbiota and correlates with peritoneal macrophage phenotype (A),** Mouse model of IP *Serratia marcescens* injection. **(B-D),** Surviving mice are characterized by lower sepsis score **(B),** higher core body temperature **(C),** and lower *S. marcescens* density in the peritoneum **(D).** n=7 mice per group, *p<0.05, Mann-Whitney unpaired t-test. **(E)**, Kaplan-Meyer survival curves demonstrating that disruption of gut microbiota by antibiotics (clindamycin) increases mortality

**Figure 22 (continued):** in infected mice while replenishment of the gut microbiota via FMT protects against mortality. n=10 per group, p<0.0001, Log-rank (Mantel-Cox) test. (**F**), Kaplan-Meyer survival curves demonstrating that depletion of macrophages by clodronate liposomes increases mortality of mice following intraperitoneal (IP) injection of *S. marcescens*. n=5 per group, p<0.0001, Log-rank (Mantel-Cox) test. (**G,H**). pMACs isolated from survivors have lower expression of canonical M1 gene *Nos2*, (**G**) associated with higher expression of the canonical M2 gene *Arg1* (**H**). n=4 mice per group for 8 hrs. time point. *p=0.0286; n=6 mice per group for 15 hrs. time point. **p=0.0022, Mann-Whitney unpaired t-test. Sm, *S. marcescens*; Cldr, clodronate liposomes; Lpsm, liposomes (vehicle control); FMT, fecal microbial transplant.

*Survival from S. marcescens peritonitis is associated with alterations in gut microbiota production of tryptophan metabolites*

In order to characterize the gut microbiota associated with survival in this model, we compared the gut microbiota of surviving versus non-surviving mice 15 hours following the standardized IP $LD_{50}$ dose of *S. marcescens*. Utilizing 16S rRNA sequencing, analysis of the cecal microbiota demonstrated no changes between surviving and non-surviving mice as judged by alpha diversity (i.e., Shannon index) (**Figure 23A)**, beta-diversity (**Figure 23B**), and composition (**Figure 24A)**.

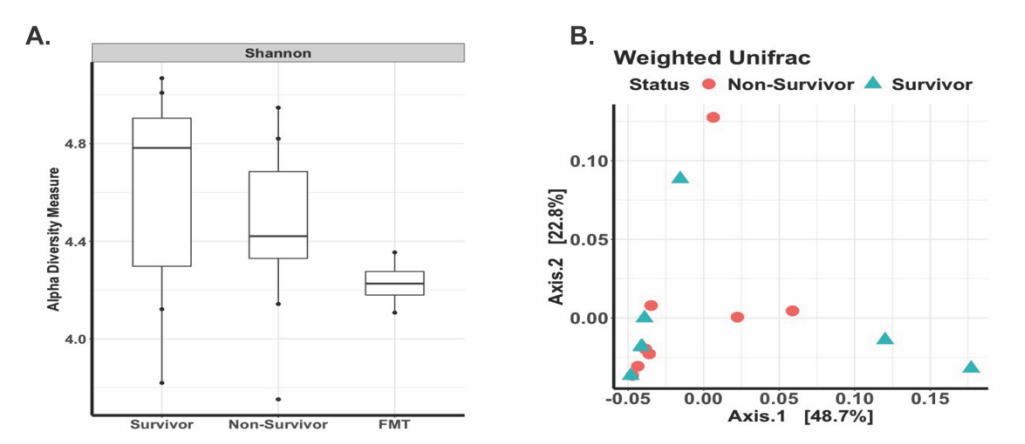



**Figure 23: Survival does not correlate with alpha or beta diversity of the cecal microbiota** Alpha diversity (A) and Beta diversity (B) determined from16S sequencing of cecal microbiota of surviving, non-surviving and FMT treated mice after *S.* marcescens peritonitis

However, a significant difference in cecal metabolites was observed between survivors and non-survivor mice when normalized to uninfected mice (**Figure 24B**), including an increase in indole metabolites, known products of tryptophan metabolism. Consistent with this finding was a reduction of cecal tryptophan during *S. marcescens* infection in both surviving and non-surviving mice, indicating increased tryptophan metabolism (**Figure 24C**). Yet, by comparison, the concentration of tryptophan was higher in the FMT-treated group (**Figure 24C**, **Table S2**). When the total relative amounts of indole metabolites were accounted for, a significant increase in indoles was observed among surviving mice and mice treated with an FMT (**Figure 24D**). These results indicated that surviving mice had a significant increase in tryptophan-derived metabolites despite no alterations in microbiota composition. We next tested whether tryptophan-derived indoles alter peritoneal macrophages in this model, as others have described[329,330]. First, we assayed the peritoneal exudate for the presence of indole metabolites and observed a significant increase in several indole metabolites in survivors relative to non-survivors (**Figure 24E**). Similarly, there was a significant increase in serum concentrations of indole-3 acrylic acid and indole-3 propionic acid (**Figure 24F).** Also, consistent with results from our prior study, a significant increase in cecal butyrate levels was also observed in surviving mice (**Figure 25A-C**); however, there were no differences in any of the short chain fatty acids (SCFAs) in the peritoneum between surviving and non-surviving mice (**Figure 24D-F**). Given the finding that indole metabolites in the gut and peritoneal cavity was associated with survival, we next attempted to determine the mechanism by which indoles produced by gut microbiota from tryptophan contribute to survival in this model.

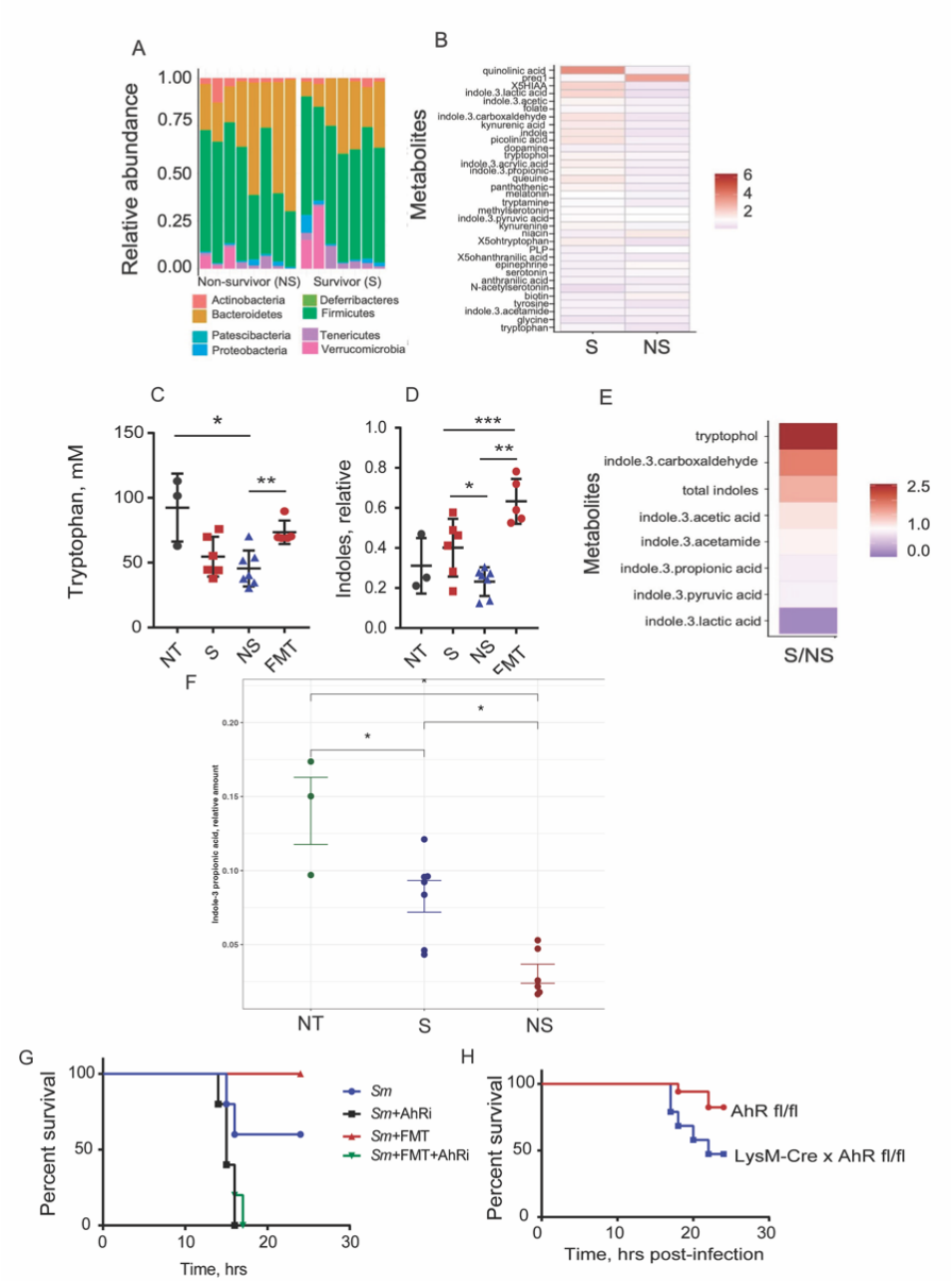



**Figure 24: Gut microbiota-derived tryptophan metabolites are increased in surviving mice and the aryl hydrocarbon receptor is required for survival in this model** (**A**), Relative abundance of cecal microbiota at the phyla level. (**B**), Gut metabolites abundance relative to uninfected mice. (**C,D**), Tryptophan (**C**) and relative total indoles (**D**) in the cecum of mice after 8 hrs of IP injection *with S. marcescens*. n=3 mice in control non-treated group (NT), n=6 mice per group with IP injection of *S. marcescens*. S, surviving mice; NS, non-surviving mice. (**C**): p=0.0049, One-way ANOVA; *p=0.0333, NT vs NS; **p=0.0303, NS vs FMT, Mann-Whitney unpaired t-test. (**D**): *p=0.035, S vs NS; **p= 0.0025, NS vs FMT; ***p=0.0173, S vs FMT, Mann-Whitney unpaired t-test. (**E**), Microbial metabolites of tryptophan in the peritoneum of surviving mice relative to non-surviving mice. **(F)**, Serum indole-3 propionic acid levels between

**Figure 24 (continued):** NT, S, and NS mice (n = 5). **(G)** Kaplan-Meyer survival curves demonstrating the abrogation of the FMT rescue effect during AhR inhibition (AhRi). n=5 per group, p<0.0001, Log-rank (Mantel-Cox) test. SM, *S. marcescens*; AhRi, AhR inhibitor StemRegennin; FMT, fecal microbial transplant. Knockout of AhR attenuates survival of mice as demonstrating by Kaplan-Meyer survival curves (**H,** p=0.0211, n = 20 mice)

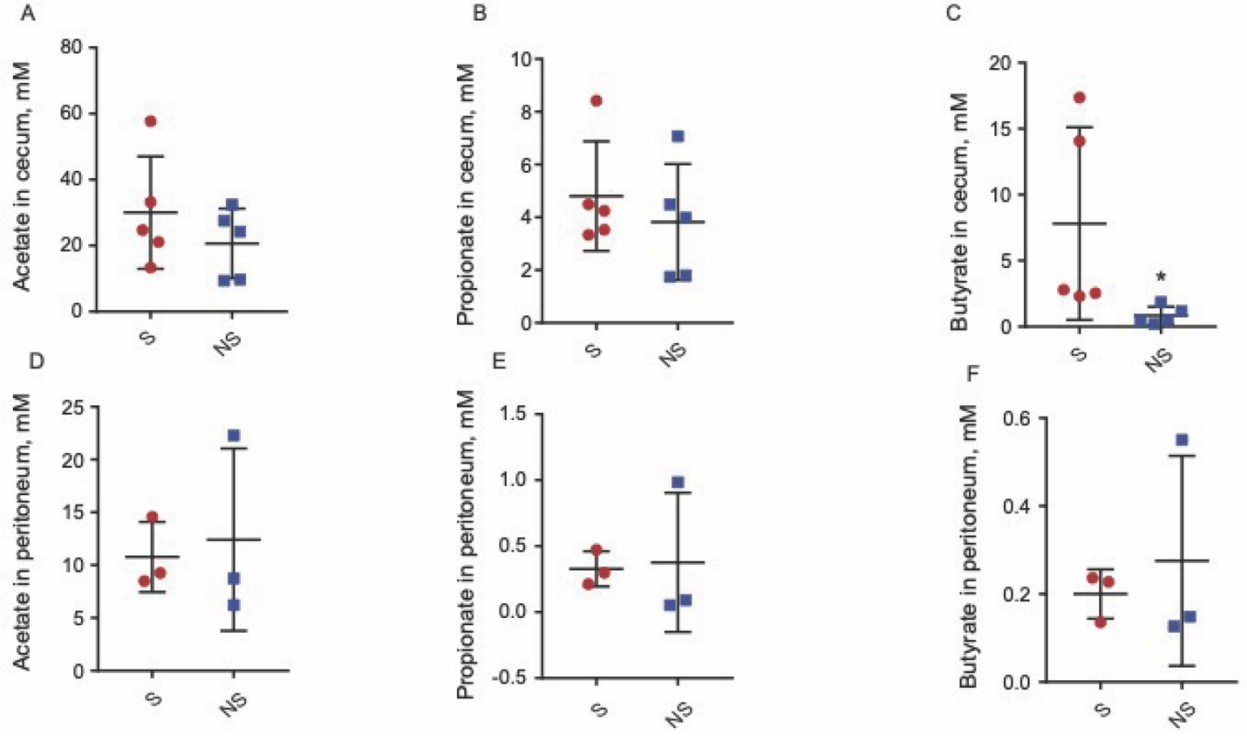



**Figure 25:Peritoneal SCFAs do not correlate with survival from Sm peritonitis** Cecal SCFAs [Acetate (**A**), Propionate (**B**), Butyrate (**C**)] in surviving and non-surviving mice (n = 5). As well as peritoneum SCFs [Acetate (**D**), Propionate (**E**), Butyrate (**F**)], n = 3 per group.

*Aryl hydrocarbon receptor is required for survival from S. marcescens peritonitis*

Indoles are known to activate the aryl hydrocarbon receptor (AhR), an intracellular receptor expressed within immune cells that has been demonstrated to impact gene expression and cell phenotype[181,322]. Therefore, we hypothesized that tryptophan metabolites are involved in the mechanisms by which the gut microbiota regulate the immune response in this model and drive survival in an AhR dependent manner[179,181].

To test this, the AhR was inhibited using the small molecule inhibitor, StemRegenin1, which resulted in increased mortality in this model. Furthermore, administration of an FMT in the presence of StemRegenin1 no longer prevented mortality (**Figure 24G**). Additionally, AhR inhibition was associated with a significant increase in *S. marcescens* in the peritoneum (**Figure 26A)** and its dissemination in blood (**Figure 26B**). Given the requirement of macrophages for survival in this model, we next determined if AhR, specifically within macrophages, was required for survival. Utilizing a macrophage specific murine AhR knockout construct (LysM-Cre x AhR fl/fl), we observed a significant increase in mortality when macrophages lacked AhR (**Figure 24H**), a significant lowering of body temperature (**Figure 26C**) and a higher sepsis score (**Figure 26D**).

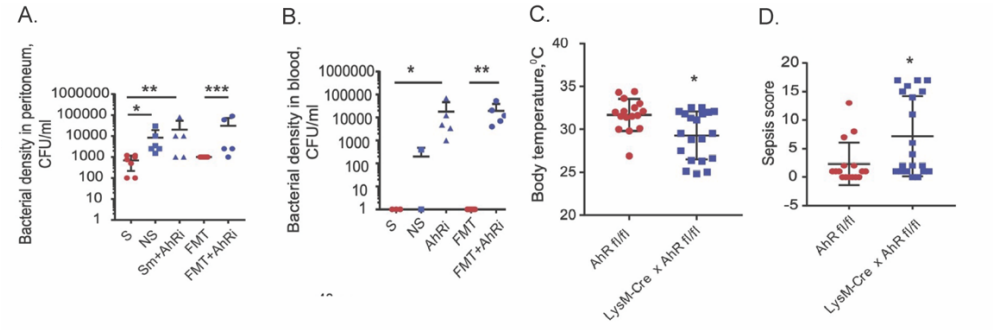



**Figure 26: AhR inhibition increases bacterial dissemination and clinical severity of Sm peritonitis** Differences in bacterial dissemination in the peritoneum (A) and blood (B) of mice treated with AhR inhibitor (n = 5 per group). Differences in body temperature (C) and sepsis score (D) in macrophage specific knockout mice (LysM-Cre x AhR fl/fl), n = 20 per group. * = $p < 0.05$

*AhR signaling in peritoneal macrophages results in increased M2 associated transcription factors and increased IL-10 signaling in surviving mice*

Given that both AhR signaling and macrophages are required for survival following an $LD_{50}$ dose of intraperitoneal *S. marcescens*, we performed a transcriptional analysis using RNA

sequencing of peritoneal macrophages at 8 hours post- IP injection of *S. marcescens* across the following groups: surviving mice, non-surviving mice, mice treated with AhRi, mice administered an FMT, and mice treated with AhRi and FMT. Peritoneal macrophages from uninfected mice were used to normalize data to controls. Volcano plots demonstrate differentially expressed genes (logFC >1.5 and FDR < 0.05) between pMACs from surviving mice compared to pMACs from non-surviving mice (**Figure 27A**) and pMACs from FMT treated mice compared to non-surviving mice (**Figure 28A**). Further, DEGs were compared between Survivors, Non-Survivors, and FMT treated mice to identify individual genes associated with survival. There were 826 unique genes to survivors, 468 genes unique to non-survivors, and over 2,300 unique to FMT treated mice (**Figure 27B**).

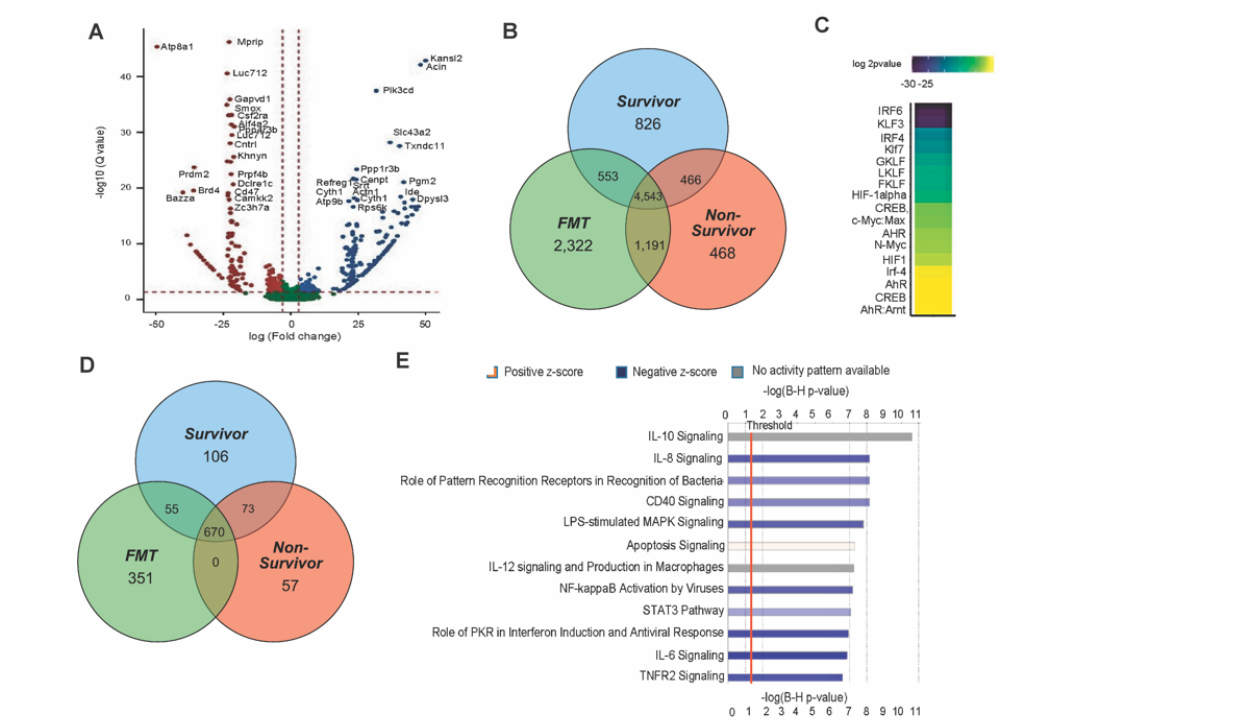



**Figure 27:Transcriptional analysis of peritoneal macrophages**

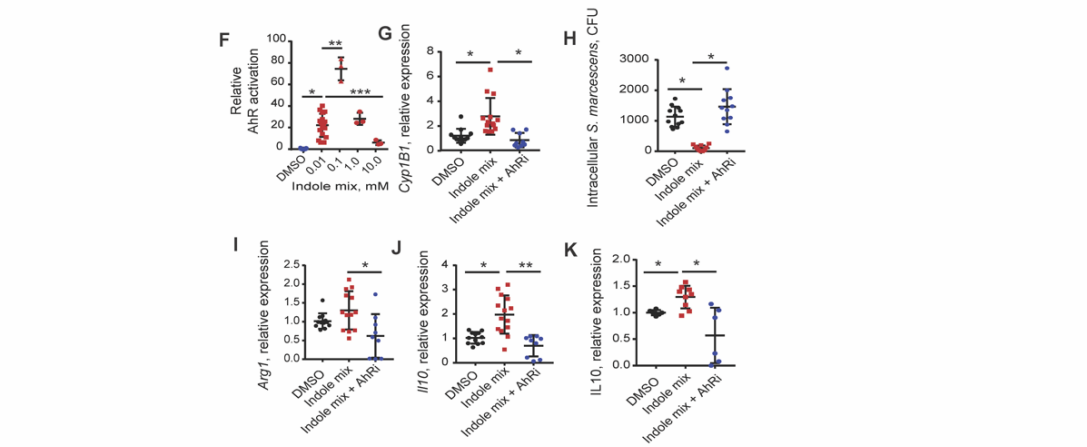



**Figure 27 (continued): Transcriptional analysis of peritoneal macrophages isolated at 8h post i.p. S. marcescens demonstrates distinct profiles of surviving and FMT treated mice in an AhR dependent manner** RNA sequencing was performed on pMACs isolated from survivors, non-survivors, n=5 per group. (**A**), Volcano plots demonstrating differentially expressed genes (logFC >1.5 and FDR < 0.05) between pMACs from surviving mice compared to pMACs from non-surviving mice (**A**). A Venn diagram demonstrating number of distinct DEG between Survivors, Non-Survivors, and FMT treated mice when expression is normalized to uninfected mice (NT). (**C**), Gene set enrichment with g:Profiler utilizing differentially regulated genes in survivors compared to non-survivors which demonstrated significant differences in transcription factors important for macrophage polarization and AhR. (**D**),A Venn diagram demonstrating shared ImmuneSigDB Gene sets shared between Survivors, Non-Survivors and FMT treated mice utilizing DEGs for each group when compared to NT mice. (**E**), On ingenuity pathway analysis, comparing survivors to AhRi resulted in significant differences in macrophage response to *S. marcescens* including IL-10 signaling. (**F**), Concentration- dependent effect of indoles on AhR activation using a mouse hepatoma H1L1.1c2 AhR reporter cell line. n=12 for DMSO (vehicle control) and indoles mix at 0.01 mM concentration, and n=3 for each of 0.1, 1.0 and 10 mM concentrations. Results are pooled from 7 independent experiments. p<0.0001, One-way ANOVA; *p<0.0001, **p=0.0018, ***p=0.0123, Mann-Whitney unpaired t-test. (**F**), Relative expression of *Cyp1b1* representing AhR activation in BMDMs following stimulation with indoles. (**G**), Intracellular killing of *Serratia marcescens* by BMDMs. Gentamicin protection assays were conducted using bone marrow derived macrophages (BMDM) exposed to a 0.01 mM indole mixture at the time of exposure to *Serratia marcescens* (multiplicity of infection of 5) and resulted in a significant increase in macrophage killing which was abrogated during AhR inhibition. n= 11 per group, p<0.0001, One-way ANOVA test; *p<0.0001, Mann-Whitney unpaired t-test. (**I-K**), Relative expression of *Arg1* (**I**), *Il10* gene expression (**J**), and IL-10 protein (**K**) in BMDMs after exposure to *Sm*. (**C**): p<0.0001, One-way ANOVA test; *p<0.0001, Mann-Whitney unpaired t-test. (**H**): p=0.0058, One-way ANOVA test; *p-0.0089, Mann-Whitney unpaired t-test. (**I**): p=0.0001, One-way ANOVA test; *p=0.0001, **p=0.0008, Mann-Whitney unpaired t-test. (**J**): p<0.0001, One-way ANOVA test; *p=0.0004, **p=0.0002, Mann-Whitney unpaired t-test. (**K**), IL10 protein expression by ELISA. p=0.014, One-way ANOVA test; *p-0.0176, Mann-Whitney unpaired t-test.

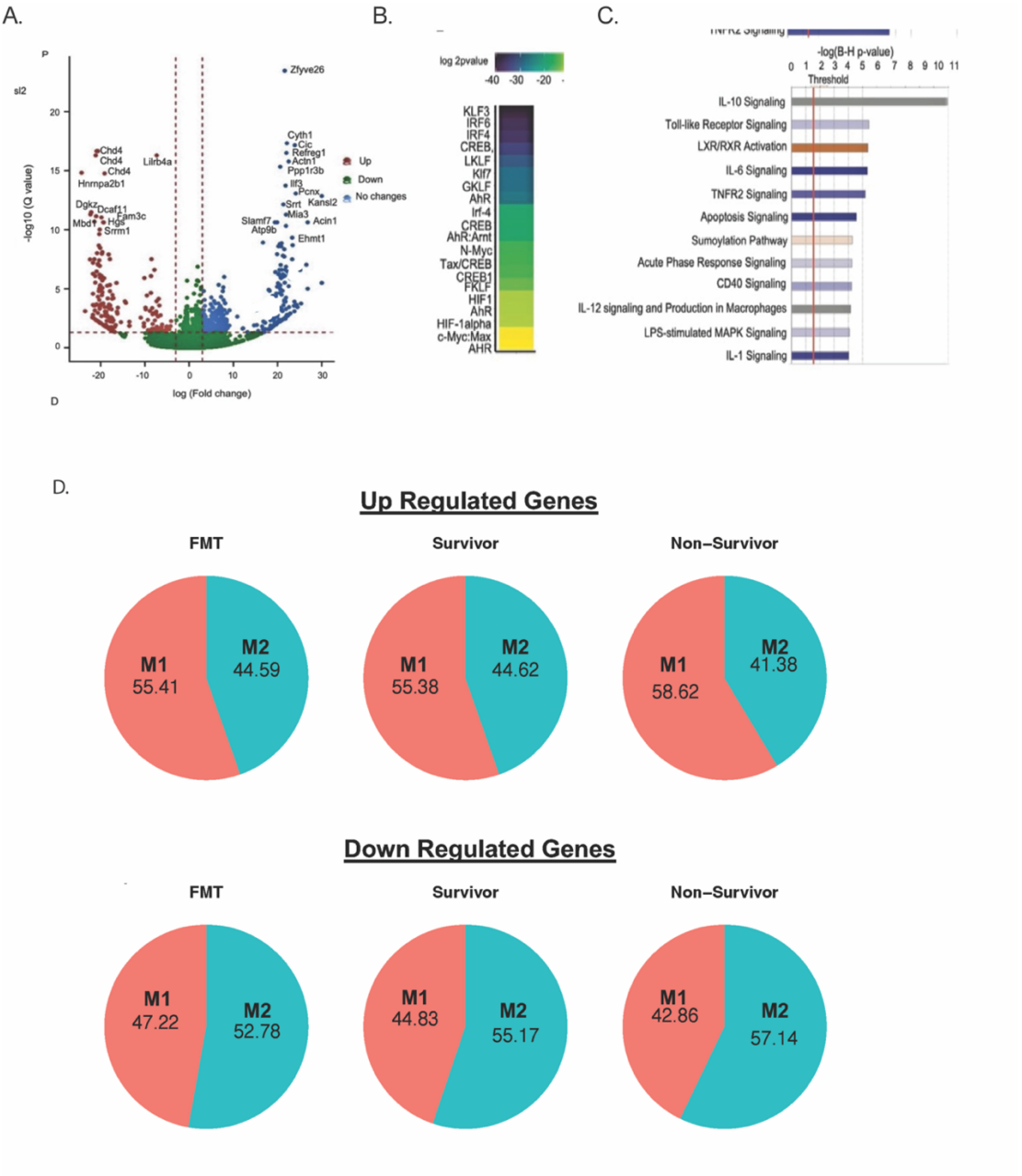



**Figure 28: RNA sequencing demonstrates unique macrophage phenotype that correlates with survival from Sm peritonitis** RNA sequencing analysis for macrophages isolated at 8h post infection in FMT mice compared to macrophages from non-surviving mice (**A**). Gene set enrichment analysis of FMT macrophages and g:Profiler to determining significantly altered transcription factors (**B**). Ingenuity pathway analysis comparing significant pathways determined between FMT and FMT + AhRi mice (**C**). Using genes previously identified to be associated with M1/M2 macrophage phenotype, the percentage of genes significantly up regulated and down-regulated were compared survivors, non-survivors, and FMT treated mice (**D**).

Interestingly, DEGs that were specific to macrophages from surviving mice included genes that have previously been associated with endotoxin tolerance and regulation of inflammation. For example, Ripk2 limits inflammation related to inflammasome activation [331], *Dcp1 and Trdmt1* have both been associated with endotoxin tolerance [332,333], and increased *Clu* expression which prior studies demonstrated reduced expression in patients who were non-survivors of sepsis [334]. Furthermore, individual genes that were oppositely expressed between survivors and non-survivors were notable to include: *Pik3cd* which is an important regulator of NF-kB and M1 polarization [335], *Lpin1* which is an important regulator of M2 macrophages[336], *Sec14l1* which is a negative regulator of RIG and M1 polarization [337], and *Slamf7* which has bene shown to limit inflammation in the setting of polymicrobial sepsis [338], *Cd47* which negatively regulates NF-kb [339], and *Hif1a* which is important for metabolic regulation of macrophages [340]. These findings of individual gene expression demonstrate that overall there is some suppression of the immunopathology associated with the septic immune response within macrophages of surviving mice (**Figure 30)**.

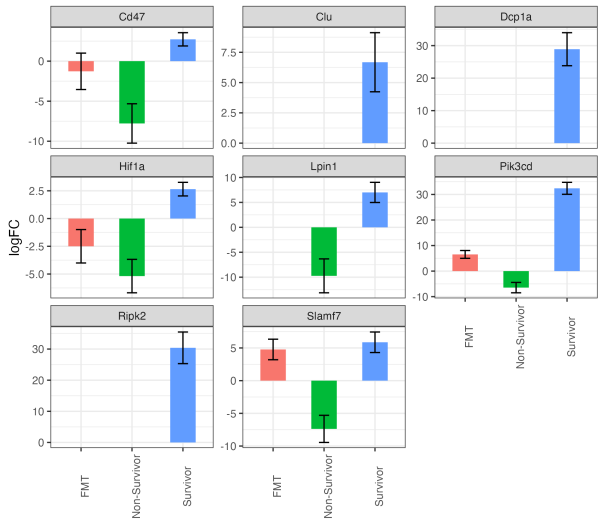



**Figure 29: Gene expression in macrophages associated with survival** Significant differentially expressed genes that were unique to survivors (*Clu, Ripk2, Dcp1a*) and oppositely expressed in survivors compared to non-survivors (*Cd47*, *Hif1a, Lpin1, Pik3cd, and Slamf7)*

Results indicate a distinct peritoneal macrophage gene expression pattern is associated with survival in this model. Using gene set enrichment analysis of differentially regulated genes (DRGs) between the various groups (**Figure 27B, Figure 28B**), we observed significant differences in predicted transcription factors regulating gene expression, including AhR, among macrophages from surviving and FMT treated mice. Transcription factors known to be important for macrophage polarization including IRF6, IRF4, KLF family of transcription factors, HIF-1a, c-Myc and CREB were found to be significantly altered between surviving and non-surviving mice (**Figure 27B)** and FMT treated mice compared to non-surviving mice (**Figure 28B)**. Furthermore, when gene set enrichment analysis was used utilizing ImmuneSigDB, there were significantly altered gene sets unique to each individual group (**Figure 27C).** Genes specific to M1/M2 macrophages [341,342] were compared between survivors, non-survivors, and FMT treated macrophages demonstrating a higher percentage of M2 up-regulated genes and M1 down-regulated genes in survivor and FMT treated mice compared to non-survivors (**Figure 28D)** To

better understand how AhR-regulated macrophages are characterized in surviving mice, Ingenuity Pathway Analysis (IPA) was applied to the transcriptional profiles of pMACs isolated from survivor versus AhRi treated mice (**Figure 27D)** as well as to pMACs isolated from FMT treated mice versus FMT+AhRi (**Figure 28C)**. IPA demonstrated that a significant impact of AhR on IL-10 signaling, toll like signaling, IL-6 and IL-12 production, and apoptosis within macrophages in both groups (**Figure 27D, Figure 28C**). Of note, CREB has been previously demonstrated to interact with AhR and regulate NF-kB signaling and IL-10 production [343–345]. AhR has the ability to affect the gene expression profile of pMACS which may interact with a number of different transcription factors, but has previously been demonstrated to regulate IL-10 production [207,330]. Therefore, AhR's role in macrophage-mediated clearance of infecting bacteria appears to be critical for survival in this model.

*Indole ligands alter macrophage phenotype and increase <u>in vitro</u> killing of <u>S. marcescens</u> via AhR activation*

In order to confirm the ability of the indole ligands to activate AhR *in vivo*, we tested the activity of the identified indoles *in vitro* using an AhR-luciferase reporter cell line. To recapitulate the composition of indoles observed within the peritoneum *in vivo* into the *in vitro* reporter system, we assembled a multi-component "indole mix" consisting of indole-3-carboxaldehyde, indole-3-acetic acid, indole-3-lactic acid, and tryptophol at various concentrations. We observed an indole-dependent increase in AhR signaling at lower doses between 0.01 mM and 0.1 mM (**Figure 27F**). Paradoxically, at high concentrations, suppression of AhR signaling was observed beginning at 5 mM (**Figure 27F**). This pattern of activation and inhibition was also observed when individual metabolites were similarly tested (**Figure 30A**).

However, unlike indoles, butyrate was observed to function as a very weak AhR agonist (**Figure 30B**).

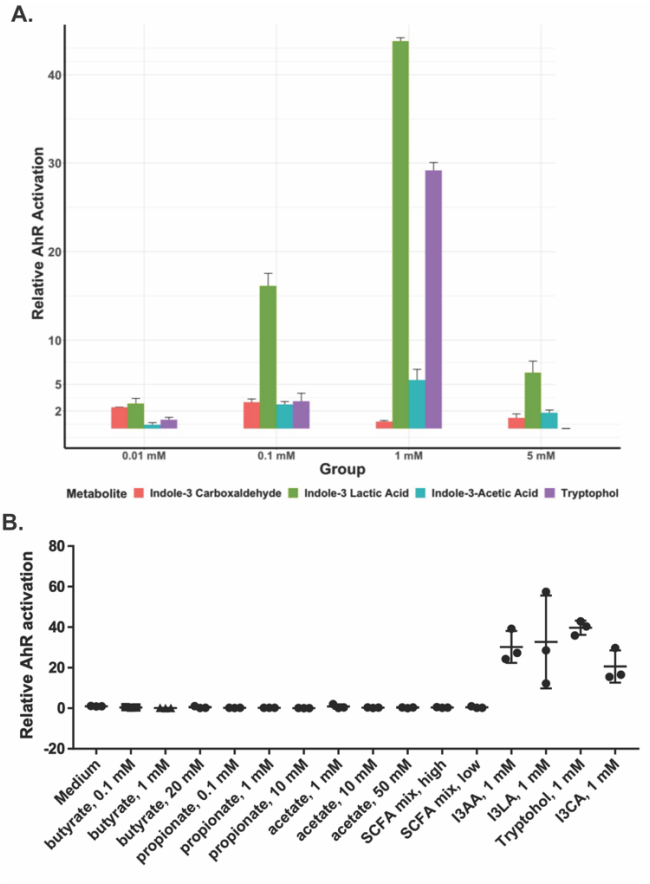



**Figure 30: SCFAs do not activate AhR *in vitro*** (A), A mouse hepatoma AhR reporter cell line was utilized comparing different doses of indoles. (B), The AhR reporter cell line was utilized to test different concentrations of SCFAs on their ability to activate AhR.

To elucidate the impact of the selected indole metabolites and AhR signaling on the macrophage response to *S. marcescens,* bone marrow derived macrophages (BMDMs) were exposed to *S. marcescens* in the presence and absence of the indole mix. Activation of AhR in BMDMs in response to the indoles was confirmed by examining the expression of *Cyp1b*, a canonical AhR-activated downstream gene (**Figure 29G**). Bactericidal activity was then measured utilizing gentamicin protection assays in which BMDMs were exposed to the indole

mix in the presence of *S. marcescens.* When exposed to the indole mix, BMDMs had a significant increase in intracellular killing of *S. marcescens*, which in turn was abrogated by AhR inhibition (**Figure 29H**). Next, BMDMs were exposed to lysates of *S. marcescens* in the presence and absence of the indole mixture. Similarly to the transcriptional alterations observed *in vivo*, indole metabolites significantly increased the expression of *Arg1* and *Il10* (**Figure 29I,J)** without affecting the production of pro-inflammatory genes (**Figure 31).** Taken together, these results suggest that AhR signaling by indole metabolites can enhance bacterial clearance while potentially limiting any associated immunopathology. AhR is known to interact with the transcription factor CREB in macrophage signaling, and this was confirmed in our model in further *in vitro* experiments in which BMDMs were co-cultured with the indole mix and *S. marcescens* lysates demonstrating increased IL-10 protein production by BMDMs (**Figure 29K**). These data demonstrate that indole metabolites can modify macrophage responses to *S. marcescens*.

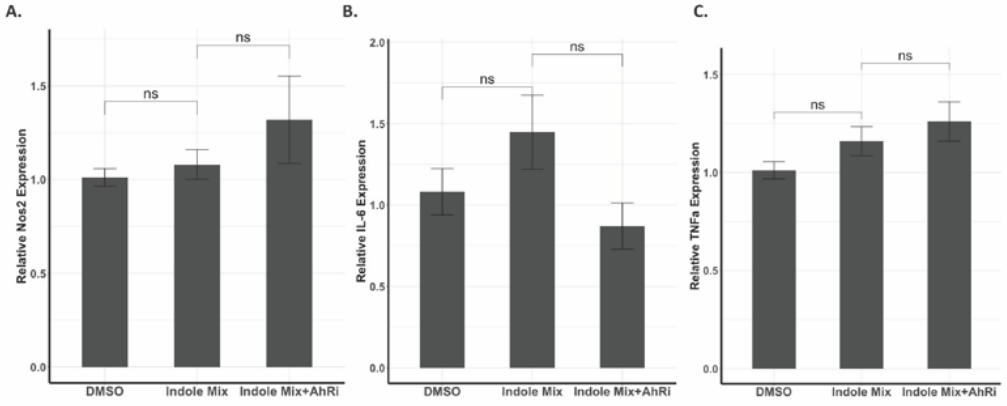



**Figure 31: AhR does not impact macrophage pro-inflammatory response to Sm *in vitro***
BMDMs were exposed to SM lysate in the presence of DMSO, 0.01mM Indole Mix, or 0.01mM Indole Mix + AhR inhibitor and gene expression of *Nos2*, *Il6*, and *Tnfa* was compared.

*Secreted products of S. marcescens and other clinically relevant pathogens inhibit indole-induced AhR activation*

Multiple lines of evidence are accumulating to demonstrate that the success of a pathogen to cause lethality in its host is dependent on its ability to subvert the immune system [346]. As such, we next determined whether the infecting pathogen in this model, *S. marcescens*, could itself adversely affect AhR activation by interfering with the effect of indoles on macrophage function. Therefore *S. marcescens* lysates, surface proteins, and supernatant were tested for their effect on AhR activation *in vitro*. *S. marcescens* lysate was demonstrated to be a very weak agonist, whereas *S. marcescens* supernatant and surface proteins did not activate AhR (data not shown). Therefore, we next determined if the exoproduct of *S. marcescens* (i.e., its supernatant) was able to suppress AhR activation in response to the presence of the indoles, given our observations that indoles are potent activators of AhR. Results indicated that when the AhR reporter cell line was first exposed to *S. marcescens* supernatant, indole activation of AhR was significantly inhibited in a manner dependent on the growth phase of *S. marcescens*, with escalating AhR inhibition using supernatant derived from bacteria at late log phase growth versus early log phase growth (**Figure 32A,B**). When *S. marcescens* supernatant was fractionated by molecular weight, the < 3kD fraction maintained its ability to inhibit indole activation of AhR whereas fractions > 3kD fraction did not (**Figure 32C**). These findings suggest that *S. marcescens* itself can secrete a low molecular weight compound capable of counteracting the AhR- activating gut microbiota-derived indole metabolites. Therefore, it is possible that the extent to which products produced by infecting pathogen inhibit AhR activity, and the extent to which this effect can be mitigated by products from the gut microbiota (i.e., indoles), may determine survival versus mortality in this model. However, further elucidation of the molecular details of this interaction is needed to define a more

generalizable principle to explain within-group variability to a standardized infectious challenge [346].

In order to determine if the observed AhR inhibition in response to gut microbiota-derived indoles was generalizable to other pathogens relevant to human sepsis, we performed reiterative assays in response to additional pathogens of interest. We utilized our AhR reporter cell line and exposed the cells to indoles in the presence of supernatant isolated from other bacteria. We first tested if the supernatants isolated from the bacteria were cytotoxic (**Figure 32D**). We observed that supernatant from *Pseudomonas aeruginosa* strains were all cytotoxic which likely confounded their ability to suppress AhR; whereas, the remaining pathogens tested were not cytotoxic. Of the strains tested, both multidrug resistant *S. marcescens* (previously designated as ICU1-2 [224] and a virulent strain of *S. marcescens* MVI inhibited indole activation of AhR. Both *Klebsiella oxytoca* (previously designated as ICU1-2) *and Klebsiella pneumoniae* (previously labeled as ICU3-1) also inhibited the activation of AhR in a manner similar to *S. marcescens* (**Figure 32E)**. AhR inhibition was also seen with gram positive pathogens, *Staphylococcus aureus* and *Enterococcus faecalis*, as well as the common fungal pathogen, *Candida albicans* (**Figure 32E)**. Similar to *Serratia* marcescens, inhibition of AhR by both *Klebsiella* species and *Enterococcus faecalis* was dependent on the phase of growth, with increased AhR suppression at later phases of growth (**Figure 32F)**. Additionally, supernatants of selected pathogens were fractionated by molecular weight, and similar to our findings seen with *S. marcescens*, inhibition of AhR activation only occurred within the < 3kD fraction and not within the > 3kD fraction (**Figure 32G**). The conserved response of AhR inhibition herein observed supports an important

role by which both gut microbiota-derived metabolites of tryptophan (i.e. indoles) and those of the pathogen, converge on AhR signaling as a potential determinant of their lethality.

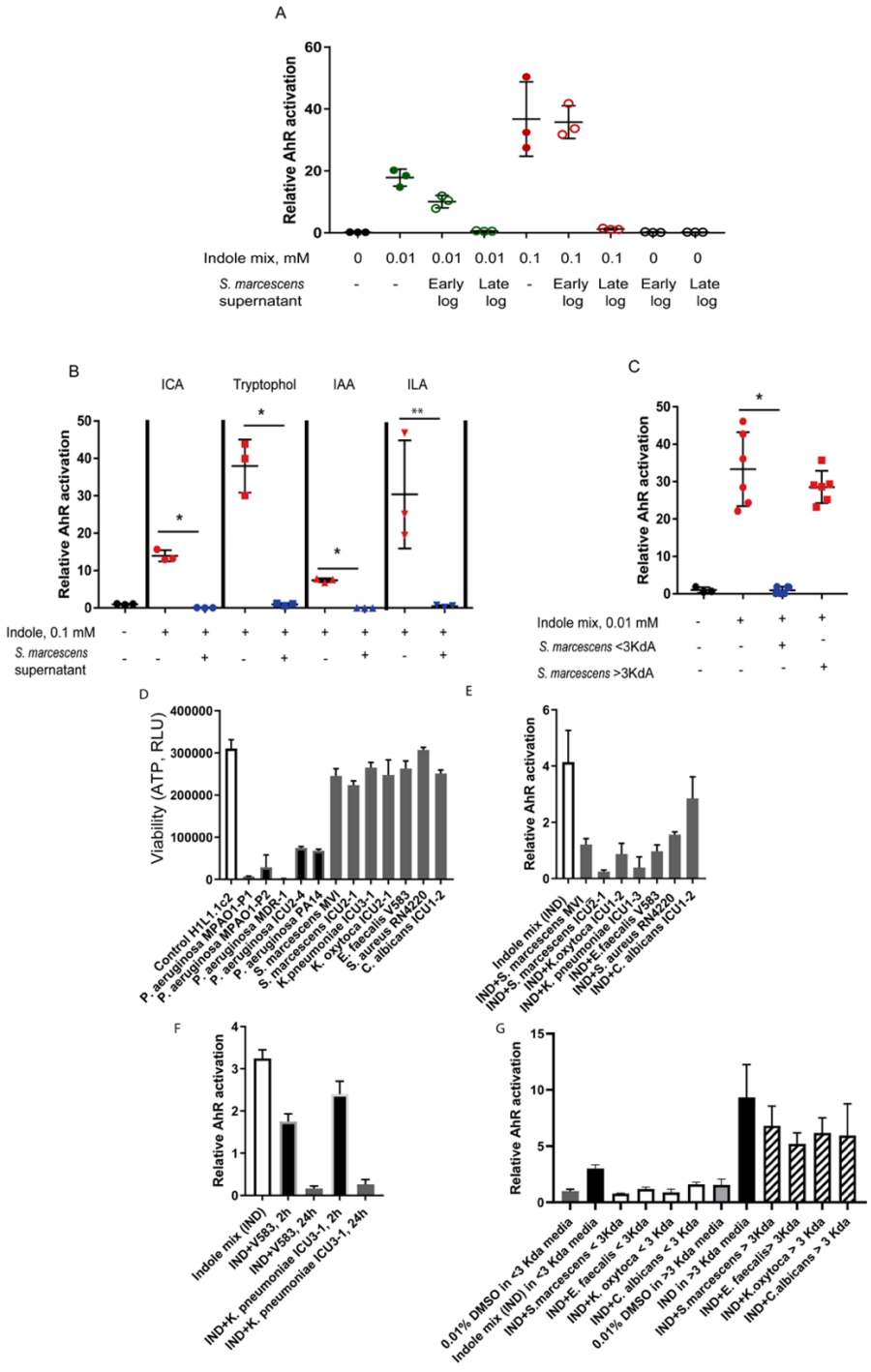



**Figure 32: S. marcescens exoproduct inhibits indole-mediated AhR activation** The Impact of *S. marcescens* on AhR signaling was studied *in vitro*. Filtered supernatant was collected from *S.*

**Figure 32 (continued):** *marcescens* at early and late log phase. The AhR reporter cell line was exposed to filtered supernatant in the presence and absence of the indole mixture to determine its impact on AhR signaling. (**A**) Supernatant from the late log phase resulted in significant repression of AhR activation in the presence of the indole mix at 0.01 mM and 0.1 mM. $p<0.0001$, One-way ANOVA. *p = 0.0004, **p=0.0062, unpaired t-test. (**B**) Supernatant from the late log phase resulted in significant repression of AhR activation in the presence of the individual indoles at 0.1 mM. $p<0.0001$, One-way ANOVA. *p <0.0001, **p=0.0231, unpaired t-test. (**C**), The <3 kDa fraction of the supernatant represses the activation of AhR by the indole mix at 0.01 mM. $p<0.0001$, One-way ANOVA. *p <0.0001, unpaired t-test. Supernatant was collected from different clinically relevant pathogens to determine their ability to inhibit indole activation of AhR *in vitro*. NOTE: Supernatants were non-cytotoxic to the cell line (D). The AhR reporter cell line (H1L1.1c2) was then exposed to the indole mixture in the presence of supernatant from pathogens that were not cytotoxic to determine their ability to inhibit indole activation of AhR (E). *Enterococcus faecalis* and *Klebsiella pneumoniae* demonstrated an AhR inhibition that was strongly dependent on growth phase (F). Pathogen supernatants were then fractionated by molecular weight to determine if size (i.e., < 3kD) was a discriminatory factor in the observed inhibition (G).

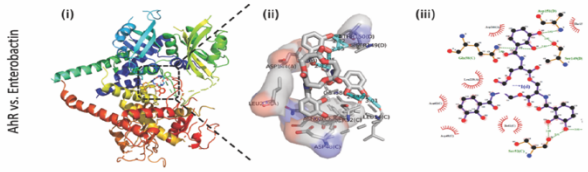
*The secreted virulence metabolite, enterobactin, is capable of suppressing indole-mediated activation of the aryl hydrocarbon receptor in vitro and increases mortality in vivo following intraperitoneal Serratia marcescens infection.*

Given the observation that a soluble factor from various pathogens can inhibit indole-mediated AhR activation, further experiments were conducted to identify the inhibitory ligand. *In silico* modeling of AhR interactions with potential microbial ligands was determined via molecular docking. Based on the docking score, we identified several potential microbial candidates capable of interacting with AhR (**Figure 33)** including enterobactin, prodigiosin, tilimycin, tilivalline and melanin, common secondary metabolites of the microorganisms studied.

A.

| Ligand | Receptor | Docking score | | B.E. (AD Vienna) (kcal/mol) |
|---|---|---|---|---|
| | AhR | | | |
| Prodigiosin | | -7.2 | | -7.6 |
| Serratomolide | | -5.1 | | -6.5 |
| Melanin | | -9.1 | | -9.2 |
| Threonine | | -5 | | -4 |
| **Indole-3-acetic acid** | | **-6.2** | **Activator (known)** | **-5.8** |
| L-onithine | | -4.8 | | -4 |
| Biliverdine | | -6.5 | | -7.7 |
| Tilimycin | | -7.8 | | -7.4 |
| Tilivalline | | -7.3 | | -10.1 |
| Enterobactin | | -8.4 | | -9.4 |
| Serratiochelin | | -5.4 | | -7.9 |
| N-acyl homoserine lactone | | -5.4 | | -4.2 |
| Farnesol | | -5.4 | | -5.3 |
| Tyrosole | | -4.9 | | -4.7 |
| Serrawetins (exolipid) <Serrawetins W2> | | -5.3 | | |
| Serrawetins (exolipid) <Serrawetins W1> | | -5.7 | | |
| | | | | |
| **AhR agonist** | | | | |
| Indole acetic acid (Indole-3-acetic acid) | | **-6.2** | **Activator (known)** | -5.8 |
| Indole lactic acid | | **-6.9** | | |
| Tryptophol | | **-5.7** | | -5.8 |
| Indole carbaldehyde <1-Methyl-1H-indole-3-carbaldehyde> | | **-6** | | -5.6 |
| | | | | |
| **AhR antagonist** | | | | |
| Stemregenin | | **-8.5** | | -8.8 |

B.




C.

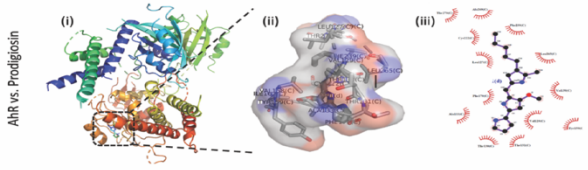



**Figure 33: *In silico* prediction of microbial inhibitors of AhR** Molecular docking was used to predict potential AhR antagonist secreted by bacteria. Table lists known agonists alongside predicted antagonists with docking score and binding energy (B.E.) shown (**A**). Representative (i) three-dimensional binding mode, (ii) surface representation, and (iii) two-dimensional illustration of molecular docking interaction between **(B)** enterobactin with AhR (binding energy = -9.4 kCal/mol) and **(C)** prodigiosin with AhR (binding energy = -7.6 kCal/mol; using AutoDock Vina. Two-dimensional illustration of molecular docking interaction shows the binding of specific ligand with various residues of AhR using LigPlot$^{+}$. The PDB ID for AhR is 5NJ8.

Interestingly, out of the candidate compounds, whole genome sequencing demonstrated that both *Klebsiella* [224] and *Serratia* species utilized in the above experiments contained the genes

necessary to produce enterobactin. Furthermore, when the binding between enterobactin and AhR was evaluated *in silico*, it was determined that enterobactin, similar to the AhR antagonist Stemregenin, had more stable binding to AhR than indole-3-acetic acid, further implicating its ability to strongly inhibit indole activation of AhR (**Figure 33A**, **Figure 34A)**. Mass spectrometry was utilized to determine the ability of *Serratia marcescens* MVI and *Klebsiella oxytoca* to produce enterobactin, and enterobactin was detected in supernatants collected from both *Klebsiella oxytoca* and *Serratia marcescens* (**Figure 34B)**. When tested *in vitro*, enterobactin significantly inhibited indole activation of AhR (**Figure 34C**) Importantly, the concentrations at which enterobactin suppressed AhR was not cytotoxic to the cells (**Figure 34D)**. These findings demonstrate, for the first time, that the virulence factor enterobactin inhibits host AhR signaling in response to indoles. We next determined if enterobactin was capable of increasing disease severity *in vivo*. When enterobactin was delivered at the time of infection with *Serratia marcescens,* there was a significant increase in mortality (**Figure 34E**). Enterobactin delivered at the doses tested did not cause mortality by itself. Taken together, these results indicate that pathogen secretion of enterobactin can subvert the host immune response by suppressing gut microbiota-derived, indole-mediated activation of AhR.

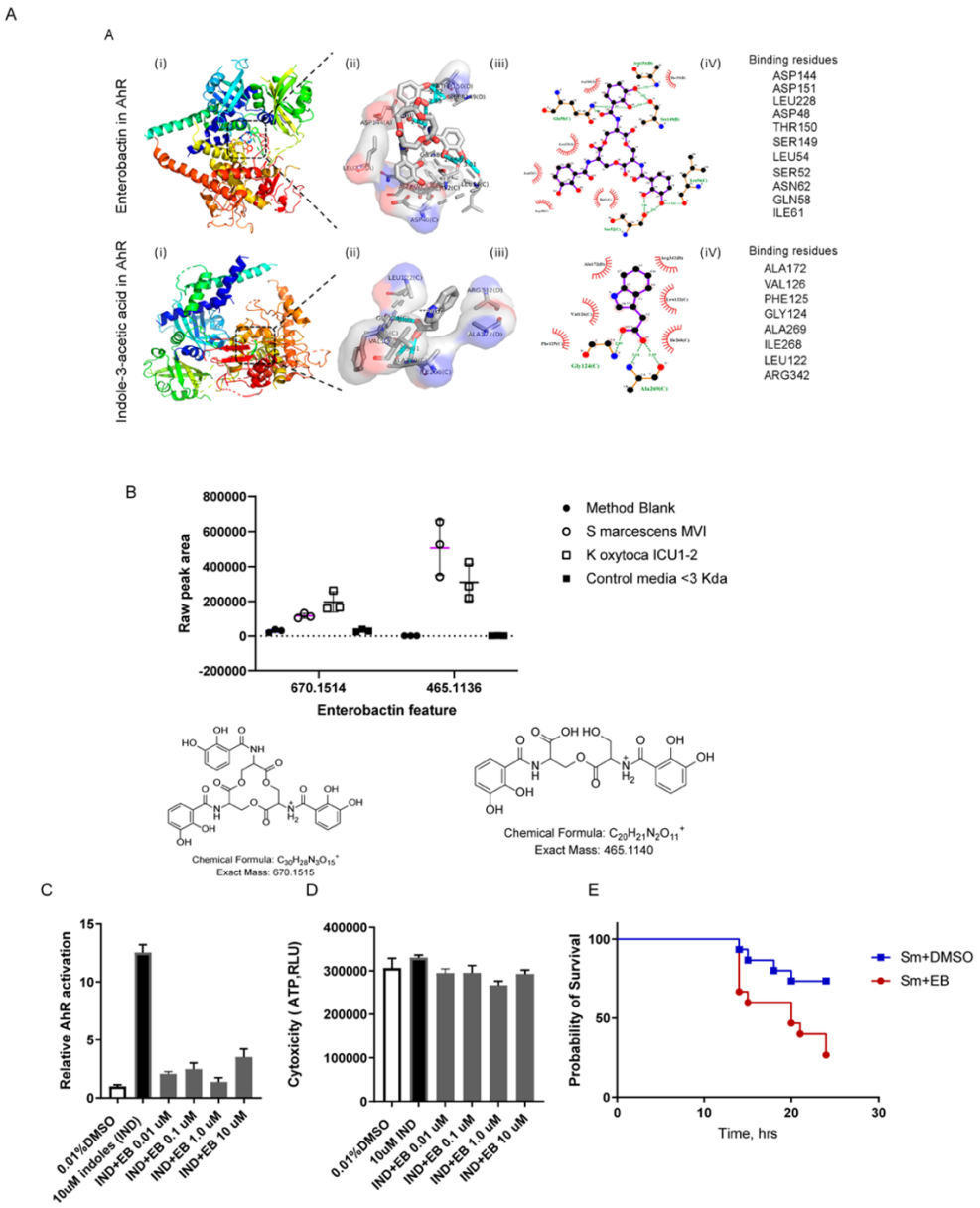



**Figure 34: Enterobactin secretion by pathogens can subvert the host immune response and plays a role in the mortality observed following i.p. *S. marcescens*** Autodock was utilized to determine potential microbial metabolites capable of interacting with AhR. Three-dimensional binding mode (i), surface representation (ii), and two-dimensional illustration of molecular docking between enterobactin and AhR and indole-3-acetic acid and AhR are demonstrated (**A**). Mass spectrometry demonstrates that both *Klebsiella oxytoca* and *Serratia marcescens* are capable of producing enterobactin as both complete enterobactin and components of enterobactin were detected in liquid culture (**B**). Enterobactin significantly inhibited indole activation of AhR *in* vitro without being cytotoxic (**C,D**). When enterobactin was delivered IP, simultaneously with *S. marcescens in vivo,* there was a significant increase in mortality compared to DMSO control, n = 15 per group (**E**).

Enterobactin, an iron chelator, has been repeatedly demonstrated to play an important role in iron chelation and pathogenicity of gram negative bacteria[347,348]. To determine whether or not iron chelation played a role in enterobactin's ability to inhibit AhR, we tested enterobactin's ability to inhibit indole activation of AhR in the presence or absence of iron *in vitro* (**Figure 35A**). Iron chelation did impact enterobactin's ability to inhibit AhR activation. Furthermore, dihydroxybenzoic acid (DHBA) is an enterobactin intermediate that also has the ability to play a role in bacterial virulence and can inhibit host metalloproteinase[349]. When DHBA was tested *in vitro*, we found that varying concentrations of DHBA could also inhibit AhR activation by indoles (**Figure 35B)**.

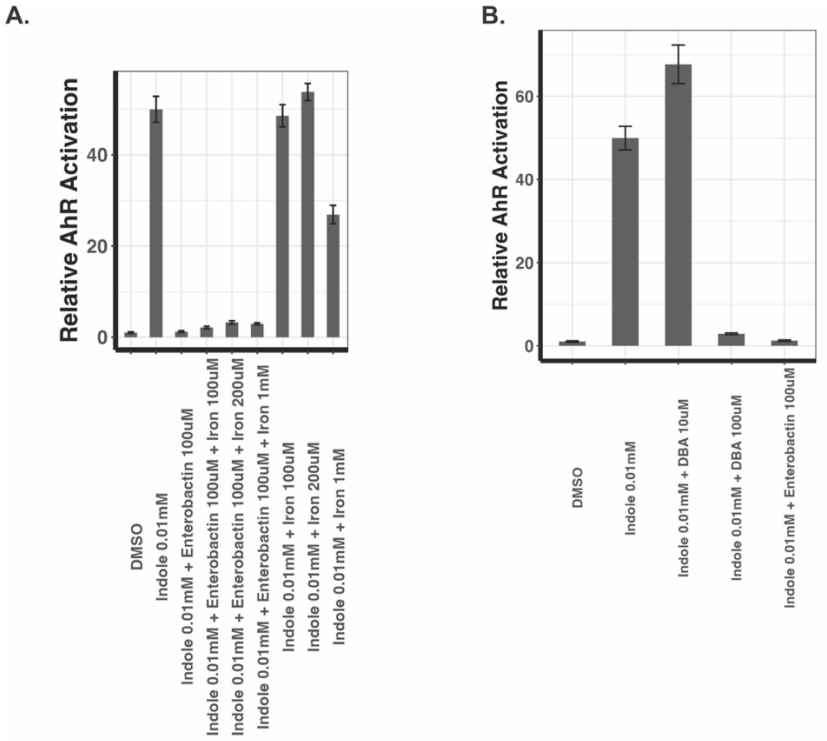



**Figure 35: Iron chelation does not impact Enterobactin inhibition of AhR *in vitro***
Enterobactin inhibition of indole activation of AhR is not impacted by iron saturation (**A**) and enterobactin intermediate DHBA inhibits indole activation AhR

*Oral tryptophan administration and systemic administration of indole metabolites improves survival from AhR inhibiting pathogens*

Despite testing various doses and dose schedules, oral indole administration did not improve survival in this model (data not shown). We speculated that this result may be a function of its complex consumption and metabolism within the gut microbiota as it passes from the mouth to the colon. Therefore, we next hypothesized that oral supplementation with tryptophan, as others have shown[16], would lead to increased indole production by the gut microbiota and improved survival from bacterial peritonitis with *S. marcescens, Klebsiella oxytoca*, and polymicrobial peritonitis (*S. marcescens*, *K. oxytoca*, *E. faecalis*, and *Candida albicans*). *K. oxytoca* was utilized as a second monomicrobial pathogen that produces enterobactin similarly to *S. marcescens. K. oxytoca is also* an emerging as a major health threat to humans with sepsis, and the pathogen community consisted of pathogens isolated from critically ill patients with sepsis [82,350,351]. We supplemented the drinking water of mice with 1 mM oral tryptophan for 14 days prior to peritoneal infection with AhR inhibiting pathogens. Supplementation with oral tryptophan resulted in a significant improvement in survival in *S. marcescens* (**Figure 36A)**, *K. oxytoca* (**Figure 36B)**, and polymicrobial peritonitis (**Figure 36C**). In mice that were not supplemented with oral tryptophan, there was a significant reduction in tryptophan and indole metabolites in the cecum following infection (**Figure 36D,F)**. Oral tryptophan supplementation maintained gut tryptophan and indole levels following infection (**Figure 36E,G)**.

We next determined whether systemic administration of indole metabolites would be sufficient to prevent mortality in mice following infection with pathogens capable of AhR inhibition (*S. marcescens, K. oxytoca,* and the polymicrobial community*)*. To test this, we injected the indole mix (indole-3-carboxaldehyde, indole-3-acetic acid, indole-3-lactic acid, and

tryptophol) intraperitoneally simultaneous with a lethal dose of *S. marcescens, Klebsiella oxytoca, or PC*. The indole mix administered into the peritoneum at 0.01 mM significantly increased survival compared to DMSO vehicle-treated mice after intraperitoneally inoculation with *S. marcescens* and *K. oxytoca* (**Figure 36H,K**). As previously established, surviving mice displayed lower sepsis scores and higher core body temperatures (**Figure 36I,J**). Reiterative experiments using the human polymicrobial pathogen community (*C. albicans, E. faecalis, S. marcescens, K. oxytoca*) did not demonstrate enhanced survival (data not shown). The indole mix was unable to rescue LysM-Cre x AhR fl mice demonstrating AhR is required for its survival effect (**Figure 36L)**.

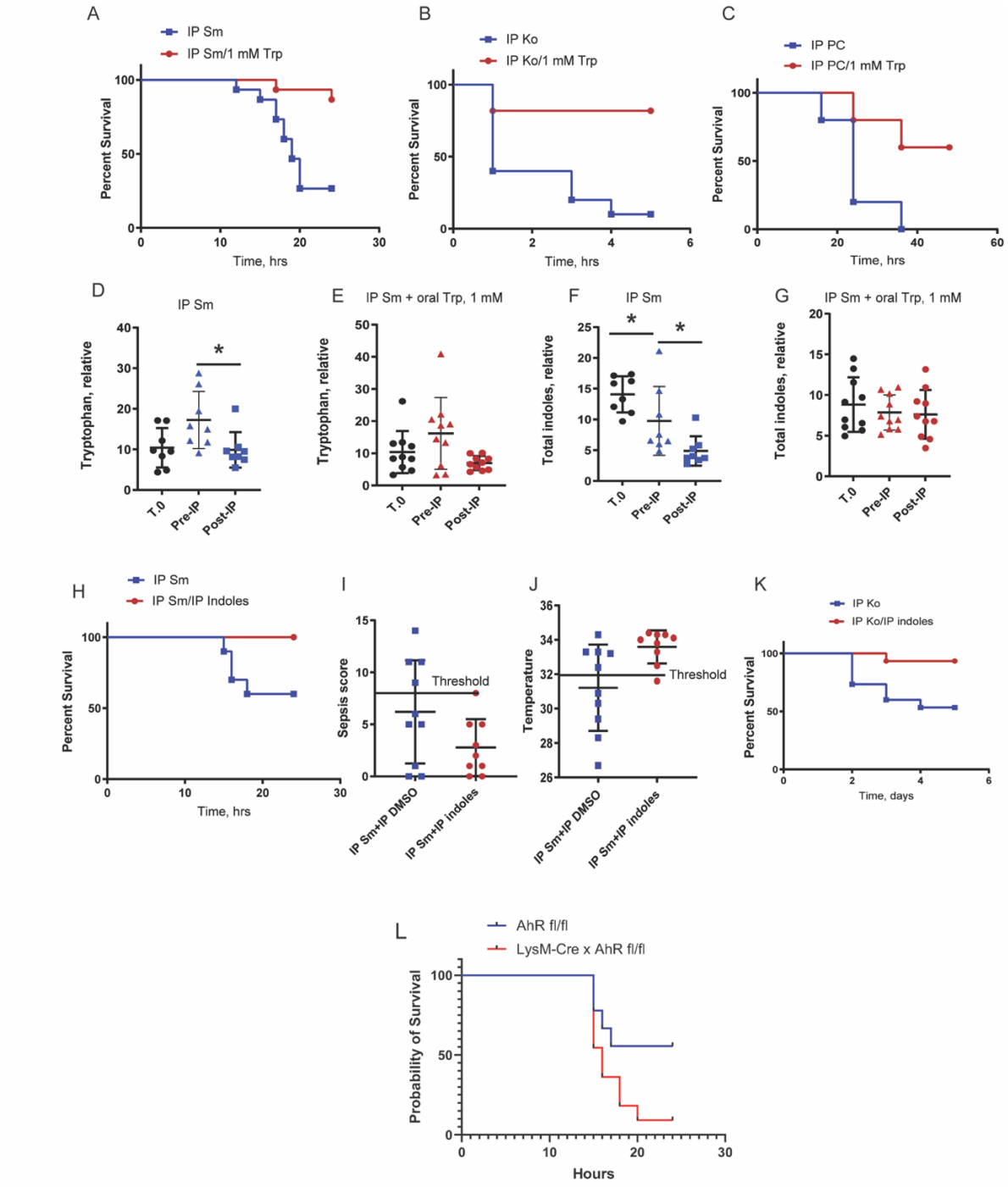



**Figure 36: Systemic delivery of the indole mixture or oral supplementation with tryptophan prevents mortality in mice following both monomicrobial and polymicrobial peritonitis** Mice were supplemented with oral tryptophan (1mM) in their drinking water for 2 weeks prior to IP infection demonstrated a significant improvement in survival folloiwng IP *S. marcecens* (**A**), *K. oxytoca* (**B**), and polymicrobial peritonitis (PC: *Klebsiella oxytoca, Enterococcus faecalis, Serratia marcescens, and Candida albicans)* (**C**). Metabolomics analysis of the stool

**Figure 36 (continued):** demonstrated that that tryptophan supplementation did not significantly increase stool tryptophan prior to infection (**D**), however a significant decrease in gut tryptophan was observed following IP pathogen injection (**D**) that was prevented by tryptophan supplementation (**E**). Similarly,we observed a significant decrease of indoles following i.p pathogen injection (F) that was not observed with Trp supplementation (**G**). Systemic delivery of the indole mixture of 0.01 mM delivered IP at the time of infection with Sm peritonitis also demosntrated a significant improvement in survival. Kaplan-Meyer survival curves, n=10 per group, p=0.0291, Log-rank (Mantel-Cox) test (**H**). (**I**), Sepsis score. All indole-treated mice sepsis scores were below threshold (sepsis score=8). (**J**), Core body temperature. All indole-treated mice body temperatures were over the threshold (32°C). (**K**), Mice were rescued with 0.01 mM indole mixture delivered i.p at the time of infection with *Klebsiella oxytoca* peritonitis. (**L**), LysM-Cre x AhR fl/fl mice and AhR fl/fl control mice were injected i.p with Sm and treated with 0.01mM indole mix at the time of infection.

Stool and serum from two separate groups of septic patients were studied to determine if similar trends were seen in indole metabolites in humans. Serum isolated from septic patients demonstrated a significant reduction in indole propionic acid and indole acrylic acid (**Figure 37A, Table 2)**. Similarly, stool samples from septic patients admitted to a separate ICU demonstrated significantly higher stool indoles in patients who survived sepsis compared to those who were non-survivors (**Figure 37B, Table 3**). These findings indicate microbiota production of indole metabolites may play a role in survival of septic patients.

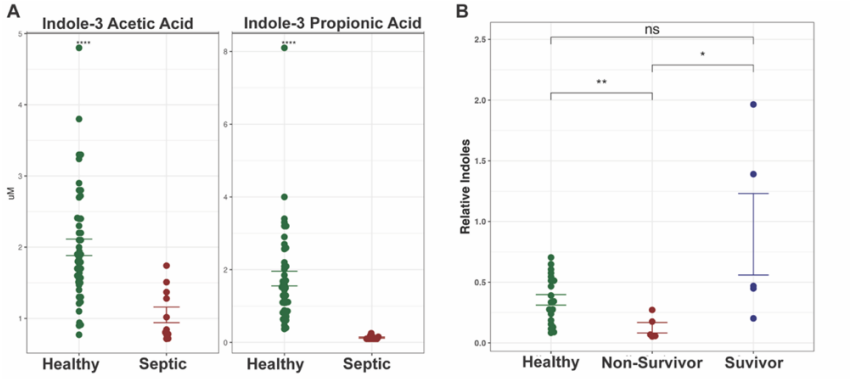



**Figure 37: Indole metabolite production in septic patients** (**A**) Serum was collected from patients diagnosed with sepsis in the ICU (n = 11) and healthy controls (n = 48) and indoles were measured by UPLC-MS/MS. **(B)** Stool was collected from patients on admission to the SICU and outcomes were compared between patients that survived sepsis (n = 5), non-survivors of sepsis (n = 5), and healthy controls (n = 21). **** - $p < 0.0001$, ** - $p < 0.01$, * - $p < 0.01$

| StudyID | Age | Sex | Race | PCT | APACHE-II | qSOFA |
|---|---|---|---|---|---|---|
| AAG- 16/01-01 | 50 | Male | White | 0.54 | 11 | 1 |
| EAA-17/01-01 | 39 | Male | White | 0.75 | 9 | 1 |
| PAB-19/01-01 | 36 | Male | White | 1.13 | 9 | 2 |
| DEA-19/01-01 | 66 | Male | White | 1.01 | 17 | 1 |
| KHI-01/02-01 | 67 | Female | White | 0.83 | 14 | 4 |
| SHM-05/02-01 | 84 | Female | White | ND | 15 | 1 |
| MAI-07/02-01 | 38 | Male | White | 0.53 | 15 | 4 |
| MVI-08/02-01 | 87 | Male | White | 1.23 | 17 | 2 |
| VRI-08/02-01 | 88 | Female | White | 1.24 | 28 | 8 |
| MMS-08/02-01 | 63 | Male | White | 0.9 | 11 | 1 |
| PAI-08/02-01 | 57 | Male | White | 1.69 | 14 | 3 |

Table 2: ICU Patients utilized for serum metabolites. PCT = procalcitonin, qSOFA = quick sequential organ failure assessment

| StudyID | Race | Sex | Age | qSOFA | Charcleston Comorbidity | Outcome |
|---|---|---|---|---|---|---|
| MICU-444 | Black or African-American | Female | 91 | 3 | 4 | Survivor |
| MICU-014 | Black or African-American | Male | 87 | 2 | 4 | Non-Survivor |
| MICU-214 | Black or African-American | Female | 40 | 3 | 0 | Survivor |
| MICU-128 | Black or African-American | Male | 84 | 3 | 5 | Non-Survivor |
| MICU-390 | Black or African-American | Male | 75 | 3 | 11 | Survivor |
| MICU-009 | Black or African-American | Female | 92 | 3 | 7 | Survivor |
| MICU-020 | White | Male | 75 | 2 | 6 | Survivor |
| MICU-397 | Black or African-American | Male | 88 | 2 | 8 | Non-Survivor |
| MICU-063 | White | Male | 58 | 3 | 1 | Non-Survivor |
| MICU-043 | Unknown | Female | 45 | 2 | 2 | Non-Survivor |

Table 3: ICU Patient demographics for stool samples

### *3.4.4 Discussion:*

Findings from the present study demonstrate that the dual activity of metabolites from the gut microbiota and the infecting pathogen can be deterministic of survival in mice systemically infected with lethal pathogens of relevance to human sepsis. We demonstrate here that gut microbiota-derived indole-mediated activation of AhR is a major determinant of survival from a lethal systemic infection (i.e intraperitoneal *S. marcescens, K. oxytoca*, and a pathogen community). The finding that oral supplementation with tryptophan may preemptively prevent the

subversion of AhR activity when the host is confronted by an infecting pathogen, is important given that the pathogens herein tested are often those that contribute to "late-onset sepsis," now the most common cause of organ failure and mortality during critical illness[8]. When patients suffer major injury or complications from elective procedures and become critically ill, life-support measures such as ventilator support and dialysis, and the application of polypharmacy such as vasoactive agents, opioids and antibiotics, not only impact the gut microbiome community membership and functional output over time [352], but also allow for the proliferation of healthcare-acquired pathogens (HAPs) that are often multidrug resistant and highly virulent [77,81,82,353]. An important mechanism by which these "overgrowth pathogens" are able to survive and reproduce, may underlie their ability to subvert the immune system [354]. Results from this study suggest that a lack of opposing forces on the AhR by metabolites from the gut microbiota may indeed play a role in the virulence and lethality of HAPs once they predominate. It may be for this reason that the indiscriminate use of antibiotics is associated with adverse outcomes in critically ill patients [77].

The gut microbiome is emerging as an important element involved in infectious disease pathogenesis and sepsis physiology. Traditionally, the conceptual framework around the pathobiology of life-threatening infection has consisted of highly polarized investigations focusing on either the pathogen or the host. In general, the microbiologic approach has attempted to hold the host constant while varying the genes or phenotype (i.e. via mutant constructs) of the pathogen of interest in an effort to define the determinants of outcome as they relate to pathogen genotype/phenotype. Conversely, the immunologic approach often holds the pathogen constant, while varying the host genotype/phenotype (i.e via knockout constructs) to invoke the host response as a key determinant of outcome. Studies that emphasize the host-pathogen interaction as a determinant of virulence (i.e harmfulness, clinical infection, organ failure, mortality) and

acceptance of the conceptual framework that virulence is neither a property of the pathogen nor that of the host, but rather a property of their interaction [355], may be needed to advance the field. For example, it is well established that secondary metabolites produced by *Pseudomonas aeruginosa* can interact with and differentially impact AhR signaling to allow for fine tuning of the host immune response [217,218]. Additionally, AhR has been demonstrated to play an important role in the innate immune response to endotoxin controlling both the pro-inflammatory response as well as the development of endotoxin tolerance and guiding resolution of inflammation through production of IL-10 [207,322]. It is likely that the anti-inflammatory impact of AhR signaling in our model is occurring via the non-canonical AhR pathway in which AhR is not required to bind to its canonical dioxin response elements to induce an anti-inflammatory response[356]. However, no work has been done to determine how the microbes within the gut microbiota and the pathogen compete for AhR activation and the extent to which this interaction influences survival.

Considering tryptophan as a unique substrate that can be metabolized by both gut bacteria and host cells to produce various immune-activating metabolites, may be an important modifiable factor in the battle against life-threatening infection [181]. Consistent with our findings are the findings of others that have demonstrated that oral supplementation with tryptophan can increase systemic levels of microbiota-derived indole metabolites with the capacity to alter the host immune response [357]. It is unclear the exact mechanism by which metabolites, such as indoles, are produced by the gut microbiota and then are delivered systemically. Prior studies, have demonstrated that metabolites produced by the gut microbiota are capable of disseminating systemically and can be detected in tissues throughout the body [358], thereby supporting our

findings of increased indole metabolites in the peritoneal cavity and serum of septic mice in our model.

Our study suggests that there may be a central role for AhR activity as a determinant of "within-group" mortality following a systemic (intraperitoneal) $LD_{50}$ dose of a virulent pathogen. To our knowledge, this is the first study demonstrating that AhR lies at the center of host-pathogen-microbiota interaction as its activation may be dually influenced by metabolites from both the infecting pathogen and the gut microbiota. The degree and direction by which AhR signaling is affected may play a role in discriminating between those mice that survive versus those that die within a group exposed to a standardized infectious inoculum. While there likely exists a fine spatial scale and time-dependent interplay between the concentration of the pathogen produced exoproduct(s) such as enterobactin, the concentration and composition of the gut microbiota-derived metabolites, and AhR activation that predicts survival versus mortality in this model, identifying each and every interaction will be necessary to fully elucidate these interactions. Competition for AhR signaling between the host microbiota and *S. marcescens*, as identified in the current report, adds to the growing examples of the "defense/counter-defense" paradigm and complexity within the host-pathogen interaction [354]. Furthermore, for the first time we have demonstrated that pathogen secretion of enterobactin, a common pathogen virulence factor, can inhibit indole-mediated AhR signaling in association with impaired survival. The host-pathogen interactions between pathogen secreted enterobactin and host lipocalin 2 has been well described in its importance for iron sequestration [359–361], but here we describe the competition between microbiota produced indoles and enterobactin for host AhR activation and the importance of this competition in determining survival from lethal bacterial infection.

There are several limitations to this study. As AhR is present on many immune cells beyond macrophages and is considered to be a promiscuous receptor based on its ability to respond to various ligands, other immune cells may be involved in the response herein described. Prior studies have demonstrated the importance of AhR signaling in both the innate and adaptive immune system. In the adaptive immune system, AhR is highly expressed in $T_H17$ and $T_{Reg}$ cells influencing their expansion and response to environmental stimuli [362,363]. Furthermore, AhR has been demonstrated to play an important role in innate lymphoid cells, dendritic cells, and macrophages, typically playing an important role in the anti-inflammatory response of these cells [203,364]. Therefore, it is likely that many of these cells may also have some degree of influence in our model and were not directly examined.

In summary, the aryl hydrocarbon receptor plays a central role in how host-pathogen-microbiota interactions determine survival from lethal sepsis. A conceptual framework may be emerging in which the extent to which a pathogen can subvert the host's immune clearance mechanisms must be balanced by the extent to which gut microbiota derived metabolites can enhance immunity. Given that all mammalian infections are treated by broad spectrum antibiotics which carry the unintended consequences of eliminating both the offending pathogen as well as multiple components of the gut microbiome, in the context of the present study, considering how to eliminate offending pathogens in a more focused and narrow spectrum may be desired.

# 4. CONCLUSION AND FUTURE DIRECTIONS:

We have shown that the gut microbiota has a major influence on the host response to surgical stress and infection, whether by shaping the host immune response, altering pathogen colonization, or modifying the interaction between the host and the pathogen. In all such interactions, the gut microbiota plays a central role.

In the first chapter, we demonstrated the broad impact of diet induced alterations to the microbiota in the host response to clinical stress (starvation, antibiotics, and surgical injury). Prior studies have emphasized the importance of diet induced changes to the microbiota and their relationship to host health. For example, mice fed a high fat diet have increased intestinal barrier permeability that results due to increased hyperglycemia causing GLUT2 mediated barrier disruption and increased susceptibility to enteric pathogen as well as increased systemic inflammation. Furthermore, antibiotic depletion of the microbiota eliminated the dissemination of microbial products [365]. Additionally, prior studies have shown that WD fed mice have increased sensitivity to endotoxemia believed to a microbiota independent mechanism [366]. The limitation of this study results from the lack of delivering LPS without a pathogen, eliminating the normal host-pathogen interaction response needed to truly understand the role of western diet in the setting of a systemic infection. Furthermore, it is impossible to separate out the role the microbiome plays in conditioning the host immune system. Exposure to microbial ligands or direct interaction between bacteria and the mucosal immune system within well-defined niches have major downstream impacts on the host. The most commonly described microbial conditioning of the host immune response is exposure to segmented filamentous bacteria (SFB*)* which in turn promotes a Th17 immune response [367]. Similarly, high sugar diets have a major impact on the abundance of

*Bacteroidetes theta* which plays an important role in conditioning peripheral Treg cells [368]. Outside of direct interactions, western diet has been associated with inducing trained immunity via NLRP3 resulting in a higher level of basal inflammation [369]. When western diet fed mice are shifted back to a standard chow diet, the basal inflammation resolves [369]. Furthermore, the presence of butyrate, which is virtually absent in the microbiome when feeding on a western diet, can result in trained immunity in bone marrow derived macrophages altering how they respond to pathogens [172]. Diet alterations to the microbiota also play an important role in the circadian variation of the mucosal immune system [370]. Diurnal variation in dietary and microbial content results in alterations in the small intestinal epithelial MHCII expression which in turn impacts the diurnal modulation of IL-10 which is important for maintaining barrier function [371]. There are numerous microbiota independent and microbiota dependent mechanisms by which a western diet shapes the host immune response and likely contributed to our findings [275,372]. These studies in combination with our findings support the notion that diet induced changes play an important role in shaping the host immune system and may in part explain the patient heterogeneity seen in response to infection. In order for us to find new ways to combat sepsis and mitigate postinfectious complications it is essential for us to find ways we might be able to take into account the life history of our patients and in particular how diet and diet induced changes to the microbiota may impact a patient's disease course.

Our second chapter, focused on how both dietary fat and fiber impact on microbiota resiliency to antibiotic exposure which correlated with decreased antibiotic induced diarrhea and weight loss. Dietary changes to the microbiota can result in both compositional and functional changes that can have a wide array of impact on the microbiota [373]. It is clear that the gut microbiota have complex communities of bacteria that are dependent on each other for

biochemical processes and maintaining a gut environment suitable for survival [374]. The utilization of dietary substrates allows bacteria to outcompete potential pathogens and prevent colonization[133]. Additionally, utilizing dietary substrates also alters the host environment to further prevent pathogen colonization. We are beginning to appreciate the complex communities of bacteria that naturally coexist and interact where there is mutual benefit from commensalism. The evolution of bacterial communities includes their ability to communicate with one another. Quorum sensing molecules are one of the most studied means of communication between bacteria [375]. Quorum sensing allows bacteria to engage in group assemblage type behaviors. One of the most studied quorum sensing molecules is AI-2 as it is one of the most widely conserved quorum sensing molecule within the bacterial kingdom [375]. One of the first studies investigating the role of AI-2 in shaping the gut microbiota, found that AI-2 played an important role in determining the resiliency of the microbiota. In particular, they found that when mice were colonized with an engineered strain of *E. coli* that constitutively produced AI-2, there was an increased resiliency of Firmicutes when mice were exposed to Streptomycin [240]. It has been projected that over 80% of Firmicutes within the gut microbiota are capable of producing AI-2 and over 20% of Bacteroidetes are predicted to be AI-2 producers [375]. Both Firmicutes and Bacteroidetes have been shown to play an important role in shaping the host, particularly through the production of SCFAs. AI-2 has also been shown to inhibit biofilm formation and virulence of *C. difficile* [376]. AI-2 can also act as a chemoattractant or chemorepellent and shape the microbiota community structure [377–379]. However, it is important to note that AI-2 has also been shown to increase virulence factors in some bacterial species [375]. Quorum sensing molecules may offer a new avenue for microbiota directed therapies; whether it is to decrease pathogen virulence or increase microbiota resistance to antibiotic exposure. Both of these functions of AI-2 could have major implications in potentially

alleviating subsequent infection following antibiotic exposure and maintain bacterial species capable of producing health-relevant metabolites. As bacteria communicate and function in a community structure within the gut, polysaccharide utilization loci (PUL) are produce which are essential for the degradation of fiber into SCFAs [380]. However, the complex communication that occurs between bacteria that leads to PUL production remains to be elucidated. It is noteworthy that the regulation of PULs within bacteria is quite complex and depends on substrate availability within the gut [381]. Future work will be necessary to advance an understanding of how quorum sensing molecules regulate the production of health-relevant metabolites. Further elucidation of these mechanisms will inform microbiota directed therapies that can regulate the production of health relevant metabolites.

The third chapter focused on how diet shapes antibiotic resistance genes within the gut microbiota. We determined that the gut resistome can be influenced by diet alone without prior exposure to antibiotics. The antibiotic resistance genes were found in bacteria that are typically considered commensals; however, this genotype could have an impact on the composition and function of other microbiota which may have impact the host. These findings were consistent with prior studies demonstrating that many commensals including Bacteroidetes and Parabacteroides harbor antibiotic resistance genes against common antibiotics including macrolides, cephalosporins, and tetracyclines [299,316]. When administering antibiotics, if a particular bacterium harbors antibiotic resistance, then they can dominate and destabilize communities of bacteria that play essential roles in maintaining the function of the gut microbiota, thus impacting the host. As discussed previously, when communities of bacteria are destabilized, there is a loss of the ability of the gut microbiota to prevent pathogen colonization as well as a change in how the microbiota interacts with the host immune system. Finally, there is also the chance that the antibiotic resistant

commensals can act as a sink for administered antibiotics and shield potential pathogens [320]. These findings support the observation that antibiotic exposure can major impact patient outcome and increase the risk for subsequent infections. Currently, antibiotic practices in the hospital consist of providing empiric antibiotics until a pathogen is identified and an antibiotic susceptibility profile is determined. This practice can be problematic when individual patients harbor differences in their gut resistome based on their life history and environmental and drug exposures [382]. Trying to narrow antibiotics appropriately is a difficult, but important goal, as prior studies have demonstrated that both too broad of antibiotic coverage and too narrow of antibiotic coverage can result in worse patient outcomes [18,383]. Further research needs to be completed to understand the response of the gut resistome following antibiotic treatment and its implication for both pathogen colonization and the host immune system. This area of inquiry could allow for advancing our understanding of the gut resistome with the goal of targeting and treating patients at risk for infections with gut-origin pathogens. Such studies could inform approaches that increase the precision of antibiotic treatments while minimize the collateral damage of antibiotics on the gut microbiota.

The final chapter connects within group differences in response to lethal infection with production of indole metabolites by the gut microbiota, host macrophage AhR, and pathogen subversion of the host immune response through the secretion of enterobactin. Host-pathogen-microbiota interactions are just beginning to be appreciated in the setting of infection and sepsis. Prior to these studies, alterations in the microbiota have been correlated with outcomes in patients with sepsis and critical illness; however, we are beginning to unlock the mechanistic role the microbiota on the outcome of life-threatening infection. The gut microbiota has the ability to improve the host immune response in disseminated infection. A recent study demonstrated that

SFB colonization in the gut promotes type 17 immune response in the lungs which results in increased IL-22 production and protection from *S. aureus* pneumonia [384]. Similarly, production of D-lactate by the gut microbiota has been demonstrated to increase clearance of disseminated pathogens by Kupffer cells in a mouse model of *S. aureus* bacteremia [385]. Similarly, gut microbiota can protect mice from viral meningitis either through stimulation of microglial by SCFAs or through the presence of gut TLR ligands which maintain microglial cells ability to clear CMV [386,387]. Our previous study demonstrated that administration of a FMT in the setting of bacterial peritonitis increased microbiota production of SCFAs which in turn modulated IRF3 expression and improved survival [224]. Although, these studies demonstrate significant correlations between alterations in the gut microbiota and the extra-intestinal immune system to drive the clearance of pathogens, they still rely on interventions to manipulate the microbiota which can often have off target effects. Here we demonstrate that the production of indole metabolites by the gut microbiota can directly interact with AhR on peritoneal macrophage and aid in clearance of a lethal pathogen and resolution of inflammation. The unique point of this study is that the differences occurring in the microbiota function driving survival occur stochastically and may explain within group survival difference to lethal infection. In support of this study, indole-3 propionic acid has been demonstrated to improve survival in the setting of cecal ligation and puncture [216]. Indoles have also been demonstrated to play an important role in regulating IL-22 production by ILC3 and protecting mice from enteric infection as well as DSS colitis [203,388].

The role of indoles and the host-pathogen interaction plays out in two ways: the first occurs through interaction with AhR, described here in detail, and the second is the potential interactions of indoles on pats described in detail in the introduction, AhR has the ability to

interact with indole metabolites and is expressed by a wide array of immune cells and can regulate immune signaling pathways such as NF-kB [206]: NF-kB induces AhR expression and AhR can modulate NF-kB by interacting with other transcription factors such as RelA and RelB to interact with NF-kB binding sites [389–391]. Although the focus of this study was on the role of AhR in macrophages, AhR is expressed in other cells that can also influence the immune response to infection. AhR activation has been associated with the regulation of T lymphocytes including: Th17, Treg cells, type 1 regulatory cells (Tr1), and innate lymphoid cells (ILC) in the gut [203,362,392]. AhR expression in Tr1 cells is important for regulating tissue inflammation and loss of AhR expression in Tr1 cells results in chronic inflammation [392]. AhR activation in Th17 cells regulates IL-22 which as described earlier can play in important role in pathogen dissemination in models of pneumonia as well as enteric infection. Furthermore, AhR activation is important for the maintenance of ILCs and control production of IL-22 and IL-17 which in turn prevent pathogen colonization and decrease susceptibility [203,204,393]. When it comes to innate immune cells, AhR has been shown to play an important role in myeloid cell development [394] and is important in dendritic cell (DC) antigen presentation [395]. AhR activation in bone marrow derived DCs has been shown to increase MHCII, CD86, IL-6, and TNF-a production. In mouse models, AhR activation in DCs seem to mainly play an anti-inflammatory role through inducing expansion Tregs and increasing Tr1 activation [396]. The role of AhR in host-pathogen-microbiota interactions observed in our study was is likely to be exclusively confined to macrophages, although macrophages were indeed required for survival in this model. Prior to our study, an already existing body of literature demonstrating the ability of AhR to regulate macrophages [330,364,397] has been reported. Yet our study was one of the first of its kind to demonstrate that indole metabolites can activate AhR in macrophages and drive a recovery directed immune

response in the face of lethal bacterial infection. Further studies are needed to connected and understand the temporal and spatial dynamics that occur between macrophages and microbial metabolites.

In addition to the impact of indole on the host immune system through its interaction with the aryl hydrocarbon receptor, indole is also thought of as a signaling molecule utilized by bacteria [208]. Indole metabolites function as signaling molecules and can have varying effects on bacteria dependenting on whether or not the bacteria produce indoles. In indole producing bacteria, indoles seem to increase biofilm formation and enhance virulence. In *E. coli*, indole has been demonstrated to increase antibiotic resistance and antibiotic tolerant persister cells [398,399]. Indole at higher concentrations can inhibit bacterial growth in indole producing organisms [400]. In non-indole producing bacteria, indoles alter bacterial physiology by impeding virulence. In *Pseudomonas aeruginosa*, indoles inhibit the quorum sensing molecule acyl homoserine lactones (AHLs) thereby decreasing its virulence [401(p7)]. Similarly, indoles have been shown to decrease the virulence of other non-indole producing bacteria including *Candida albicans* and *Staph aureus* [402,403]. Indole derivatives produced by bacteria also have been shown to be potent inhibitors of biofilm formation in non-indole producing bacteria [404]. Therefore the central role of indoles in terms of their production by the microbiota, their activation of AhR on host cells, and their ability to signal potential pathogens to alter their virulence places them in a unique position within host-microbiota-pathogen interaction, given that both bacteria and the host have evolved to detect and respond to these molecules. In our study, we found that *Serratia marcescens* had a reduction in growth and biofilm formation at higher concentrations (1mM) than what was utilized in our model. The wide arrange of response seen by both the host and pathogen demonstrate that multiple variables within context of indole concentration and spatial location

can drive a recovery directed immune response following a major physiologic and infection-related perturbation.

These studies provide support for the significant role of the gut microbiota in shaping the complex interaction between the host and the infecting pathogen in a manner which has a important role in survival. However, there are several limitations to these studies. First, the findings and mechanistic studies were all performed in mice which may not necessarily translate to human patients. Although we provide some evidence of alterations in indole metabolites in septic patients, additional work needs to be done to determine if these alterations correlate with similar AhR induced changes in macrophages in humans. There is also the possibility that a different set of metabolites or only select indole metabolites not found to be significant in mice could be responsible for a microbiota directed recovery in humans. Furthermore, as mentioned above, the impact of indoles on host-pathogen interactions is dependent on both the host and the pathogen and although we demonstrated the importance of indoles in *S. marcescens, K. oxytoca*, and polymicrobial peritonitis it may not play a major role in all infections in all patients as indoles have a varying impact on pathogens. The other major limitation of these studies is the utilization of a single sex and strain of mice. Although, controlling for these experimental variables provided consistency to study the interactions between the host, pathogen, and microbiota, there are likely sex and genetic components that will also contribute to microbiota directed outcomes in lethal infection. As we attempt to improve our understanding of the gut microbiota in sepsis and infection, it will be important to perform more clinical studies considering the individual history of the patient as well as the infecting pathogen to continue to build on how the gut-microbiota-pathogen relationship can be leveraged to improve treatment.

Manipulations of the gut microbiota have a clear impact on both the host immune system, pathogen colonization, and pathogen virulence that in turn may dictate outcomes following infection. Over the last decade of microbiota research, we have continued to find ways in which the microbiota correlates with clinical outcomes, but until recently lacked mechanistic details on how the microbiota can shape host immune system responsiveness and pathogen behavior. As the medical community continues seek ways to improve our understanding of life-threatening infection and develops new therapies to combat antibiotic resistant infections, microbiota directed therapies may offer new insight and hope to improve patient care.

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