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The Role of Gut Microbiota Urease in the Host With Liver Disease

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Abstract
Significant metabolic interactions exist between the gut microbiota and the mammalian host, one prime example of which is nitrogen metabolism. In the colon, bacterial urease hydrolyzes host-derived urea into carbon dioxide and ammonia. Colonic ammonia can subsequently be absorbed by the host or utilized by the gut microbiota for additional nitrogen metabolism. In patients with liver disease such as cirrhosis and congenital urea cycle disorders, hepatic abnormalities prevent the normal processing of ammonia, leading to hyperammonemia and hepatic encephalopathy (HE). Although circulating ammonia levels are correlated with damage to the central nervous system, the pathogenesis of HE is complex and not fully elucidated, hindering progress in treatment. Current treatment options including antibiotics, lactulose, and a low protein diet (LPD) are complicated by issues such as side effects, concerns of safety and efficacy in long-term use, and poor adherence. Our goal is to develop a safe, durable, and efficacious treatment for HE through the inoculation of a urease-free bacterial consortium. We show that we are able to engineer the gut microbiota to reduce fecal urease activity and ammonia levels in mice. Depletion of the endogenous gut microbiota

followed by transplantation with Altered Schaedler Flora (ASF), a defined consortium of eight murine gut commensal bacteria with minimal urease gene content, established a persistent new community that exhibited long-term reduction in fecal urease activity and fecal ammonia production. ASF transplantation was associated with a decrease in morbidity and mortality in the thioacetamide (TAA) murine model of hepatic injury and fibrosis. Although the ASF consortium demonstrated reduced resilience in response to dietary stress, ASF transplantation led to further reductions in fecal ammonia on a LPD without exacerbating host metabolic dysfunction. These findings point to the potential use of a human urease-free bacterial consortium to alter clinical management and outcome in HE. Furthermore, they provide proof of concept that microbiota transplantation with a defined microbial consortium can lead to durable metabolic changes with therapeutic utility.

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DEDICATION

To my parents, for their unending love and support, and for teaching me the importance of lifelong learning.

And to my siblings, for being my best and closest friends.

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ABSTRACT

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Significant metabolic interactions exist between the gut microbiota and the mammalian host, one prime example of which is nitrogen metabolism. In the colon, bacterial urease hydrolyzes host-derived urea into carbon dioxide and ammonia. Colonic ammonia can subsequently be absorbed by the host or utilized by the gut microbiota for additional nitrogen metabolism. In patients with liver disease such as cirrhosis and congenital urea cycle disorders, hepatic abnormalities prevent the normal processing of ammonia, leading to hyperammonemia and hepatic encephalopathy (HE). Although circulating ammonia levels are correlated with damage to the central nervous system, the pathogenesis of HE is complex and not fully elucidated, hindering progress in treatment. Current treatment options including antibiotics, lactulose, and a low protein diet (LPD) are complicated by issues such as side effects, concerns of safety and efficacy in long-term use, and poor adherence. Our goal is to develop a safe, durable, and efficacious treatment for HE through the inoculation of a urease-free bacterial consortium. We show that we are able to engineer the gut microbiota to reduce fecal urease activity and ammonia levels in mice. Depletion of the endogenous gut microbiota followed by transplantation with Altered Schaedler Flora (ASF), a defined consortium of eight murine gut commensal bacteria with minimal urease gene content, established a persistent new community that exhibited long-term reduction in fecal urease activity and fecal ammonia production. ASF transplantation was associated with a decrease in morbidity and mortality in the thioacetamide (TAA) murine model of hepatic injury and fibrosis. Although the ASF consortium demonstrated reduced resilience in response to

dietary stress, ASF transplantation led to further reductions in fecal ammonia on a LPD without exacerbating host metabolic dysfunction. These findings point to the potential use of a human urease-free bacterial consortium to alter clinical management and outcome in HE. Furthermore, they provide proof of concept that microbiota transplantation with a defined microbial consortium can lead to durable metabolic changes with therapeutic utility.

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CHAPTER 1. Introduction

1.1 The gut microbiota and its metabolic interactions with the mammalian host

The human gastrointestinal tract is home to approximately 100 trillion microorganisms, which exceed the total number of human cells by ten-fold[1]. The biomass reaches up to 10^{12} cells per gram in the distal colon, making it one of the most densely populated microbial habitats on earth[2]. This microbial community is known as the gut microbiota, and it comprises ~500-1000 bacterial species along with fungi, viruses, and archaea[3]. The microbes and their genomes, collectively known as the gut microbiome, significantly impact various host physiologic processes, including nutrition and metabolism[4, 5], immune regulation[6], inflammation and cancer[7, 8], and even neurodevelopment and behavior[9]. They perform these functions through mutualistic interactions with the host, mediated primarily by the exchange of metabolites derived from microbial and/or host metabolism. Specific metabolites may be produced by specific microbes, the presence of which may thus be essential. But different microbes may have evolved similar functions through divergent pathways, leading to functional overlap or even a competitive relationship with one another for limited environment and resources. Over the past few decades, the scientific community has seen a dramatic increase in our knowledge of the composition and function of the gut microbiome. This was made possible largely through technological advancements in culture-independent methods for isolating and identifying microbes (e.g. 16S rRNA gene sequencing) as well laboratory and computational methods to characterize their functions (e.g. gnotobiotic mice and gene network association studies). Nevertheless, the future challenges of this field lie in our ability to identify specific microbes and/or their functions associated with particular diseases and to develop methods to modulate their unwanted effects. One

approach would be to replace these “pathogenic” microbes with “beneficial” microbes targeted toward specific biological processes without impairing other physiological functions. This dissertation represents the work of a series of experiments designed to test the feasibility and efficacy of such an approach. We replaced the endogenous gut microbiota in mice with a defined bacterial consortium that lacks the bacterial enzyme urease, which hydrolyzes host-derived urea to carbon dioxide and ammonia in the colon (see **Section 1.2**). We hypothesized that such a bacterial consortium would decrease colonic ammonia production and alter host nitrogen metabolism, with the durability of its effects dependent upon the resilience of the bacterial consortium to environmental perturbations such as dietary changes.

A large body of scientific literature supports the important role that the gut microbiota plays in host metabolism. As one example, the gut microbiota is thought to contribute the rising epidemic of metabolic diseases such as diabetes and obesity in the developed world[10]. Pioneering work involving obese individuals and genetically obese mice (*ob/ob*) found that the obese phenotype is associated with changes in the relative abundance of two dominant bacterial phyla, Bacteroidetes and Firmicutes, whereby Bacteroidetes were found to be decreased and Firmicutes increased[11, 12]. Using metagenomic and biochemical analyses, the investigators demonstrated that the microbiome associated with the obese phenotype had an increased capacity to harvest energy from the diet. Furthermore, by colonizing germ-free mice with the gut microbiota of an *ob/ob* mouse and showing that it led to a greater increase in total body fat than with the gut microbiota of its lean littermate, they demonstrated that these effects were transmissible. Similar findings were reproduced by transplanting the gut microbiota of human twins discordant for obesity into germ-free mice[13]. Conversely, by transplanting the gut microbiota of Malawian twins discordant for kwashiorkor into germ-free mice, a

form of severe acute malnutrition, the same group of investigators demonstrated that the combination of a Malawian diet and the kwashiorkor microbiome produced marked weight loss in mice accompanied by perturbations in amino acid, carbohydrate, and intermediary metabolism[14]. These finding strongly implicate the role that the gut microbiota plays in host nutrition and metabolism by affecting energy extraction.

Three important classes of biological molecules illustrate the dynamic and complex metabolic interactions between the mammalian host and its gut microbiota: bile acids, short chain fatty acids (SCFAs), and choline. The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized in the liver from cholesterol. Primary bile acids are generally conjugated to glycine and taurine in the liver, stored in the gallbladder, and then secreted into the small intestine in order to facilitate the digestion and absorption of dietary fats and fat-soluble vitamins. Upon reaching the terminal ileum, approximately 90-95% of the bile acids are reabsorbed across the intestinal epithelium and recycled to the liver, completing the enterohepatic cycle 5-15 times daily[5]. However, the remainder 5-10% of conjugated primary bile acids are either deconjugated by the small intestinal bacteria to produce secondary bile acids such as deoxycholic acid (from cholic acid) and lithocholic acid (from chenodeoxycholic acid) or lost in the feces[4]. Bacteria that deconjugate primary bile acids to form secondary bile acids are generally anaerobic bacteria of the genera *Bacteroides*, *Eubacterium*, and *Clostridium*[5]. Both primary bile acids and secondary bile acids can act as signaling molecules by binding to cellular receptors such as the farnesoid X receptor (FXR, activated by primary bile acids) and G-protein-coupled-receptor (GPCR) TGR5 (activated by secondary bile acids) with various downstream effects. FXR and TGR5 both affect glucose metabolism, but FXR impairs glucose homeostasis, while TGR5 improves glucose homeostasis[4]. Circulation of primary and secondary bile acids throughout the body with activation of FXR and

TGR5 in peripheral organs can also affect overall host metabolism. For example, TGR5 activation in brown adipose tissue and muscle can increase energy expenditure and ameliorate diet-induced obesity. The gut microbiota may thus modulate obesity and type 2 diabetes through its effects on lipid and glucose metabolism by altering the composition of the bile acid pool and FXR and TGR5 signaling.

As opposed to bile acids that are primarily produced by the liver, SCFAs such as acetate, butyrate, and propionate are products of complex carbohydrate fermentation by colonic bacteria. Certain bacterial species, including *B. thetaiotaomicron* and *Bacteroides ovatus*, contain more glycosidases and lyases than humans and thereby are able to metabolize nearly all the glycans in dietary fiber[4]. SCFAs perform a variety of functions. Butyrate serves as an important energy substrate for the colonic epithelium, where it can also affect proliferation, differentiation, and modulation of gene expression by inhibiting histone deacetylase[4]. Acetate and propionate can reach various organs through the bloodstream and serve as substrates for lipogenesis and gluconeogenesis as well as regulate different gene expressions by binding to GPCRs GPR41 and GPR43[5]. The end-organ effects of SCFAs through GPCRs depend on the cell type. For example, SCFAs can modulate secretion of the hormone glucagon-like peptide-1 (GLP-1) through effects on enteroendocrine L-cells in the distal small intestine and colon, thereby improving insulin secretion[5]. On the other hand, SCFAs suppress inflammation in neutrophils through GPR43 signaling. As acids, SCFAs can further lower pH in the colonic luminal environment and affect microbiota composition and gut motility.

Unlike bile acids and SCFAs, choline is predominantly obtained from the diet in foods such as red meat and eggs, although it can also be synthesized by the host[4]. Choline is an essential component of the cell membrane, and it is primarily metabolized in the liver, where it functions in lipid metabolism and the synthesis of very-low-density

lipoprotein. The gut microbiota can metabolize dietary choline to produce trimethylamine (TMA), which is further metabolized in the liver by the flavine monooxygenase system to produce trimethylamine-N-oxide (TMAO), shown to be associated with the development of atherosclerosis and cardiovascular diseases[5]. Both SCFAs and choline are discussed further in **Section 1.5**.

1.2 Mammalian nitrogen metabolism and the significance of bacterial urease

As a key component of amino acids and nucleic acids, nitrogen is essential to life. The body obtains most of its nitrogen through dietary protein intake, which are hydrolyzed into peptides and amino acids by proteases and peptidases in the gastrointestinal tract[15]. Most of the amino acids and peptides are absorbed into the body via specialized transporters in the intestinal epithelium, but significant amounts of undigested dietary as well as endogenous proteins (e.g. gastric and pancreatic secretions, sloughed intestinal epithelial cells) enter the colon[16], where they serve as nitrogenous substrates for the colonic microbiota[17]. The absorbed amino acids can function as building blocks for enzymes that catalyze critical cellular and molecular processes in the body or undergo alternative metabolic pathways to act as precursors for purines, pyrimidines, neurotransmitters, hormones, and other nitrogen-containing compounds[15]. The catabolism of nitrogenous compounds, in particular amino acids, results in the formation of ammonia, which can be toxic to the central nervous system if elevated above normal (see **Section 1.3**). As a result, ammonia is generally combined with glutamate to form glutamine via glutamine synthetase for transport in the blood[18]. Circulating glutamine is subsequently deaminated by glutaminase to regenerate glutamate and free ammonia in the liver and the kidneys.

To dispose of excess nitrogen in the form of ammonia, the body utilizes a series of enzymatically-mediated reactions known as the urea cycle to convert ammonia into urea, $CO(NH_2)_2$, a non-toxic waste product. One nitrogen of the urea molecule is derived from ammonia, and the other nitrogen is derived from aspartate; the carbon and oxygen are derived from CO_2. The urea cycle consists of five reactions involving five enzymes, two of which are mitochondrial and three are cytosolic[19]. The urea cycle begins with 1) the formation of carbamoyl phosphate by carbamoyl phosphate synthetase I, which requires N-acetylglutamate as an allosteric activator. The synthesis of N-acetylglutamate from acetyl coenzyme A and glutamate by N-acetylglutamate synthase is thus often considered the sixth reaction of the urea cycle. 2) The carbamoyl portion of carbamoyl phosphate is combined with ornithine via ornithine transcarbamoylase to form citrulline. 3) Citrulline is then transported from the mitochondrion into the cytosol and combined with aspartate to form argininosuccinate via argininosuccinate synthetase. 4) Argininosuccinate is cleaved by argininosuccinate lyase to produce fumarate and arginine. Finally, 5) arginase cleaves arginine to regenerate ornithine and form urea. Since arginase occurs almost exclusively in the liver, only the liver can synthesize urea by cleaving arginine, but other tissues such as the kidneys can synthesize arginine via the above reactions. Hepatic urea is primarily transported via the bloodstream to the kidneys for urinary excretion, but approximately 15-30% of hepatic urea enters the colon, where it is hydrolyzed back into ammonia and carbon dioxide by the bacterial enzyme urease[20]. Colonic ammonia is then 1) reabsorbed by the host for additional hepatic nitrogen metabolism, 2) utilized by the gut microbiota to synthesize amino acids and other nitrogenous compounds, or 3) excreted in the feces. Bacterial urease is thus critical for colonic urea nitrogen utilization and recycling by the healthy host and the gut microbiota. However, colonic ammonia production via bacterial urease activity can

exacerbate conditions of hyperammonemia, such as hepatic encephalopathy (HE) in cirrhosis and urea cycle disorders (see **Section 1.3**).

Urea and urease both represent important landmark molecules in the history of scientific investigation. Urea was the first organic molecule synthesized, and urease from jack bean was the first enzyme crystallized as well as the first enzyme shown to contain nickel[21]. A variety of bacteria, fungi, plants, and invertebrates produce urease, but no mammalian urease gene has been identified[22, 23]. Therefore, the hydrolysis of colonic urea via urease activity is exclusively a function of the gut microbiome. By hydrolyzing urea to carbon dioxide and ammonia, bacterial urease provides bacteria with their preferred nitrogen substrate to synthesize amino acids, amines, indoles, and other nitrogenous metabolites[22, 24]. At the same time, bacterial urease can function as a virulence factor and contribute to the development of pathogenic conditions such as HE, nephrolithiasis, pyelonephritis, and peptic ulceration[22]. As one example, *Helicobacter pylori* generates ammonia via urease to neutralize gastric acid, allowing it to colonize and proliferate in the stomach and leading to the formation of peptic ulcer disease[22].

Bacterial ureases are cytosolic enzymes with the exception of *H. pylori* urease, which has both cytosolic and extracellular activity[25]. Most bacterial ureases are composed of three subunits (encoded by ureA, ureB, and ureC), but the urease of *Helicobacter spp.* is composed of two subunits (encoded by ureA and ureB) and has been shown to form a dodecameric complex of repeating subunits. Nevertheless, the homology of amino acid sequences in urease is highly conserved across bacterial species[25]. The urease gene cluster contains 7-9 genes that consist of both structural and accessory genes. Only one regulatory gene, ureR, has been identified and is found only in those gene clusters inducible by urea (see below)[25]. Regulation of urease expression mainly occurs by three mechanisms. Some bacteria express urease constitutively (e.g.

Morganella morganii), some bacteria express urease in the presence of urea (e.g. *Proteus mirabilis*), and still other bacteria express urease under nitrogen limiting conditions (e.g. *Klebsiella*), which is controlled by a two-component nitrogen regulatory system (see below)[22, 25]. Additionally, the urease of *Streptococcus salivarus* is reported to be regulated by pH. Urease exhibits simple Michaelis-Menten-type kinetic behavior[25]. No evidence for substrate inhibition or allosteric behavior has been detected, but there are a number of urease inhibitors that act by competitive inhibition, including hydroxamic acids, phosphoramidates, and thiols. Urease inhibitors have been used to prevent and/or treat conditions such as urinary stones and peptic ulcer disease, but their therapeutic role is largely limited by their significant side effects[26].

As mentioned above, certain bacteria express urease under nitrogen limiting conditions. This is activated by the *nac* gene product, Nac, which belongs to the LysR family of regulatory proteins[27]. Nac itself does not respond to nitrogen availability but is regulated by NtrBC, a two-component regulatory system that senses and responds to nitrogen deprivation[28]. NtrB is a sensor histidine kinase, and NtrC is a response DNA-binding transcription factor. In the setting of nitrogen limitation (perceived by bacteria as low intracellular glutamine and ammonia), NtrB phosphorylates NtrC, which subsequently activates transcription of genes that encode proteins to generate alternative nitrogen sources. These include scavenging and transport systems of nitrogenous molecules (e.g. ammonia, amino acids), glutamine synthetase, and catabolic enzymes of nitrogenous compounds[29]. Furthermore, NtrC activates transcription of relA to produce RelA, which synthesizes guanosine tetraphosphate (ppGpp), mediator of the stringent response[30]. The stringent response is another bacterial stress response system and affects a number of bacterial physiological processes in the setting of environmental stress. In the setting of nitrogen deprivation,

the stringent response promotes amino acid biosynthesis but inhibits translation and nucleotide metabolism. Thus, Ntr and stringent response systems work synergistically to increase nitrogen availability but inhibit cellular processes that promote bacterial proliferation when environmental nitrogen is scarce.

1.3 The gut microbiota and bacterial urease in hepatic encephalopathy

As mentioned previously, colonic ammonia production via urea hydrolysis by bacterial urease exacerbates conditions of hyperammonemia such as hepatic encephalopathy (HE) in liver disease and congenital urea cycle disorders. HE manifests as a spectrum of neurological and psychiatric dysfunctions that range from mild memory impairment to confusion, coma, and brain edema, potentially leading to death. The pathogenesis of HE is complex and remains to be fully elucidated, but ammonia has long been implicated to play an important role. More recently, inflammation and oxidative and/or nitrosative stress are also reported to contribute to the development of HE[31]. Furthermore, there is evidence to suggest that alterations in the gut microbiota and their metabolites such as amino acid derivatives (e.g. indoles, oxindoles, false neurotransmitters precursors) and endotoxins may be involved in HE[32].

Ammonia is the best characterized neurotoxin in the pathogenesis of HE. Plasma ammonia concentrations are elevated in approximately 90 percent of the patients with HE, although levels do not always correlate with HE severity[33, 34]. In addition to host catabolism of proteins and amino acids, the gut represents the primary source of ammonia production in the body. This is due to both bacterial metabolism of nitrogenous compounds (e.g. dietary and endogenous proteins and urea) in the small intestine and colon as well as enterocyte metabolism of glutamine (see **Section 1.2**). Ammonia produced in the gut enters the systemic circulation via the portal vein, which drains

directly into the liver. A normal liver converts almost all of the ammonia into urea and/or glutamine, but hyperammonemia can occur in liver disease and urea cycle disorders due to decreased hepatic metabolism and/or portosystemic shunts[32]. Ammonia is generally transported in the blood in the form of glutamine, which provides a nontoxic form of storage and transport[18]. The formation of glutamine from glutamate and ammonia via glutamine synthetase occurs primarily in the muscle and liver, which may be impaired in the setting of chronic liver disease given muscle wasting and hepatic dysfunction, further elevating circulating ammonia levels in the blood. Ammonia passes through the blood-brain-barrier (BBB) to enter the central nervous system (CNS), where it leads to brain edema and intracellular swelling due to the formation of glutamine within astrocytes, the only cell type within the CNS that contains glutamine synthetase[35]. Indeed, one study showed that inhibition of glutamine synthetase ameliorated brain swelling in rats infused with ammonia[36].

Since hyperammonemia leads to increased formation of glutamine in the brain, it indirectly increases cerebral uptake of large neutral amino acids (LNAAs) by enhancing the activity of the L-amino transporter at the BBB that exchanges glutamine in the brain for LNAAs in the blood. LNAAs include both the aromatic amino acids (AAAs) and the branched chain amino acids (BCAAs). In the setting of liver disease, there is increased AAAs (i.e. phenylalanine, tryptophan, and tyrosine) relative to BCAAs (i.e. valine, leucine, and isoleucine) due to increased muscle consumption of BCAAs to form glutamate and decreased hepatic metabolism of AAAs[18]. Since both BCAAs and AAAs utilize the L-amino transporter to enter the CNS, decreased BCAA concentrations leads to increased transport of AAAs into the brain. AAAs not only serve as precursors to catecholamines and serotonin that function as neurotransmitters, they can also form false neurotransmitters such as octopamine, phenylethanolamine, and synephrine[37].

Normally AAAs are decarboxylated to form amines, which subsequently undergo additional metabolism in the liver to form aldehydes. When hepatic function becomes impaired, precursor AAAs and their amines are shunted away from aldehyde formation and may enter the central nervous system to become locally β-hydroxylated into false neurotransmitters and replace normal neurotransmitters[38]. Fischer et al. proposed that the ratio of BCAA to AAA (valine + leucine + isoleucine) / (phenylalanine + tyrosine) would be predictive of HE[39]. Indeed, numerous reports have verified that a decrease in this ratio is associated with hepatic dysfunction[40, 41]. Based on this rationale, BCAAs have been used to treat HE. In a meta-analysis that included 16 trials with 827 participants with HE, although treatment with BCAAs did not affect mortality, it significantly improved HE symptoms[42]. Based on preliminary data in our lab, we have also observed that altering the composition of the murine gut microbiota using a urease-deficient bacterial consortium can affect host plasma amino acid homeostasis and increase the BCAA/AAA ratio (see **Future Directions**). This suggests that in addition to its effects on colonic ammonia production via bacterial urease activity, the gut microbiota may modulate nitrogen flux leading to alterations in host and microbial amino acid metabolism via urease-dependent mechanisms.

The gut microbiota has indeed been implicated to play a role in the pathogenesis of HE. Perhaps the best evidence to support its pathogenic role comes from the efficacy of HE treatments aimed to modulate the gut microbiota and/or its metabolites. In addition to the use of BCAAs as described above, lactulose, polyethylene glycol (PEG), antibiotics, and probiotic have been used to treat HE. Lactulose is a nonabsorbable disaccharide that serves as a standard therapy for HE. It is fermented by bacteria into short chain fatty acids, thereby acidifying the colonic environment and sequestering colonic ammonia as ammonium ion, which is more easily excreted into the feces. There

is also evidence to suggest that lactulose may function as a prebiotic to increase the growth of beneficial bacteria such as *Lactobacilli*, curtailing the growth of harmful urease-producing bacterial species[43]. Nevertheless, lactulose use is poorly tolerated due to the induction of significant diarrhea, leading to poor adherence. A recent study compared the efficacy of lactulose and PEG in the treatment of HE. PEG is a purgative commonly used for constipation and/or preparation for colonoscopy. In the acute hospitalized setting, cirrhotic patients randomly assigned to receive PEG showed more improvement in HE after 24 hours than those who received lactulose based on a standardized scoring algorithm[44]. This suggests that rapid catharsis of colonic ammonia and/or other bacterial metabolites by PEG alone can be effective in the treatment of HE. Nevertheless, the chronic use of PEG as a maintenance therapy for HE has not been validated.

Rifaximin is a synthetic nonabsorbable antibiotic that has demonstrated comparable efficacy to lactulose in the treatment of HE. The mechanism by which rifaximin ameliorates HE is not well understood, as studies have found no significant change in the composition or abundance of the gut microbiota after treatment with rifaximin, except for modest decrease in *Veillonellaceae* and increase in *Eubacteriaceae*[45]. Nevertheless, rifaximin is associated with improved cognitive function and endotoxemia in HE accompanied by alteration of gut bacterial associations with metabolites, suggesting that its beneficial effects may be attributable to changes in bacterial metabolic function. Alternatively, rifaximin may act through the alteration of the small intestinal microbiota, the composition of which would not be well characterized in studies focused on the examination of feces. Although rifaximin has demonstrated adequate safety profile, the risk of bacterial resistance in long-term use remains a concern. Other antibiotics used to treat HE such as aminoglycosides, metronidazole,

and vancomycin are similarly limited by significant toxicity and/or concern for bacterial resistance. Probiotics are used to treat HE based on the notion that they may modulate the gut microbiota by decreasing pathogenic bacteria and/or urease activity. In mice, a lactobacillus probiotic reduced ammonia levels and mortality after liver injury with thioacetamide[46]. Several probiotics tested in humans with cirrhosis have reported modest evidence of efficacy[47, 48]. While promising, studies of probiotic therapies have suffered from methodological limitations, poor documentation of long-term effects, and small effects on outcome. These findings motivate efforts to engineer resilient bacterial communities that effectively and durably alters the gut microbiota to treat HE.

1.4 Fecal microbiota transplantation and use of defined bacterial consortia

With the rising incidence of *Clostridium difficile* infection (CDI) over the past few decades, clinicians and researchers have sought new ways to treat this disease entity that is frequently recurrent and refractory to standard antibiotic therapy. Fecal microbiota transplantation (FMT) has proven to be highly effective in treating recurrent and/or refractory CDI, with a cure rate over 90%[49]. This provides proof of principle that the dysbiotic microbiota can be modified to treat disease. The use of donor fecal material to treat disease is not a novel concept. Rather, it has been practiced for centuries. The Chinese most likely began the earliest known use of fecal material to treat disease. According to Chinese literary records, during the 4th century, a well-known traditional Chinese medicine doctor named Ge Hong reported the use of human fecal suspension given orally to treat food poisoning or severe diarrhea with positive results[50]. In 17th century Europe, a German physician named Christian Paullini published a book called *Heilsame Dreck-Apotheke* (Salutary Filth-Pharmacy) on the medical uses of human and animal feces[51]. In the modern era, Eiseman *et al.* reported the successful treatment of

pseudomembranous colitis using fecal enemas in 1958[52]. The idea of transferring gastrointestinal contents (i.e. transfaunation) has also been used for centuries in veterinary medicine for indications such as treating chronic diarrhea in horses and increasing resistance of newborn chicks to *Salmonella* infection[49].

As opposed to the untargeted use of transferring entire microbial communities in feces from donors to recipients, another approach is to create synthetic mixtures of defined bacterial strains cultured in the laboratory to achieve targeted physiological effects. In 1989, Tvede and Rask-Madsen reported the successful treatment of recurrent CDI using a defined mixture of ten bacterial strains isolated from healthy human feces[53], representing the first known use of a defined bacterial mixture to treat disease. The mixture contained facultative aerobic and anaerobic bacteria, including three strains of *Clostridia* species, three strains of *Bacteroides* species, two strains of *E. coli*, *Streptococcus faecalis*, and *Peptostreptococcus productus*. These strains were chosen to replace bacteria found to be diminished and/or absent in CDI (e.g *Bacteroides spp.*) and installing bacteria that inhibited *C. difficile* in vitro (e.g. *R. productus, C. bifermentans*, and one *E. coli* strain). In 2013, Petrof *et al.* isolated 33 strains of nonpathogenic bacteria from the stool of a healthy donor and named the bacterial mixture "RePOOPulate," which was used to successfully treat recurrent CDI in two patients for at least six months[54]. Although RePOOPulate bacterial strains were rare at baseline in the recipients prior to treatment, analysis of their stool samples at six-month follow-up after treatment showed that RePOOPulate constituted more than 25% of the gut microbiota. These findings demonstrate the efficacy, resiliency, and sustainability of defined bacterial mixtures in achieving targeted physiological effects to treat disease.

The success of FMT and synthetic defined bacterial mixtures in the treatment of CDI provides credence to the notion that other disease entities associated with dysbiosis

may be treated with similar approaches. These include but are not limited to inflammatory bowel disease[55], metabolic syndrome[4, 5], and even autism[9]. As discussed in **Section 1.1**, researchers haven shown that the obese and lean phenotype can be conferred to germ-free mice by transferring fecal microbiota from obese and lean human donors, respectively[13, 14]. However, it is unclear which bacterial strains and/or combination of bacteria were responsible for the observed phenotype. Identifying specific microbes and understanding their metabolic properties may be the next crucial steps toward achieving the desired physiological effects, especially in the creation and development of defined bacterial mixtures. In the case of CDI, different microbes may utilize different mechanisms to curtail the growth of *C. difficile*, among which are competitive niche exclusion, production of small molecules and/or antimicrobial peptides, modulation of bile acid metabolism, and immune regulation[49]. The specific members in a bacterial consortium and their metabolic properties required to suppress the growth of *C. difficile*, however, remain unknown. In the case of HE, a bacterial consortium that lacks urease activity would limit colonic urea hydrolysis and ammonia production, potentially ameliorating the clinical sequelae of hyperammonemia.

Defined mixtures of microbes have also been used in the creation and development of gnotobiotic mice[56]. One such example is Altered Schaedler Flora (ASF). The original Schaedler Flora was created by Russell W. Schaedler in the 1970s to colonize germfree mice with bacteria isolated from conventional mice to restore cecal morphology and enhance murine health. The chosen bacterial strains were primarily aerobic bacteria or less oxygen sensitive anaerobes that were easy to culture, consisting of the following: *Escherichia coli* var. *mutabilis*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus salivarius*, group N *Streptococcus*, *Bacteroides distasonis*, a *Clostridium* sp., and an EOS fusiform bacterium[57]. In 1978, the National Cancer Institute

revised and standardized the Schaedler Flora (renamed “Altered Schaedler Flora”) by keeping four of the original bacterial strains (the two lactobacilli, *B. distasonis*, and the EOS fusiform bacterium) while replacing the other four bacterial strains with a spiral-shaped bacterium and three new fusiform EOS bacteria[57, 58]. ASF has now been used for decades by commercial mouse vendors to colonize rodent colonies and enhance the health of immunodeficient mouse strains. We found that ASF is low urease activity, making it useful as a prototype consortium for studies of metabolic engineering described to treat HE (see **Chapter 2**).

The use of FMT and synthetic microbial mixtures for the treatment of diseases entail preparation of the gut environment for proper inoculation and colonization of the transplanted bacterial species. In the case of CDI, there is generally antecedent use of antibiotics that have already disrupted the endogenous microbiota, leading to dysbiosis, proliferation of *C. difficile*, and subsequent replacement and suppression of *C. difficile* by the transplanted microbiota. This is not necessarily the case for other disease conditions. One reason that current generation of probiotics may not effectively or sustainably replace the endogenous microbiota is because of the large endogenous biomass already present in the gut environment, occupying niches and preventing the effective colonization by new bacterial taxa. Thereby, developing a systematic and reproducible method to dramatically reduce the endogenous gut microbial load will be crucial for the successful inoculation and engraftment of defined bacterial consortia in the treatment of disease (see **Chapter 2**).

1.5 The impact of diet on the composition and function of the gut microbiota

To develop stable and resilient microbial communities that can exert durable and targeted physiological effects, we must first understand how they respond to

environmental stressors. Antibiotics and diet, in particular, have been shown to exert strong effects on the composition and function of the gut microbiota. By depleting certain endogenous bacterial populations, antibiotics can lead to dysbiosis. This predisposes to the proliferation of pathogenic bacterial strains such as *C. difficile* as discussed previously. Dietary changes, on the other hand, exert influences over the gut microbiota by altering nutrient and substrate availabilities. By increasing substrates preferred by one microbial species over another, diet not only alters the composition of the gut microbiota, but also microbial metabolic functions as well as host physiological responses. One example is the concept of enterotypes. Several studies have shown that the ratio between two main genera in the human gut microbiota, *Bacteroides* and *Prevotella*, is strongly dependent on diet. The relative abundance of *Prevotella* was found to be high in populations that consumed a plant-based diet high in carbohydrates and simple sugars, such as rural African village in Malawi and Venezuela[59, 60]. On the other hand, the relative abundance of *Bacteroides* was found to be high in populations that consumed more animal proteins and fats, such as the United States[61, 62]. *Bacteroides* and *Prevotella* may exhibit co-exclusion based on their taxonomic and functional similarities[55, 63]. Although other studies have challenged the concept of enterotypes to suggest that it represents more of a gradient rather than distinct clusters of taxonomic representations across populations, it nevertheless emphasizes the importance of diet in shaping the gut microbiota[55, 64]. In **Chapter 3**, we will examine how systemic nitrogen limitation in the setting of a low protein diet (LPD) can affect the composition and function of a defined bacterial consortium with minimal urease activity. This is relevant because bacterial urease is critical in host-gut microbiota nitrogen flux, but also because a LPD is used clinically to treat conditions of hyperammonemia.

Therefore, investigation of the stability and resilience of a urease-deficient bacterial consortium in the setting of a LPD has significant clinical implications.

Exactly how significant and rapid are the effects that diet exerts on the gut microbiota? In a controlled-feeding experiment, ten human subjects were randomized to receive either a high-fat, low-fiber diet or a low-fat, high-fiber diet for ten days[62]. 16S tagged sequencing analysis of fecal samples revealed significant inter-individual differences that accounted for most of the variances in gut microbial composition. Over the course of experiment, however, the study did not find a significant reduction in inter-individual differences within the same diet group. This suggests that short-term dietary changes do not overcome inter-individual differences in gut microbial composition. Nevertheless, the study found that changes in gut microbiota from baseline were detectable within 24 hours of initiating controlled feeding, indicating rapid effects from the dietary interventions. Alterations in the composition of the human gut microbiota due to dietary perturbations may be modest compared to those observed in rodents. In a human study comparing the microbial composition between omnivores and vegans, the differences were found to be a relatively modest[65]. On the other hand, in a recent murine study, the investigators used either a low-fat, high-plant-polysaccharide diet or a high-fat, high-sugar diet to examine the effects of dietary perturbations on the gut microbiota of five inbred mouse strains, mice deficient for genes relevant to host-microbial interactions, and >200 outbred mice[66]. The high-fat, high-sugar diet rapidly and reproducibly altered the gut microbiota despite differences in host genotype, taking an average of 3.5 days for each diet-responsive bacterial groups to reach a new steady state.

Specific dietary nutrients may affect the relative abundances of specific microbes based on their metabolic properties and preferences. For example, the main saccharolytic genera in the human gut microbiota include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Lactobacillus*, and *Ruminococcus*[67]. The most abundant amino acid fermenting bacteria in the human small intestine include the genera *Clostridium*, *Bacillus-Lactobacillus-Streptococcus* groups, and *Proteobacteria*, whereas bacteria belonging to the genera *Clostridium* and *Peptostreptococcus* appear to be the most prevalent species involved in amino acid fermentation in the large intestine[68]. In a murine study, investigators generated gnotobiotic mice by inoculating ten sequenced human bacterial strains into germfree mice then assessed the changes in species abundance in response to systematic variations in four defined dietary ingredients representing four macronutrients (i.e. casein=protein, corn oil=fat, corn starch=polysaccharide, and sucrose=simple sugar)[69]. Based on the responses, they developed a statistical model that was able to predict over 60% of the variation in species abundance caused by dietary changes and identify the dietary factor that best explained the changes seen for each community member. For example, the abundances of *E. coli* and *C. symbiosum* correlated with changes in the diet variables sucrose and starch, respectively. They also found that total community biomass and the abundance of each community member were best explained by changes in the diet variable casein, potentially because a component of casein, likely amino acids and/or nitrogen, influences the growth of the microbial community.

Dietary nutrients not only affect the composition of the gut microbiota, but also its function and, in turn, microbial metabolites and the host metabolome. One prime example is the fermentation of complex carbohydrates in the diet by the gut microbiota to produce short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate as

discussed in **Section 1.1**. Another example is bacterial metabolism of dietary lipid phosphatidylcholine that is associated with the development of atherosclerosis and increased risk for cardiovascular disease, also discussed in **Section 1.1**[70, 71]. Furthermore, in a human study that examined gut microbiota composition and metabolomics analyses between omnivores and vegans in an urban US population, several important differences were found[65]. First, diet has a strong impact on the plasma metabolome of omnivores and vegans, but the effect on the composition of the gut microbiota was quite modest. Plasma metabolomes between omnivores and vegans differed significantly, but the microbiota composition was not strongly associated with the plasma metabolome. Second, the production of microbial metabolites from dietary substrates is constrained by the composition of the gut microbiota. Unlike prior studies of populations living in agrarian societies where increased SCFA levels have been attributed to both increased consumption of indigestive carbohydrates and increased polysaccharide-fermenting microbiota[59], this study did not detect significant differences in fecal SCFA levels between omnivores and vegans despite increased ingestion of fermentable substrates in the latter. Although numerous studies have examined the impact of dietary interventions involving alterations in carbohydrate and fat contents, the effect of altering protein content is relatively under-investigated especially in light of its importance in host and gut microbiota nitrogen metabolism. In **Chapter 3**, we will discuss the impact of a low protein diet on the gut microbiota and host in terms of nitrogen flux given relevance to the treatment of hyperammonemic inborn errors of metabolism such as urea cycle disorders.

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CHAPTER 2. Engineering the Gut Microbiota to Treat Hyperammonemia

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> *equal contribution

Personal contribution:

> 1) Experiment implementation, sample collection and analyses, and data interpretation to characterize the effects of ASF transplantation on urease activity
>
> 2) Experimental design and implementation, data collection and analyses to determine the effects of ASF transplantation on neurobehavioral performance and mortality in the thiocetamide murine model of hepatic injury and fibrosis
>
> 3) Biochemical assessment of fecal and plasma ammonia levels in all related experiments, including sample collection and processing
>
> 4) Assistance with drafting and revising the relevant sections of the manuscript

2.1 ABSTRACT

Engineering the gut microbiota for therapeutic modulation of host metabolism is an emerging goal of microbiome research. In the intestine, bacterial urease converts host-derived urea to ammonia and carbon dioxide, contributing to hyperammonemia-associated neurotoxicity and encephalopathy in patients with liver disease. Here we report engineering the gut microbiota in mice for therapeutic reduction of urease activity. Depletion of the pre-existing gut microbiota followed by inoculation with Altered Schaedler's Flora (ASF), a defined consortium of 8 bacteria with minimal urease gene content, established a persistent new community that exhibited long-term reduction in fecal urease activity and ammonia production. ASF transplantation was associated with decreased morbidity and mortality in a murine model of hepatic injury. These results provide proof-of-concept that inoculation of a prepared host with a defined gut microbiota can lead to durable metabolic changes with therapeutic utility.

2.2 INTRODUCTION

Dysbiosis, an abnormal and pathogenic state of the human microbiome, has been implicated in inflammatory bowel diseases, atherosclerosis, obesity, diabetes, colon cancer, and other diseases [1, 2]. Fecal microbiota transplantation (FMT) is highly effective in the treatment of refractory *Clostridium difficile* infection (CDI), providing proof-of-principle that a human disease can be treated by engineering the gut microbiota [3], and further studies indicate that a healthy microbiota can prevent disease acquisition [4].

Bacteria residing in the human gut produce urease, the activity of which is beneficial in healthy hosts but pathogenic in hosts with liver disease. Urea produced by the liver as a waste product is both excreted in urine and transported into the colon, where it is hydrolyzed by bacterial urease into carbon dioxide and ammonia. Ammonia is

then 1) utilized by the microbiota for protein synthesis, 2) reabsorbed by the host where it is incorporated into the nitrogen pool by hepatic metabolism, or 3) excreted in the feces. Mammalian genomes do not encode urease genes, so ammonia production results from bacterial urease activity acting on host-produced urea [5, 6]. Ammonia is also largely responsible for the alkaline pH of the colonic luminal environment, acting to buffer the short chain fatty acids also produced by the microbiota. Systemic ammonia levels are elevated in patients with liver injury, chronic liver disease, or urea cycle defects, where hepatic abnormalities prevent the normal processing of ammonia delivered to the liver from the intestinal tract. Circulating ammonia is correlated with damage to the central nervous system in patients with chronic liver disease or inborn errors of metabolism, resulting in hepatic encephalopathy (HE) [7, 8].

Current treatments for hepatic encephalopathy and hyperammonemia are inadequate [8]. Antibiotics traditionally used to treat hepatic encephalopathy, including aminoglycosides and metronidazole, are limited by side effects and concerns for safety including ototoxicity, nephrotoxicity, and peripheral neuropathy [9, 10]. Although rifaximin, a minimally absorbed antibiotic, has shown efficacy in the treatment and prevention of hepatic encephalopathy [11, 12], potential development of antimicrobial resistance with long-term use remains a concern. Lactulose is used to acidify feces and sequester ammonia as ammonium, but lactulose is poorly tolerated, resulting in poor adherence [13]. In a mouse-model of thioacetamide-induced liver injury, a lactobacillus probiotic has been reported to reduce ammonia levels and mortality [14], but these benefits have not been extended to human studies [15, 16]. While promising, studies of probiotic therapies in humans described to date have suffered from methodological limitations, did not document long term effects, and showed consistently small effects on outcome, motivating efforts to engineer more resilient and effective bacterial communities.

Here we show that a synthetic microbial community lacking urease activity can be installed in the gut to reduce the production of ammonia long-term and thereby mitigate HE in a mouse model. For proof of concept studies, we used Altered Schaedler's Flora (ASF), which consists of eight murine gut commensal bacterial strains that were assembled in the 1970s and standardized by the National Cancer Institute in 1978 [17]. The strains were originally selected based on their persistence from generation to generation in germ-free mice and their ability to restore the cecal morphology comparable to that of conventional mice. The ASF community is innocuous, known to have a beneficial effect of inducing immune tolerance [18], and is used by commercial mouse vendors to enhance the health of immunodeficient mouse strains. We found that ASF is low in urease activity, making it useful for studies of metabolic engineering described here. Mixed results have been reported regarding the transfer of conventional microbiota between rodents, with some studies reporting successful transfer with repetitive inoculation [19] but others that the use of antibiotics prevented transfer [20]. We developed methods for purging the gut microbiota from normally colonized mice, then transplanted in ASF by gavage. Transplantation of ASF was monitored longitudinally using deep sequencing of DNA from fecal pellets, revealing highly efficient colonization in properly prepared hosts. Over four weeks of monitoring, ASF was partially displaced by selective colonization with environmental Firmicutes, reaching a new steady state, but fecal ammonia levels remained low. The engineered gut community was tested in thioacetamide (TAA) models of acute and chronic liver injury (**Fig. 2-1A)** where we show that it reduced fecal ammonia levels, mortality, and neurobehavioral deficits**.** These results show that transplantation of a minimal defined microbial community can alter metabolism in a pre-determined fashion by establishing a new gut microbiome, resulting in therapeutic benefits in disease models.

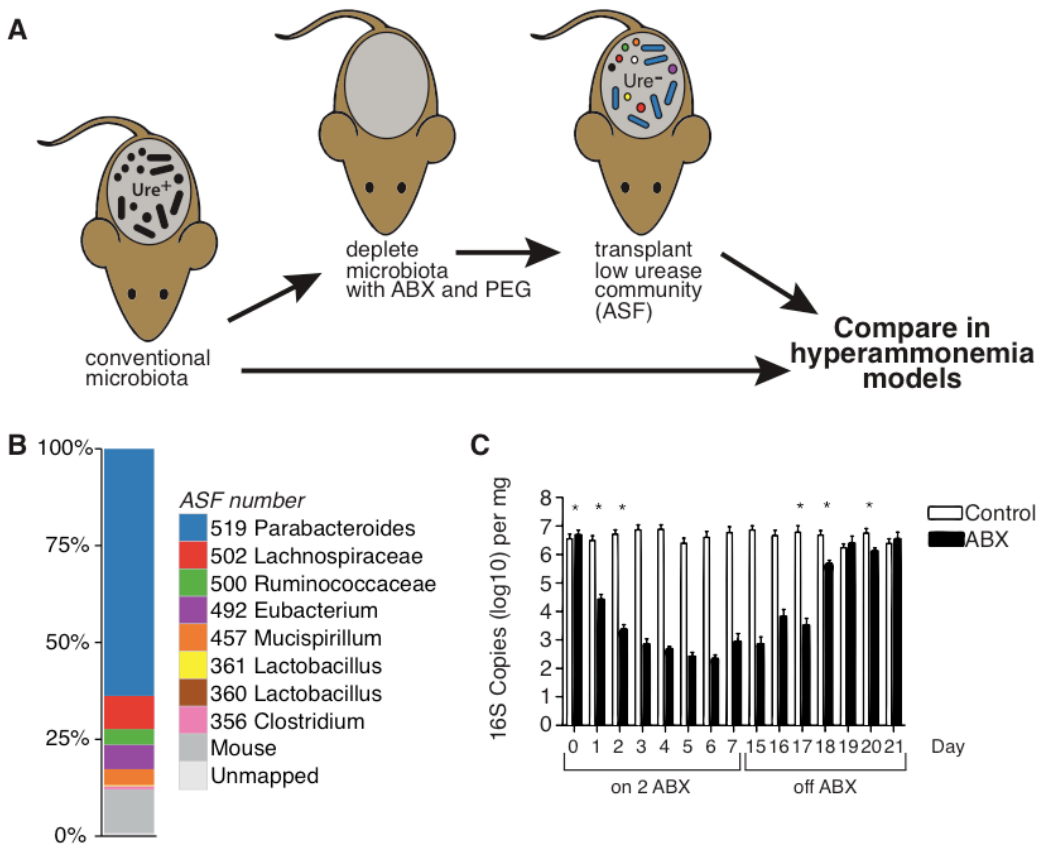

Figure 2-1. Transferring ASF into a previously colonized murine host. (**A**) Diagram of the experimental method. (**B**) Shotgun metagenomic analysis of stool from ASF-colonized animals used for gavage in this study. Proportions of the different ASF lineages and other organisms are shown by the color code to the right. **(C)** Time course of 16S rRNA gene copy number during oral antibiotic treatment (14 days, vancomycin and neomycin) and upon discontinuing antibiotics on day 15 (n=3 per group). Asterisks on left indicate $p<0.0001$ on Days 0-2 compared to average of Days 5-15 in ABX group. Asterisks on right indicate $p<0.05$ between ABX and control group. Paired-sample t-test and two-tailed Student's t-test.

2.3 MATERIALS AND METHODS

Animals: Feces from CB17 SCID mice colonized with ASF (Taconic, Tarrytown NY) were the source of ASF in transplantation experiments. Mice in this study were maintained in standard SPF barrier facility and were fed irradiated AIN-76 (Research Diets, 21% protein by kilocalories) chow. The non-irradiated diet experiment used

Laboratory Rodent Diet 5001 containing 29% protein by kilocalories. For microbiota transfer experiments, 0.1 g of feces was diluted 10-fold in PBS. Where indicated, germ-free mouse recipients were purchased from Taconic as were conventionally-housed Swiss Webster mice in the first experiment. All other experiments used either male or female 8-week-old C57BL6 mice (Jackson Labs). Pre-treated conventional mice were prepared for inoculation by the oral delivery of antibiotics in drinking water (1.125 g of aspartame, 0.15 g of vancomycin, and 0.3 g of neomycin in 300 ml of sterile water) for 72 hours. During the final 12 hours water supply was exchanged with a 10% PEG solution (Merck, Whitehouse NJ) and the mice were fasted. Mice were then inoculated with feces by oral gavage daily for 5-7 days. Fecal pellets were collected during the indicated time points for bacterial taxonomic and biochemical analyses.

DNA isolation, quantitative PCR, sequencing, and analysis: DNA was isolated from stool as described [21, 22]. Bacterial 16S rRNA gene sequences were PCR amplified using primers binding to the V1V2 region [21, 22] using bar coded primers [23, 24]. Shotgun metagenomic data was collected using the TruSeq library preparation method and an Illumina HiSeq instrument. Sequence reads were quality controlled and analyzed using the QIIME pipeline with default parameters [25]. Sequence data sets will be deposited at the NCBI upon acceptance of this paper for publication.

Urease activity and ammonia assays: Fecal ammonia levels were determined using an Ammonia Assay Kit (ab83360, Abcam, Cambridge, MA). Fecal pellets were suspended in the assay buffer provided at a concentration of 1 mg/10 uL and centrifuged at 13,000 x g for 10 minutes at room temperature to remove insoluble material. Ammonia concentration was then determined according to the kit protocol.

Fecal urease activity was measured by suspending fecal pellets in 0.5 mM HEPES buffer. After sonication and centrifugation the supernatant was incubated for 30 minutes at 37°C with 1µCi ^{14}C-labeled urea (19.9 mCi/mmol, ARC-0150, American Radiolabeled Chemicals, St. Louis, MO) in a sealed container. The air was purged into a trap containing 2.5 mL of 0.2 M benzethonium hydroxide in methanol (82156, Sigma, Milwaukee, WI) and $^{14}CO_2$ activity was quantified by liquid scintillation counting. A standard curve was generated using purified E. coli urease (150 IU/mg, 22060744-1, BioWorld, Dublin, OH).

The urease breath test was performed after a 4-hour fast by placing mice in a sealed glass chamber. A total of 4 cc of air was withdrawn from the chamber using a gas-tight syringe after 10 minutes and injected into 12 ml (gas-helium) Labco Exetainer glass vials (438B, Labco Limited, Lampeter, UK) to establish a baseline CO_2 level. The mouse was then injected by tail vein with [^{13}C]urea (150 µg/g body weight) (CLM-311-0, Cambridge Isotope Labs, Inc., Andover, MA). Blood was collected 15 minutes post-injection to assess total urea and isotopic enrichment in [^{13}C]urea. Enrichment in expired air was measured at 30, 60, 120, 180, and 240 min following injection of labeled urea. The $^{13}CO_2/^{12}CO_2$ ratio was measured in gas samples with a Finnigan Delta Plus isotope ratio-mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A commercial CO_2 source (Airgas, Radnor, PA) was used as the standard. The measurement of isotopic abundance in [^{13}C]urea in plasma involves the elimination of CO_2 from the sample and the subsequent conversion of urea to CO_2 with commercially available urease [26].

Induction of acute liver injury and hepatic fibrosis: Mice were given a single dose of TAA at 600 mg/kg by IP injection in the high-dose TAA acute liver injury model. In the low-dose TAA acute liver injury model, mice were given a single dose of TAA at 300 mg/kg by IP injection, with subsequent performance of the Y-maze neurobehavior test at 48 hours after TAA injection (see Neurobehavior test below). In the chronic liver fibrosis model, all mice were given TAA by IP injection three times weekly, with initial dose 100 mg/kg that was decreased to 50 mg/kg during 1st week given mortality, then dose escalation to 100 mg/kg during the 2nd week, 200 mg/kg during the 3rd week, 300 mg/kg during the 4th week, and 400 mg/kg during the 5th to 7th week of TAA administration.

Neurobehavior test: The Y-maze test was conducted to assess the rodent's memory and spatial learning [27]. It consists of three identical and equally-spaced arms, and the natural tendency of the mouse is to investigate a new arm of the maze rather than returning to the one previously visited. A mouse with cognitive deficit would exhibit less "spontaneous alternation," defined as entering all three arms in three sequential arm entries. Control mice typically exhibit >60% spontaneous alternation. In this experiment, mice were allowed to habituate to the testing room in which there are no overt visual cues for 30 minutes prior to testing. The maze was cleaned with 70% ethanol before using and between trials to eliminate odor cues. A trial started when a mouse is released into one arm of the maze (same arm for each mouse). As the mouse navigated the maze, each arm entry was noted. At the end of the timed trial (8 minutes), total arm entries are summed and spontaneous alternations are determined by the following formula: % Spontaneous Alternation= [(Number of alternations) / (Total arm entries−2)] ×100. Trials were video recorded as well as graded during the procedure. Image

analysis software was used to measure the total distance traveled during the trial for each mouse.

Statistics: Results are expressed as means ± standard error of the mean (SEM). Statistical significance among three or more groups was assessed by analysis of variance (ANOVA). Two-tailed Student's t-test and paired-sample t-test were used for direct comparison between two groups and within group, respectively. Tukey's test was used to adjust for multiple comparisons. Kaplan-Meier survival curves were compared using the Log-Rank Test. $P < 0.05$ was considered as statistically significant.

Study approval: All animal studies were performed with the approval of University of Pennsylvania's Institutional Animal Care and Use Committee.

2.4 RESULTS

Transplantation of ASF into previously colonized mice.

The original ASF was composed of eight bacterial strains. Over time, ASF has been maintained in laboratory mice by fecal-oral transmission associated with cohousing in gnotobiotic isolators. Thus the composition of the ASF donor material used here was first characterized by DNA isolation and shotgun metagenomic sequencing (16.9 Gb). Alignment to draft genome sequences of ASF strains [28] documented the presence of seven of the eight original strains in our samples (**Fig. 2-1B**). *Parabacteroides* (ASF519) was the predominant lineage present in pellets. Additional ASF strains consisted of roughly even proportions of Clostridia (ASF356, ASF492, ASF500, ASF502) and *Mucispirillum schaedleri* (ASF457), accompanied by low levels of *Lactobacillus* (ASF361). No high-quality read pairs mapped concordantly to strain ASF360. In

addition to ASF strains, 11% of reads mapped to the mouse genome, and a small fraction of alignments mapped to probable artifacts including cloning vectors, metazoans and additional bacteria. We conclude that the bacteria in our donor material were mostly or entirely ASF strains.

To assess the persistence of ASF in mice housed under non-sterile conditions, sequential fecal pellets were collected from ten ASF-colonized mice that were transferred to conventional SPF housing, DNA was purified from pellets, and the abundance and types of bacteria present assessed by QPCR and deep sequencing of 16S rRNA V1V2 gene segments (**Supplementary Fig. 2-1**). Copy numbers of 16S sequences per gram of stool showed roughly similar abundance for ASF and conventionally colonized mice. Six of the eight ASF strains were resolved at the depth of sequencing performed. ASF519 (*Parabacteroides*) accounted for ~75% of sequence reads at the time of transfer of the mice. After approximately 2 months under nonsterile conditions, ASF519 remained the dominant taxon, and non-ASF taxa accounted for almost half of the sequence reads. Evidently ASF lineages persisted but did not entirely exclude other bacteria.

We reasoned that reduction of the endogenous microbiota would promote transfer of ASF, so we investigated the response of conventional microbiota to the oral delivery of two nonabsorbable antibiotics (ABX), neomycin and vancomycin. The 16S rRNA gene copy number was reduced by ~4 logs within 72 hours of oral antibiotic initiation ($p<0.0001$ for days 0, 1, and 2 compared to the mean of days 5-15), then returned to baseline 5 days after discontinuing antibiotics (**Fig. 2-1C**; $p=0.36$ for comparison of ABX versus control at day 21), paralleling previous studies [29, 30].

Fecal slurries obtained from ASF-colonized mice (Taconic, Germantown, NY) were then gavaged into conventionally housed recipients for seven days following a 72-

hour pre-treatment with oral antibiotics and a 12-hour intestinal purge using polyethylene glycol (PEG; pretreatment with antibiotics and PEG termed “Prepared” henceforth). PEG was used in our gut cleansing protocol because the use of a purgative will likely be necessary to reduce bacterial load in the human intestinal tract due to high biomass. For comparison, ASF transplants were carried out on conventional mice without pretreatment and on germ-free mice. Mice that were treated with antibiotics and PEG, and subsequently transplanted, showed normal numbers of bacteria by 16S QPCR copy number within 10 days (data not shown).

Longitudinal fecal samples were analyzed by deep sequencing of 16S rRNA gene tags, then the proportions of bacterial lineages detected plotted as heat maps, where each row shows a bacterial lineage and each column a fecal specimen (**Fig. 2-2**). Conventional mice that were not pretreated showed no increase in ASF lineages despite ASF gavage (**Fig. 2-2**, “Conventional + ASF gavage”). A few preexisting lineages were present that were indistinguishable from ASF using the V1V2 16S sequence window, but these did not increase in abundance after ASF gavage. A second group of mice (discussed above) were colonized with ASF from birth and then moved to the non-sterile facility. These mice had high levels of ASF519 and detectable levels of four other ASF strains (**Fig. 2-2**, “ASF-colonized”).

These groups were then compared to (1) germ-free mice gavaged with ASF and (2) conventional mice pretreated with antibiotics and PEG and gavaged with ASF (**Fig. 2-2**, “Germ-free + ASF gavage” and “Prepared host + ASF gavage”). Prior to ASF gavage, both the germ-free animals and antibiotic-treated animals showed high levels of *Lactococcus*, a lineage found in high levels in mouse chow [29], indicating that endogenous gut bacteria were mostly or entirely absent. Gavage of these animals with

ASF resulted in establishment of ASF lineages that persisted for the duration of the sampling period. Communities were again dominated by ASF519.

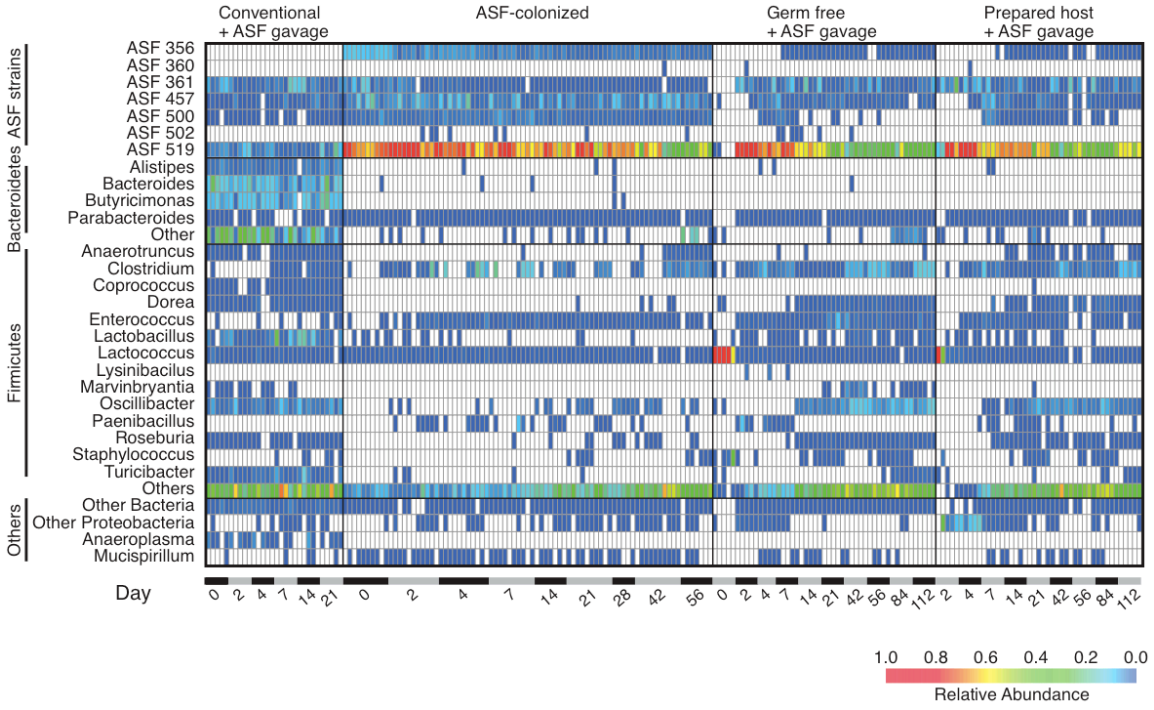

Figure 2-2. Heat map showing the relative abundance of bacterial lineages over time in ASF-colonized mice and controls. Rows indicate bacterial lineages as annotated on the left. Relative abundance is indicated by the color code at the bottom of the figure. Columns summarize sequencing results from individual fecal specimens. Elapsed time in days is shown along the bottom. Groups studied are indicated at the top of the heat map. These include (starting from the left): Conventional mice that were gavaged with ASF stool without preparation ("Conventional + ASF gavage"); mice that were ASF-colonized from birth, then transferred to a nonsterile SPF facility ("ASF-colonized"); mice that were germ-free, then gavaged with ASF ("germ free + ASF gavage"); and conventional mice that were prepared by antibiotic and PEG treatment, then gavaged with ASF (Prepared host + ASF gavage").

Longitudinal evolution of the transplanted ASF community.

Persistence of the transplanted ASF community was quantified using segmental regression, plotting time against the proportion of ASF in each sample (**Fig. 2-3A**). Prepared ASF-transplanted mice or germ-free ASF-transplanted mice transferred to nonsterile conditions were compared and found to behave similarly. Initially ASF

comprised the majority of the community. After transfer, the proportion of ASF slowly declined, becoming ~45% of the gut community after 34 days (Davies' test for change in slope $p<0.001$; 95% CI for break point was 27-41 days by segmented linear regression). After 34 days, the proportion of ASF strains did not decrease for the duration of the experiment (120 days; $p=0.86$). Comparisons of community membership using unweighted UniFrac analysis of the 16S rRNA gene sequencing data showed non-ASF lineages had colonized by day 14, and members persisted for the duration of the study (**Fig. 2-3B**), although the final abundance of non-ASF lineages was not achieved until about day 30 (**Fig. 2-3A**). Diversity of this new stable state measured by the Shannon index approached that of the starting community (**Fig. 2-3C**). ASF519 (*Parabacteroides*) was the main taxon persisting in both germ-free + ASF gavage and Prepared + ASF gavage hosts after six weeks, comprising approximately 40% and 50% in each (**Fig. 2-2**). As in humans, bacteria belonging to the *Bacteroides* genus were dominant taxa in conventionally housed mice [31, 32].

The new lineages appearing after transplantation were specific (**Fig. 2-4**). Members of the Bacteroidetes phylum did not recolonize after ASF transplantation, suggesting that ASF519, a member of the closely related *Parabacteroides* genus, may have occupied niches available to this group. Several Firmicutes lineages did colonize over time, including lineages annotating as *Oscillibacter*, *Clostridium*, and "Other" Firmicutes (mostly Order Clostridiales). Thus the community achieved a new steady state containing both ASF and environmentally-acquired lineages.

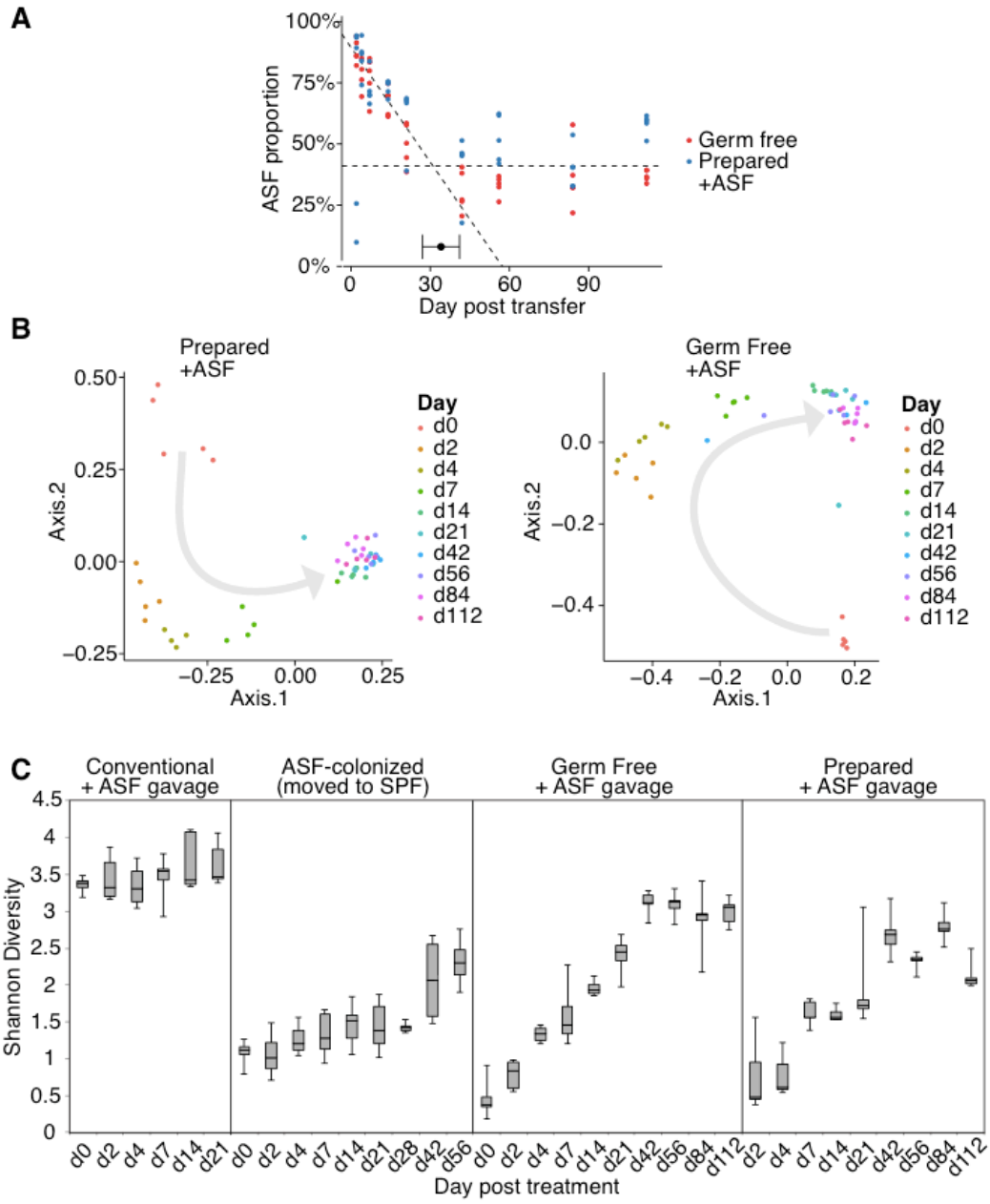

Figure 2-3. Development of a stable gut microbial community nucleated by inoculation with ASF. (A) Segmented regression analysis of communities in mice that were either germ-free or Prepared conventional mice subjected to ASF gavage. The y-axis shows the proportion of ASF lineages inferred from 16S rRNA gene tag pyrosequencing data. The x-axis shows the number of days post transfer. Segmented regression analysis showed two phases, indicating a slow decline in the ASF proportion up to about day 30, followed by establishment of a new steady state with ~40% ASF lineages. **(B)** PCoA ordination over time. Changes in community membership over time were analyzed using unweighted Unifrac [33]. The grey arrow indicates the progression of time. **(C)** Shannon diversity of the gut microbiota over time in the four hosts described in Fig. 2-2.

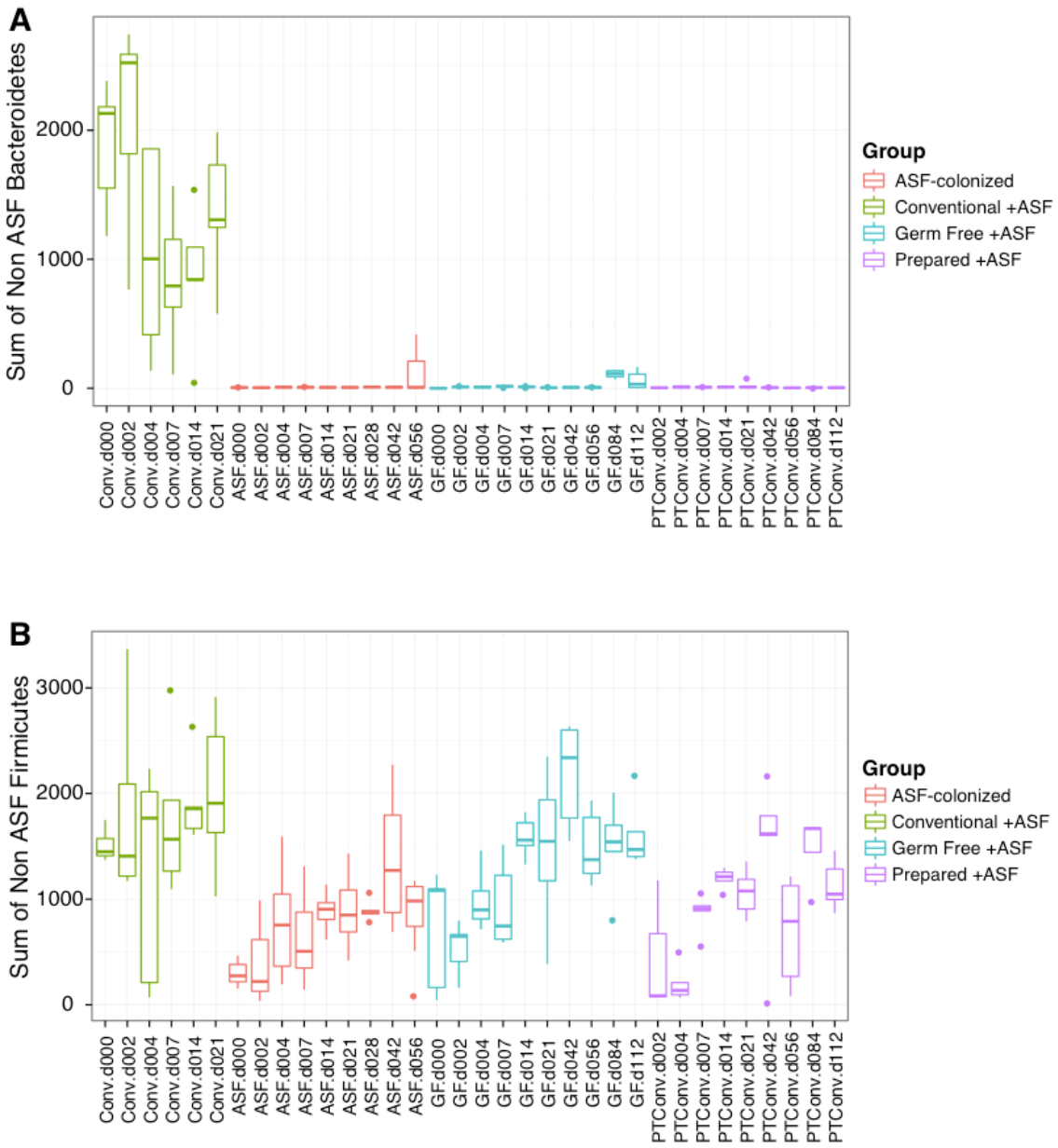

Figure 2-4. Comparison of non-ASF sequence reads from either (A) Bacteroidetes or (B) Firmicutes, illustrating selective repopulation with environmental Firmicutes. Groups are color-coded and represent change over time.

ASF has minimal urease gene content and activity.

An analysis of the complete ASF genomes showed minimal presence of urease genes. No urease genes were identified in the predominant *Parabacteroides* ASF519.

Two ASF members contained urease genes, ASF492 and ASF361, but both were minor members of the community after transplant (**Fig. 2-2**).

We then characterized urease activity. Urease activity was readily detectable in pellets from conventionally housed mice but was undetectable in pellets from mice colonized with ASF (**Fig. 2-5A**). Similar results were obtained with pellets from mice treated with oral antibiotics that reduced bacterial load 10,000-fold (**Fig. 2-5A**). Intravenous delivery of ^{13}C-urea to quantify urease activity in vivo through the production of $^{13}CO_2$ in a breath test revealed minimal hydrolysis in ASF-colonized mice (**Fig. 2-5B**).

Transplantation of ASF into prepared hosts led to a reduction in fecal urease activity lasting for at least 80 days (**Fig. 2-5C**). The novel community assembled after 30 days had low urease activity, even though new lineages became established in addition to ASF (**Fig. 2-2**). Analysis of representative genomic sequences from these newly established genera showed that *Oscillibacter*, *Dorea*, *Enterococcus*, and *Roseburia* do not encode urease genes. *Clostridium* and the group annotating as "other" Firmicutes (mostly Clostridiales), are mixed, with some representatives encoding urease genes while others do not. It is unknown whether the newly proliferating organisms lacked urease genes, or whether they encoded urease genes but expressed them at low levels. Thus the net effect of ASF colonization, together with establishment of additional lineages, was achievement of a new steady state with low urease activity. The new community persisted long term – ASF-transplanted mice showed low fecal ammonia levels for over one year in a SPF housing facility (data not shown).

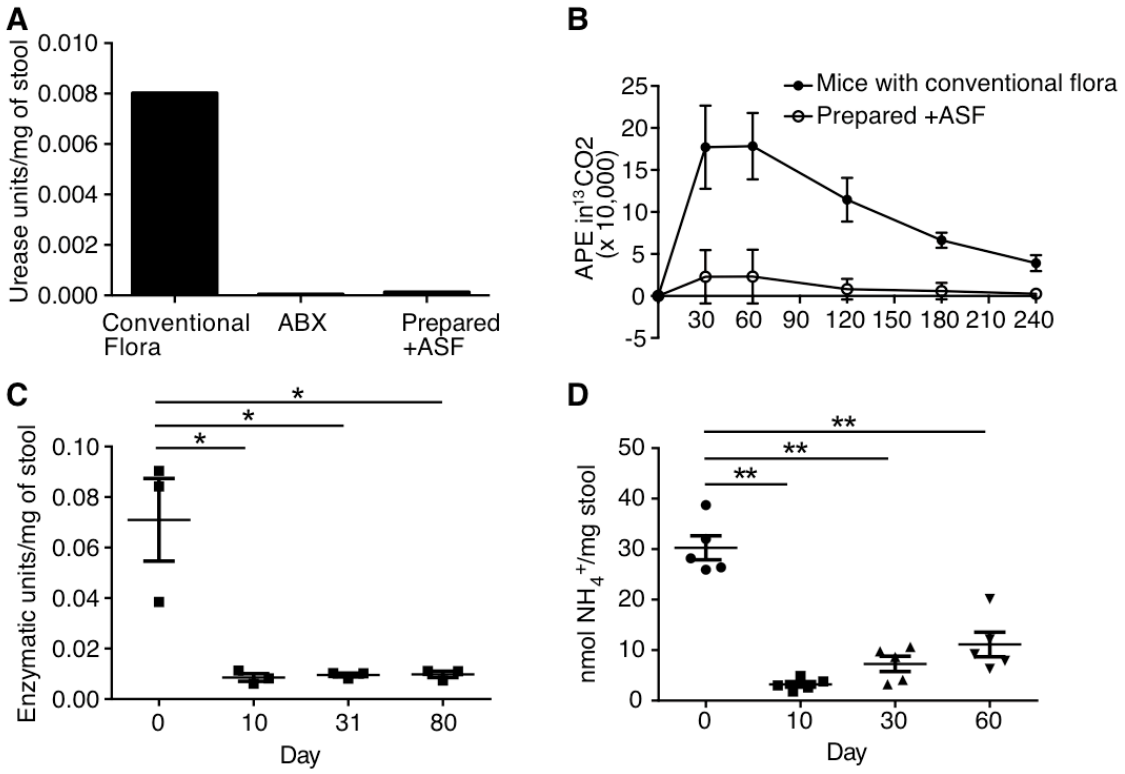

Figure 2-5. Transfer of ASF leads to reduction in urease activity and fecal ammonia levels. (A) Urease activity in the feces of a conventionally-housed mouse versus a mouse treated with antibiotics and a mouse colonized with ASF. **(B)** In vivo urease activity in conventionally-housed (n=5) and ASF-colonized mice (n=5) quantified by the release of $^{13}CO_2$ after IV injection of ^{13}C-urea. **(C)** Fecal urease activity in Prepared mice post-ASF transplantation at the indicated time points on irradiated diet (n=3). **(D)** Fecal ammonia levels pre- and post-transplant of ASF into Prepared mice fed a non-irradiated diet (n=5). *p<0.01, **p<0.001. Tukey's test for multiple comparisons.

The resilience of this new community state to dietary stress was evaluated by placing mice transplanted with ASF on a low protein diet similar to those used in patients with hyperammonemic inborn errors of metabolism [34]. Fecal ammonia levels remained much lower than was achieved by a low protein diet alone, an effect that was durable for several months (T.D.S. and G.D.W., unpublished observations). Another dietary stress, consumption of a non-irradiated diet, led to the displacement of ASF by other bacteria within weeks after FMT, with the predominant taxon *Parabacteroides* ASF519 being partially replaced by other taxa within the Bacteroidetes phylum (**Supplementary Fig. 2-**

2). Nevertheless, the reduction in fecal ammonia levels compared to baseline remained significant for several months even on a non-irradiated diet (**Fig. 2-5D**). Reductions in fecal ammonia levels have been correlated with reductions in blood ammonia [14, 35, 36], indicating that changes in colonic ammonia production and/or absorption can be associated with blood levels. The reduction in fecal ammonia may alter levels of false neurotransmitter precursors produced by the gut microbiota and/or by the host since ammonia is a substrate for both, leading to reduced formation of biogenic amines that are hypothesized to play a role in hepatic encephalopathy [37-39]. Thus we propose that fecal ammonia is a useful biomarker for response of the host to the treatment of hyperammonemia.

ASF transplantation reduced mortality and cognitive impairment in murine models of acute and chronic liver injury.

A major cause of morbidity and mortality associated with acute liver injury is the development of HE. Since hyperammonemia is associated with the development of HE in patients with impaired hepatic function [8], we asked if the transplantation of ASF might mitigate the effects of acute hepatic injury induced by thioacetamide (TAA) [40] treatment. Prepared ASF-transplanted (Prepared + ASF) mice showed both a reduction in fecal ammonia levels (**Supplementary Fig. 2-3A**) and reduced mortality in response to high-dose TAA compared to mice with conventional microbiota (**Fig. 2-6A**). This finding was also seen in the setting of chronic liver injury in mice transplanted three weeks prior to the chronic delivery of TAA at low-escalating doses for 7 weeks (**Fig. 2-6B**). Compared to control mice, Prepared + ASF mice demonstrated markedly reduced mortality that was maintained over the 7-week period during which hepatic fibrosis developed in both

groups[41, 42] (data not shown), consistent with a sustained reduction in fecal ammonia for months after ASF transplantation.

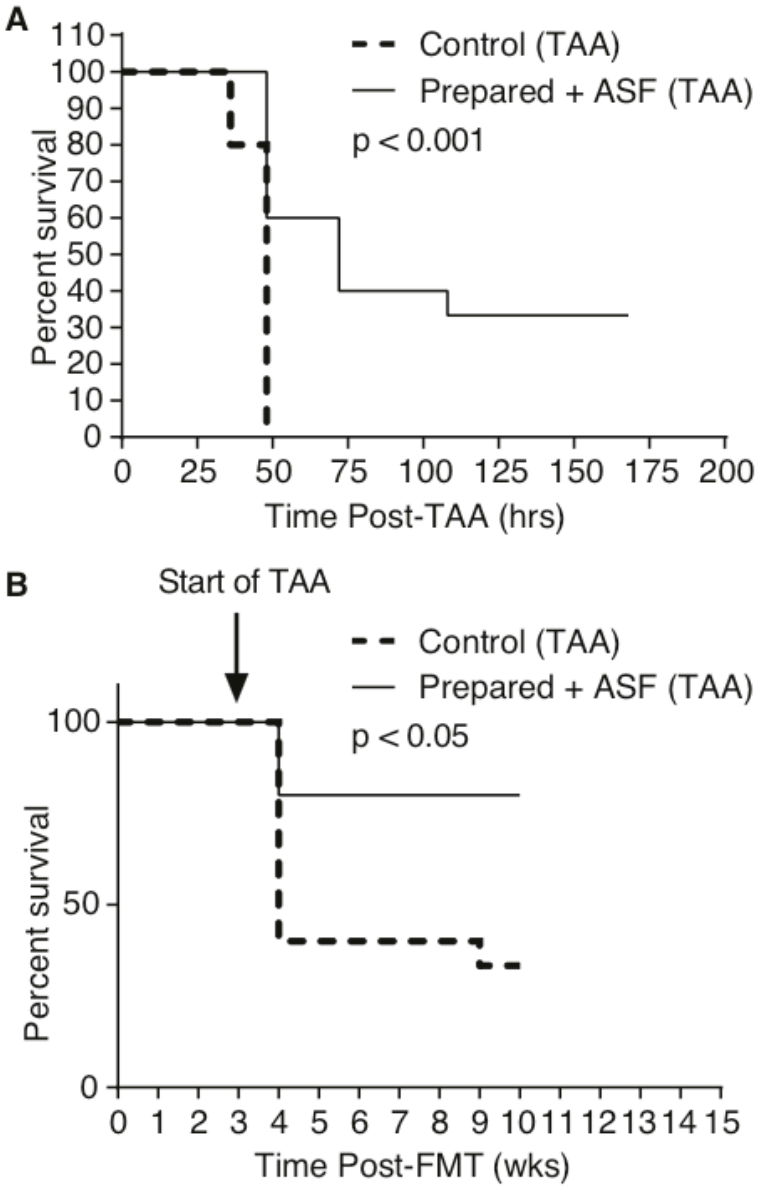

Figure 2-6. ASF transplantation into Prepared mice reduces mortality after thioacetamide-induced hepatic injury and fibrosis. (A) Kaplan-Meier survival curves of high-dose TAA-induced acute hepatic injury in conventional vs. Prepared mice transplanted with ASF (n=15 per group). **(B)** Kaplan-Meier survival curves of low escalating doses of three times weekly chronic TAA administration initiated three weeks after ASF transplantation (n=15 per group). Survival curves were analyzed by the Kaplan-Meier method using the log-rank test.

In mice, the TAA model has also been associated with neurobehavioral abnormalities resembling HE in humans [43]. Using a lower dose of TAA to reduce mortality, we analyzed memory and spatial learning in a Y-maze test comparing Prepared + ASF mice to mice transplanted with normal microbiota (Prepared + Normal Microbiota) as a control [44]. The survival rates were similar between the two groups at 80-90% at the lower TAA dose (**Supplementary Fig. 2-3B**). Fecal ammonia levels were reduced in mice transplanted with ASF compared to normal microbiota (**Supplementary Fig. 2-3C**). Prepared + Normal Microbiota mice showed a decrease in cognitive function after TAA treatment, quantified as spontaneous alternations in a Y-maze test, whereas Prepared + ASF mice treated with TAA were not different from untreated controls (**Fig. 2-7A**). Mice transplanted with ASF and normal microbiota did not differ in locomotor activity after TAA treatment (as quantified by total distance traveled and number of arm entries in the Y-maze) to account for the difference in spontaneous alternations, although both groups exhibited significantly less locomotor activity compared to untreated controls (**Figs. 2-7B and 2-7C**). To exclude the possibility that ASF transplantation directly reduced liver injury, we measured plasma alanine aminotransferase (ALT) and quantified histologic evidence of hepatocyte necrosis (**Supplementary Figs. 2-4A and 2-4B**). Both revealed that liver damage induced by TAA was not reduced by either ASF transplantation or antibiotic treatment. Thus improved survival and behavioral performance were associated with reduced ammonia levels and not improved locomotor activity or liver injury.

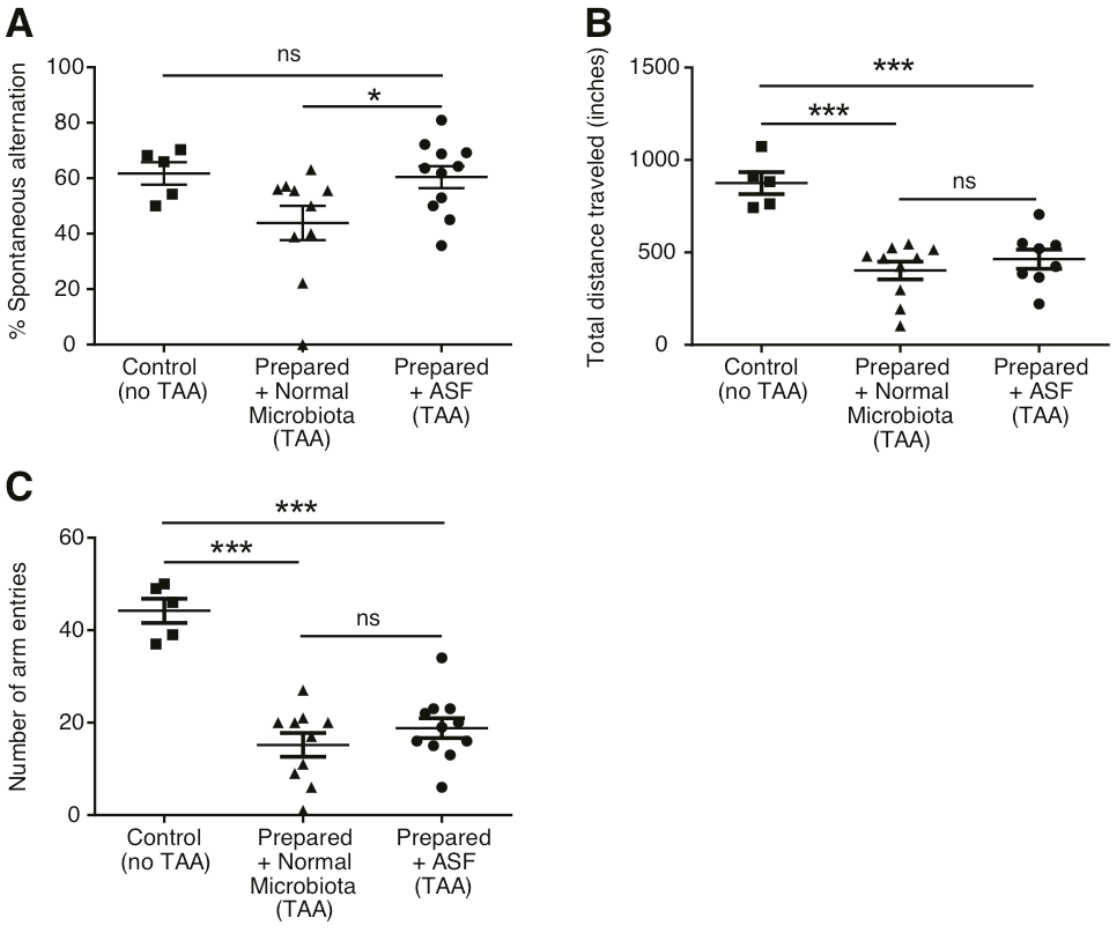

Figure 2-7. ASF transplantation into Prepared mice restores cognitive but not locomotor deficits after thioacetamide-induced hepatic injury. (A) Spontaneous alternations after TAA treatment quantified by Y-maze testing in Prepared mice that received transplantation with either Normal Microbiota (n=10) or ASF (n=11), compared to untreated control mice (n=5) (*p<0.05; ANOVA p=0.04). (**B**) Total distance traveled and (**C**) number of arm entries in the Y-maze in Prepared + ASF and Prepared + Normal Microbiota mice after TAA treatment compared to untreated control mice (***p<0.001; ANOVA p<0.001).

2.5 DISCUSSION

The success of FMT in the treatment of C. difficile infection (CDI) establishes that transplanting a resilient microbial community can alter a dysbiotic microbiota and thereby treat disease. Feces, however, contains not only bacteria but also a multitude of archaea, fungi, and viruses, so there is concern for safety [45], motivating development of defined

microbial consortia for human inoculation that have well-characterized biological properties and respond to the gut environment in predictable ways. Here we show that a defined minimal consortium of bacteria, ASF [17, 18], can durably reprogram the composition and metabolic function of the gut microbiota when inoculated into a properly prepared host. By taking advantage of the minimal urease activity in ASF, we provide evidence that reprogramming the gut microbiota can lead to lower fecal ammonia levels and mitigate the morbidity and mortality associated with liver damage.

The endogenous gut microbiota needed to be depleted by treatment with oral antibiotics and PEG for efficient transfer of the ASF community into conventionally housed mice. After the gut purge, transfer was as effective as into germ-free recipients. Tracking by sequencing suggested an orderly succession of lineages. After ASF transplantation, the predominant ASF519 strain appeared at the earliest times after gavage. *Mucispirillum schaedleri* (ASF457), and Ruminococcaceae (ASF500) also appeared early on. The last to appear was the *Clostridium* sp. (ASF356) at day 14 in some mice, one week after gavaging was complete, perhaps indicating the need for the development of a specific niche that permitted the establishment of more fastidious taxa. Most mice contained a *Lactobacillus* strain indistinguishable from ASF361 prior to transplantation, so this lineage could not be tracked with the methods used. The observed succession may be similar to the succession of bacterial taxa in human infants as consumption of oxygen by initial gut colonizers allows the expansion of bacterial clades that are obligate anaerobes [46, 47].

By monitoring the composition of the transplanted ASF community over four months by 16S rRNA gene sequencing, we were able to assess its persistence in the non-sterile SPF environment. The ASF community did not fully exclude other taxa. The community appeared to achieve a new steady state whereby both types of transplanted

hosts came to resemble the mice colonized with ASF from birth housed long-term in a SPF environment. In each of these three groups, *Parabacteroides* (ASF519) remained the dominant taxon. Bacteria of the *Bacteroides* genus commonly dominate the human and murine gut microbiota [31, 48-51], but new *Bacteroides* did not accumulate over time in ASF-colonized mice, suggesting that *Parabacteroides* may have excluded *Bacteroides*, reminiscent of the "trade-off" between *Bacteroides* and *Prevotella* in human gut [22, 31, 52, 53]. The mechanism by which *Parabacteroides* excludes *Bacteroides* is unknown but, due to their taxonomic and functional similarities, may involve competition for limiting resources [54] such as has been shown for glycans and the competition between *Bacteroides* species in the colonic crypt [55]. Over time, the *Parabacteroides* and environmental Firmicutes established a new steady state approximating the composition of the conventional microbiota in humans and mice [48, 49, 56] possibly involving a syntrophic relationship between these lineages [57].

There was a sustained reduction in fecal urease enzymatic activity and ammonia production upon transfer of ASF into Prepared mice. There was no return of urease activity several months after transfer despite a substantial increase of non-ASF taxa. Possible lack of urease genes in many of the *Clostridium* taxa that accumulated after transplantation may explain the persistently reduced urease activity, although we cannot exclude other mechanisms since the regulation of urease enzymatic activity is known to be complex [6].

Production of ammonia by the gut microbiota has been implicated in host nitrogen balance [58-60], so there is a theoretical risk that a urease-free community might have an adverse effect on protein balance and growth of the host. However, we have tracked mice for over 1 year post-ASF transplantation and have not observed any adverse effects on body weight or mortality. ASF is acknowledged to be an innocuous

bacterial consortium in mice with beneficial effects on immune tolerance [18]. Nevertheless, additional safety studies will need to be performed using a humanized version of ASF in rodent models before human studies can be contemplated.

In summary, given that HE is a major contributor to morbidity and mortality in liver disease [61], transplantation of next-generation engineered communities based on ASF, coupled with improved preparation of the host as described here, represents a promising approach to more effective therapy.

2.6 ACKNOWLEDGEMENTS

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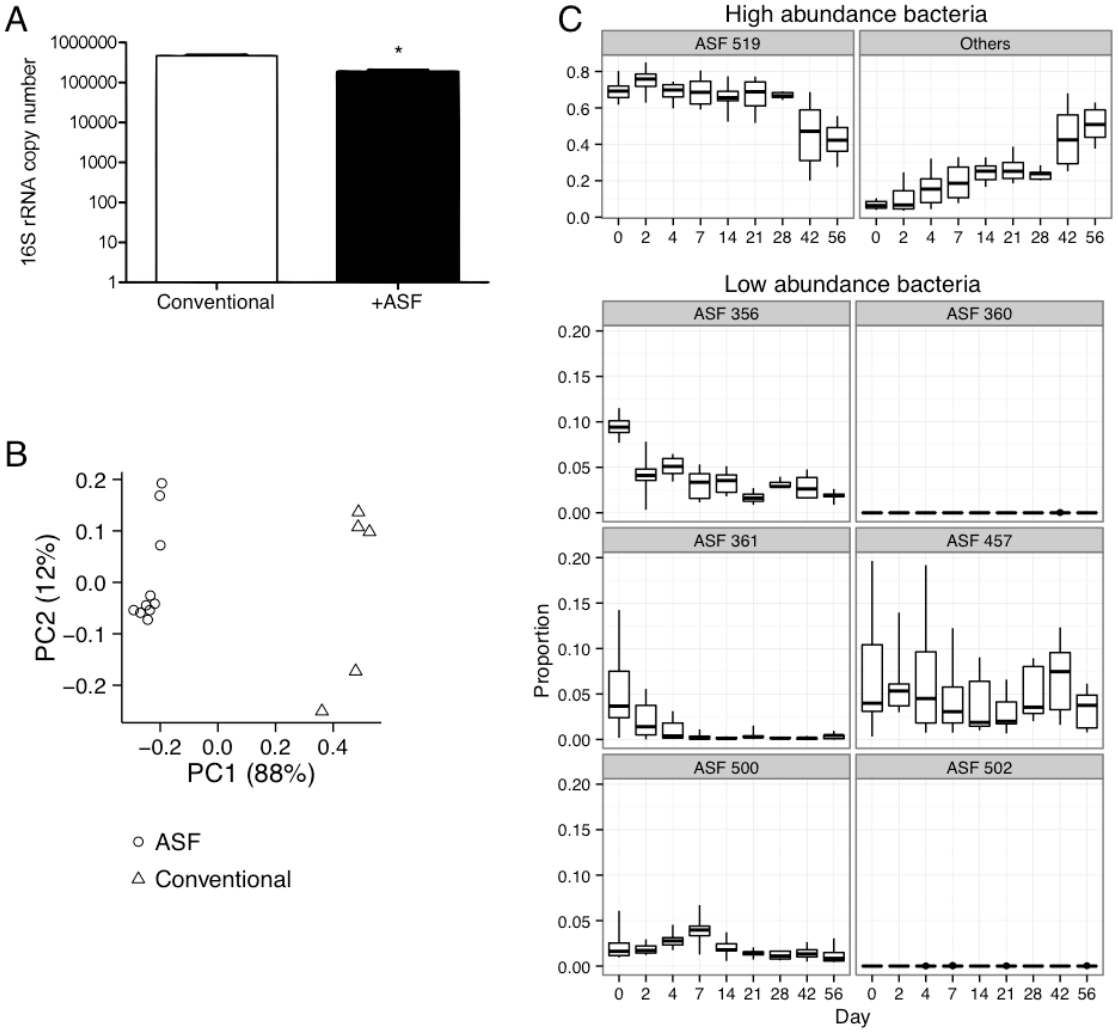
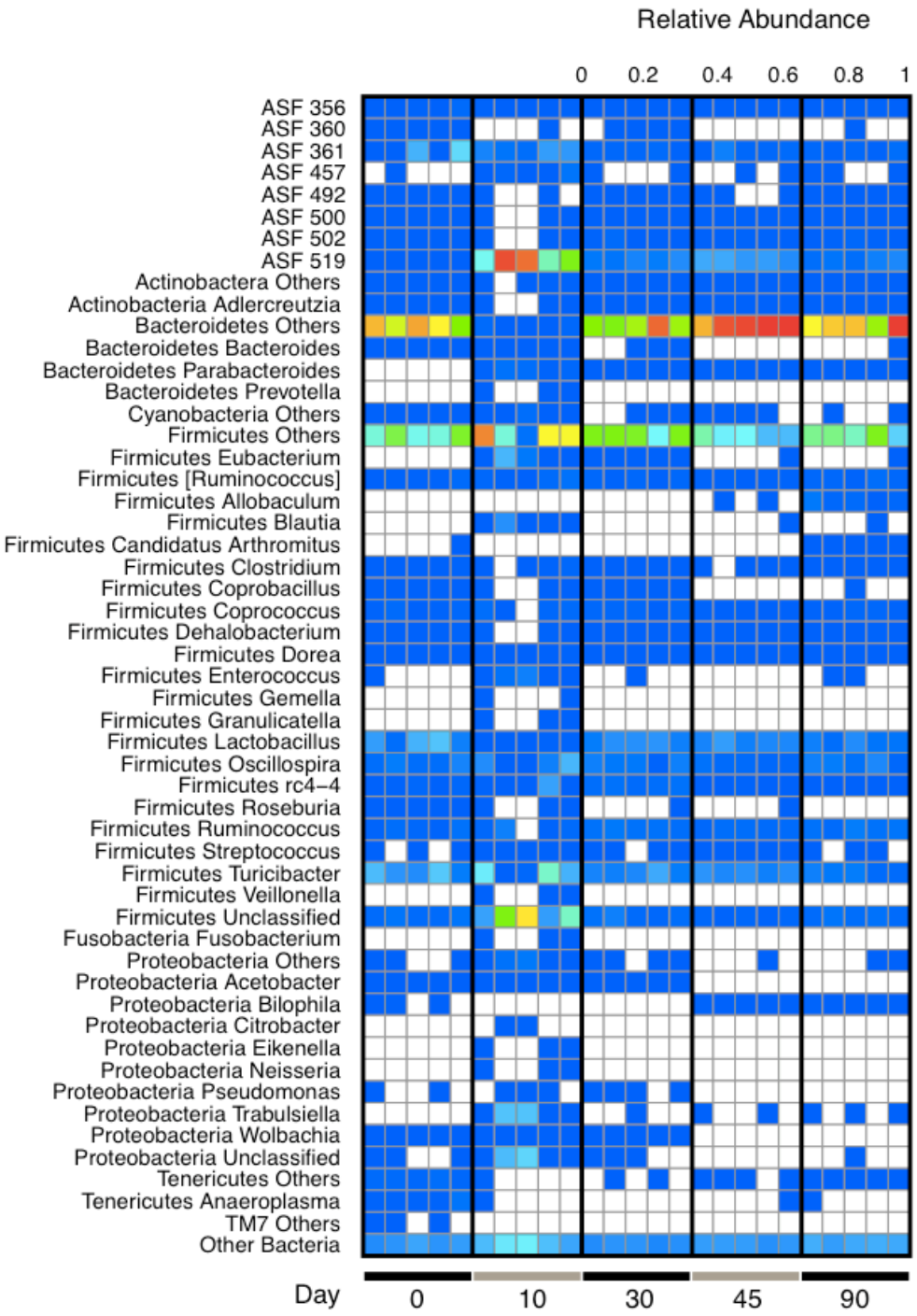
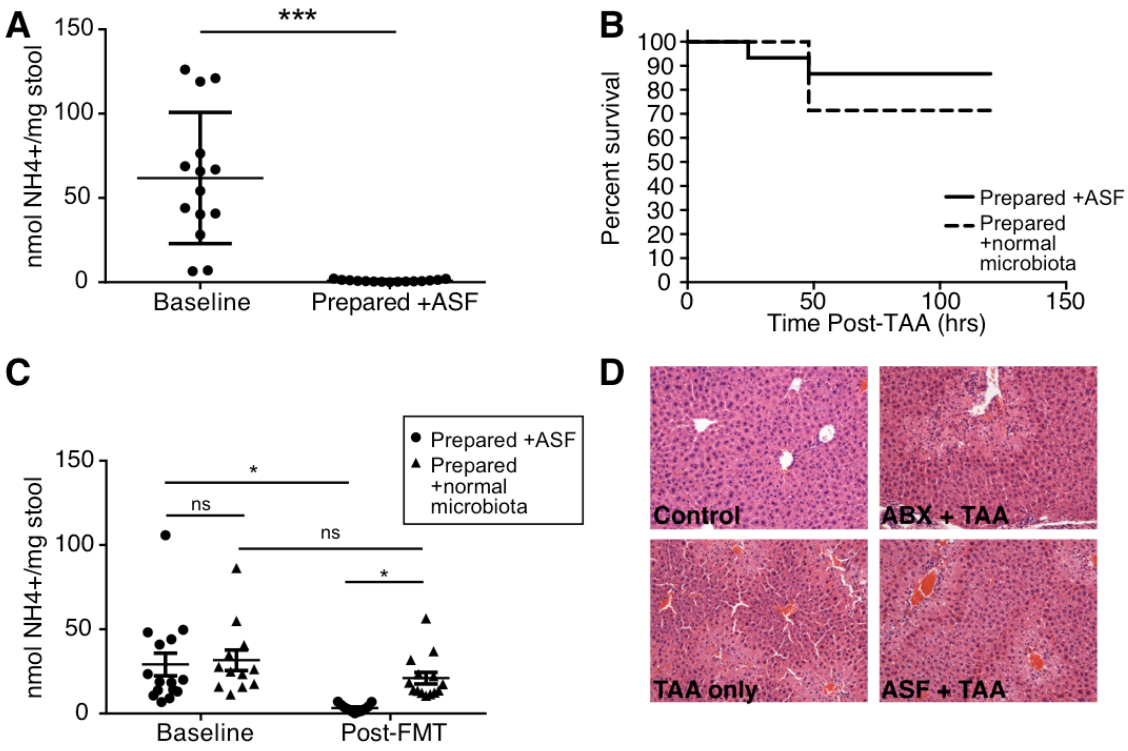
2.8 SUPPLEMENTARY INFORMATION



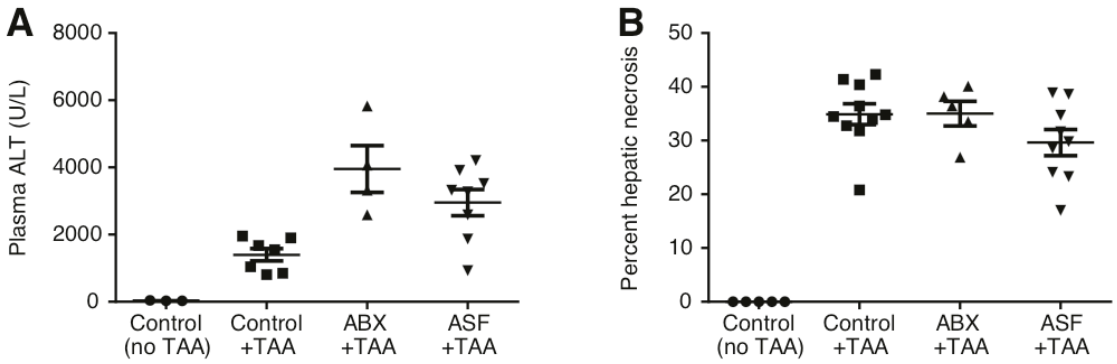
Supplementary Figure 2-1. Distinctiveness and long-term resiliency of the Altered Schaedler's Flora (ASF) in a host housed in a conventional specific pathogen free (SPF) environment. (A) 16S rRNA gene copy number of conventional (n=5) vs. ASF (n=10) fecal microbiota (p=0.015). **(B)** PCoA ordination of conventional (n=5) vs. ASF (n=10) communities based on 16S rRNA gene sequence tags. **(C)** Relative abundance of individual ASF community members in mice (n=10) housed in a SPF environment over 2 months. Two-tailed Student's t-test.



Supplementary Figure 2-2. Effect of a non-irradiated normal chow diet on ASF resilience after transplantation. Heat map showing the relative abundance of bacterial lineages over time in ASF-transplanted mice (n=5). Rows indicate bacterial lineages as annotated on the left. Relative abundance is indicated by the color code at the top of the figure, columns summarize sequencing results from individual fecal specimens, and elapsed time in days is shown along the bottom.



Supplementary Figure 2-3. Fecal ammonia levels, mortality rates and histology associated with TAA-induced hepatic injury studies. (A) Post-ASF transplant fecal ammonia levels in the high-dose TAA study shown in Figure 6A. **(B)** Kaplan-Meier survival curves and **(C)** fecal ammonia levels in the low-dose TAA study associated with results shown in Figure 7. **(D)** Photomicrographs of H&E stained hepatic sections from mice treated with high-dose TAA as indicated (200X) with quantification of hepatocellular necrosis (Supplementary Figure 4B). *p<0.01, ***p<0.001. Two-tailed Student's t-test and paired-sample t-test.



Supplementary Figure 2-4. Differential liver injury do not explain differences with or without ASF transplantation. (**A**) Plasma alanine aminotransferase (ALT) levels in control, antibiotic-treated, or ASF-transplanted mice after TAA treatment compared to untreated control mice (n=3 in untreated control group, n=7 in control + TAA group, n=4 in ABX +TAA group, n=8 in ASF + TAA group). (**B**) Liver damage quantified histologically by a pathologist reported as percent hepatic cellular necrosis (n=5 each in untreated control group and ABX + TAA group, n=10 each in control + TAA group and ASF + TAA group; 10 random 200x HPF per liver, no statistically significant difference among the three TAA-treated groups by ANOVA; blinded analysis).

CHAPTER 3. Dietary Regulation of the Gut Microbiota Engineered by a Minimal Defined Bacterial Consortium

The contents of this chapter have been submitted as:

> Shen TC, Chehoud C, Ni J, Hsu E, Chen YY, Bailey A, Laughlin A, Bittinger K, Bushman FD, Wu GD. Dietary regulation of the gut microbiota engineered by a minimal defined bacterial consortium. PLOS ONE 2016 (under review).

Personal contribution:

> 1) Assistance with overall study design
>
> 2) Experiment implementation, sample collection, and data analyses and interpretation of all murine studies in terms of metabolic phenotyping, dietary interventions, and fecal microbiota transplantation (excluding 16S rRNA gene sequencing and analyses)
>
> 3) Biochemical assessments of fecal/serum urea and fecal ammonia levels in all related experiments
>
> 4) Assistance with drafting and revising the relevant sections of the manuscript

3.1 ABSTRACT

We have recently reported that Altered Schaedler Flora (ASF) can be used to durably engineer the gut microbiota to reduce ammonia production as an effective modality to reduce morbidity and mortality in the setting of liver injury. Here we investigated the effects of a low protein diet on ASF colonization and its ability to engineer the microbiota. Initially, ASF inoculation was similar between mice fed a normal protein diet or low protein diet, but the outgrowth of gut microbiota differed over the ensuing month. Notable was the inability of the dominant *Parabacteroides* ASF taxon to exclude other taxa belonging to the Bacteroidetes phylum in the setting of a low protein diet. Instead, a poorly classified yet highly represented Bacteroidetes family, S24-7, returned within 4 weeks of inoculation in mice fed a low protein diet, demonstrating a reduction in ASF resilience in response to dietary stress. Nevertheless, fecal ammonia levels remained significantly lower than those observed in mice on the same low protein diet that received a transplant of normal feces. No deleterious effects were observed in host physiology due to ASF inoculation into mice on a low protein diet. In total, these results demonstrate that low protein diet can have a pronounced effect on engineering the gut microbiota but modulation of ammonia is preserved.

3.2 INTRODUCTION

The gut microbiota responds to multiple environmental stressors such as diet [1-4], antibiotic use [5], inflammation of the intestinal tract [6], and infection of the host with enteric pathogens [7]. By studying the gut microbiota in pediatric patients with Crohn's disease, we have recently shown that the effects of these factors may be independent even if present simultaneously [8]. Amongst these, the impact of diet has received considerable attention as a potential modifiable factor that shapes the composition and/or function of

the gut microbiota to prevent and/or treat disease [9]. The high-level efficacy of fecal microbiota transplantation (FMT) in the treatment of *Clostridium difficile* infections (CDI) is proof of concept that inoculating a host with a consortium of microbes has a meaningful effect on the composition of the gut microbiota [10]. The use of feces could be considered an untargeted approach with potential risks [11], but growing evidence suggests that the use of defined microbial consortia could be developed to treat disease [12, 13]. We have recently shown that the gut microbiota can be durably reconfigured to reduce fecal urease activity and ammonia production through oral inoculation of Altered Schaedler Flora (ASF), a defined microbial consortium that contains minimal urease gene content [13]. ASF comprises 8 murine gut commensal bacterial strains assembled in the 1970s and standardized by the National Cancer Institute in 1978 [14]. It is now commonly used to create gnotobiotic mice and/or to enhance the health of immunodeficient mouse strains.

Examples of co-metabolism between the gut microbiota and its mammalian host requiring host-derived substances include bile acids, mucous, and urea. The latter is particularly important for nitrogen flux between the host and the gut microbiota [15, 16]. As the primary source of nitrogen, dietary protein is essential to the synthesis of nucleic acids, amino acids, and other nitrogenous compounds. The catabolism of dietary protein by the host leads to hepatic formation of urea, a nitrogenous waste product that is excreted through the urine or delivered into the colon, where hydrolysis by bacterial urease results in the production of carbon dioxide and ammonia. Ammonia is a shared substrate for the synthesis of proteins, amino acids, and other small molecules by both the host and its microbiota. Although generally thought to be nutritionally beneficial to the host by enhancing nitrogen recycling, the production of ammonia by the gut microbiota can have deleterious effects in the setting of altered hepatic function, resulting in the

development of neurotoxicity [17-19]. Under such conditions, a low protein diet (LPD) can be used to reduce systemic ammonia levels [20, 21].

By inoculating mice with ASF after the endogenous microbiota has been reduced through the use of antibiotics and polyethylene glycol (PEG), the composition of the gut microbiota can be durably modified in composition as well as function. Functionally, there was a long-lasting reduction in fecal ammonia that was effective in reducing morbidity and mortality in the thioacetamide model of liver injury [13]. Since (1) the absorption of fecal ammonia produced by the gut microbiota may be an important source of nitrogen for the host especially in the setting of dietary protein restriction [15], and (2) low protein diets are used clinically in patients with hyperammonemic inborn errors of metabolism [22], there are a number of questions about the impact of diet on the engineering of the gut microbiota to reduce ammonia production. What is the effect of a LPD on the ability of a defined bacterial consortium to colonize in the gut? Does a LPD have an effect on the composition of the engineered microbiota? Will the ammonia reduction by microbiota engineering be sustained and exhibit lower levels than those achievable by a LPD alone? And lastly, will a significant reduction in gut microbiota ammonia production be deleterious to the host on a LPD?

Here, we address these questions by inoculating mice on a LPD with either feces from conventionally-reared mice (Normal Feces, or NF) or with ASF, monitoring the resultant composition of the gut microbiota over time by 16S tagged sequencing, assessing functionality by quantifying fecal ammonia levels, and investigating the impact on the host by metabolic profiling. Although a LPD has no effect on the ability of ASF to colonize the gut of the host upon inoculation, the resultant engineered state of the microbiota is altered primarily due to the re-emergence of S24-7, a specific bacterial taxonomic family within the Bacteroidetes phylum. Despite this alteration, fecal ammonia

levels remain diminished and without consequence to the metabolic physiology of the host on a LPD.

3.3 MATERIALS AND METHODS

Animals

C57B6J female mice 8 to 12 weeks old (The Jackson Laboratory) were used in this study. Fecal pellets collected from five ASF-colonized CB17 SCID mice (Taconic) served as the source of the ASF inoculum whereas five conventionally-colonized C57B6J mice (The Jackson Laboratory) served as the source of the normal feces (NF) inoculum used in the FMT procedures as previously described [13]. Fecal homogenates were prepared by diluting 0.1 g feces 10-fold in PBS. Mice were prepared for FMT by oral delivery of antibiotics in drinking water (1.125 g aspartame, 0.15 g vancomycin, and 0.3 g neomycin in 300 mL sterile water) for 72 hours. During the final 12 hours, the water supply was exchanged with a 10% PEG solution (Merck), and the mice were fasted. Mice were then inoculated daily with fecal homogenates by oral gavage for 7 days. All mice were housed five per cage in a conventional specific-pathogen free (SPF) facility (and transferred from one conventional facility to another conventional facility within the University of Pennsylvania 10 weeks after the start of experiment for NMR imaging) and fed irradiated AIN-76A (Research Diets D10001, 21% protein by kilocalories – NPD, see **S1 Table**). After one week, ten mice were switched to irradiated AIN-76A with lower protein content (Research Diets D08092201, 3% protein by kilocalories – LPD, see **S1 Table**). Fecal pellets were collected at baseline on NPD, 10 weeks after placement on LPD, 2 weeks after FMT (15 weeks on LPD), 4 weeks after FMT (17 weeks on LPD), and 10 weeks after FMT (23 weeks on LPD) for bacterial taxonomic and biochemical analyses. Fecal pellets were collected in 1.5 mL microcentrifuge tubes (Sigma-Aldrich) and immediately

placed on dry ice then stored in -80°C freezer until time of analysis. Body composition was determined after ten weeks on respective diets using NMR imaging via the Mouse Phenotyping, Physiology and Metabolism Core at the University of Pennsylvania. All animal studies were performed with the approval of the Institutional Animal Care and Use Committee of the University of Pennsylvania (Protocol Number: 803408).

16S V1-V2 Sequencing

DNA was isolated from stool as previously described [2, 23]. 100 ng of DNA was amplified with barcoded primers annealing to the V1-V2 region of the 16S rRNA gene (forward primer, 5′-AGAGTTTGATCCTGGCTCAG-3′; reverse primer, 5′-CTGCTGCCTYCCGTA-3′; [24, 25] using AccuPrime Taq DNA Polymerase System with Buffer 2 (Life Technologies). PCR reactions were performed on a thermocycler using the following conditions: initiation at 95°C for 5 min followed by 20 cycles of 95°C × 30 s, 56°C × 30 s, and 72°C × 1 min 30 s, then a final extension step at 72°C for 8 min. The amplicons from each DNA sample, which was amplified in quadruplicate, were pooled and purified with Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's instructions. Purified DNA samples were then sequenced using the 454/Roche GS FLX Titanium chemistry (454 Life Sciences).

16S rRNA Gene Sequence Analysis

16S rRNA gene sequence data was processed with QIIME v 1.8.0 [26] using default parameters. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity and then assigned Greengenes taxonomy [27] using the uclust consensus taxonomy classifier. Sequences were aligned using PyNAST [28] and a phylogenetic tree was constructed using FastTree [29]. Weighted and unweighted UniFrac [30] distances were

calculated for each pair of samples for assessment of community similarity and generation of principal coordinate analysis (PCoA) plots. Statistical analyses for bacterial abundance difference was performed using non-parametric Wilcoxon test, and p-values were corrected for multiple comparisons using the Benjamini and Hochberg procedure.

Measurement of fecal ammonia

Fecal ammonia concentrations were determined using an Ammonia Assay Kit (ab83360, Abcam, Cambridge, MA). Fecal pellets were suspended in the assay buffer provided at a concentration of 1 mg/10 uL, homogenized, and centrifuged at 13,000 x g for 10 minutes at room temperature to remove insoluble material. Ammonia concentration was then determined according to the kit protocol.

Measurement of serum and fecal urea

Urea concentrations were determined using the QuantiChrom™ Urea Assay Kit (DIUR-500, Bioassay Systems, Hayward, CA). Serum samples were assayed directly. Fecal pellets were suspended in ddH_2O at a concentration of 1 mg/10uL, homogenized, and centrifuged at 2,500 x g for 10 minutes at room temperature to remove insoluble material. Urea concentration was then determined according to the kit protocol.

3.4 RESULTS

LPD impacts host physiology and nitrogen metabolism but modestly alters the composition of the gut microbiota.

We first set out to investigate the effects of a LPD on both the murine host and the gut microbiota. Fifteen adult female C57BL6J mice were placed on an open source

irradiated purified rodent diet containing normal amount of dietary protein at 21% by kilocalories (AIN-76A), henceforth referred to as normal protein diet (NPD, **Supplementary Table 3-1**) for one week upon arrival into the University of Pennsylvania SPF vivarium. Subsequently, ten of the 15 mice were switched to an irradiated low protein diet (LPD, **Supplementary Table 3-1**) formulated using AIN-76A as the base that contains 3% protein by kilocalories. The LPD was made isocaloric by proportionally increasing carbohydrate content while keeping fat content unchanged. The remaining 5 mice continued to be fed the NPD. We monitored physiological changes in these mice using body weight and food intake measurements as well as body composition determination via nuclear magnetic resonance (NMR) imaging. We found that compared to NPD-fed mice, LPD-fed mice exhibited poor weight gain despite equivalent caloric consumption (**Fig. 3-1A**). LPD-fed mice also demonstrated increased fat mass and decreased lean mass (**Fig. 3-1B and 3-1C**). Corresponding to a reduction in serum urea concentration compared to NPD-fed mice (**Fig. 3-1D**), LPD-fed mice exhibited significant reductions in fecal urea and fecal ammonia levels after ten weeks on the LPD (**Fig. 3-1E and 3-1F**). These results are consistent with the fundamental role that dietary protein plays in host nitrogen balance. Reduction in dietary protein may have an effect on the gut microbiota by reducing the delivery of urea to the colonic environment leading to the reduction in fecal ammonia levels.

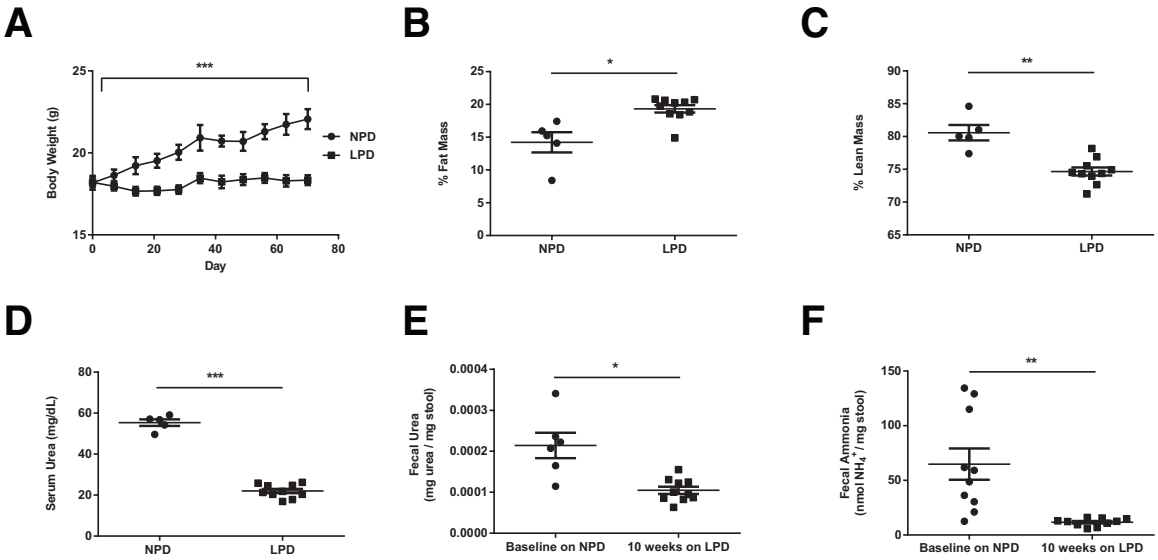


Figure 3-1. Changes in murine physiology and nitrogen metabolism on a LPD. Differences in (A) body weight (n=5 in NPD group, n=10 in LPD group), (B) % fat mass, (C) % lean mass, and (D) serum urea concentrations between NPD-fed and LPD-fed mice. (E) Fecal urea and (F) fecal ammonia levels at baseline on the NPD and after placement on the LPD. Values represent mean ± SEM. Statistical significance in body weight determined by two-way ANOVA with repeated measures; statistical significance in other parameters determined by paired and unpaired two-tailed Student's t test. *p<0.05, **p<0.01, ***p<0.001.

16S tagged sequencing (1045 to 5305 reads per sample, median = 3448 reads) revealed modest yet distinct differences in the composition of the fecal microbiota in mice after placement on the LPD. These difference can be visualized in principal coordinates analysis of weighted (**Fig. 3-2A**) and unweighted (**Fig. 3-2B**) UniFrac distance. The LPD led to a significant increase in the diversity of the gut microbial community as assessed by the Shannon diversity index (**Fig. 3-2C and Supplementary Fig. 3-1**). The LPD also led to significant increases in the relative abundance of Mollicutes and Coriobacteria and a decrease in the Firmicutes classes Erysipelotrichi and Clostridia (**Fig. 3-2D and 3-2E**).

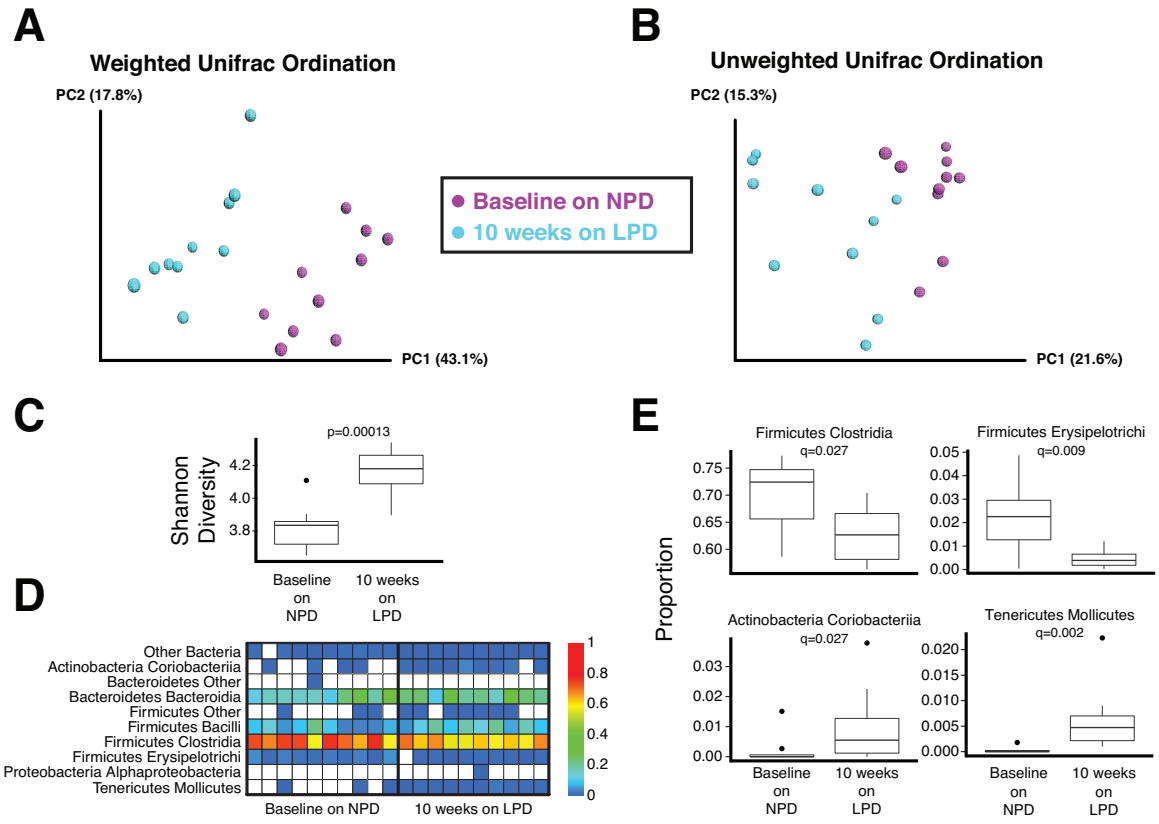


Figure 3-2. Effect of a LPD on the composition of the gut microbiota. Principal coordinates analysis (PCoA) ordination of mice before and after placement on the LPD for 10 weeks. Changes in community membership were analyzed using (A) weighted and (B) unweighted Unifrac. (C) The interquartile range of Shannon diversity values is shown for mice on the NPD who were later put on the LPD (Wilcoxon rank sum test p-value = 0.0001299). (D) Heatmap showing the relative abundance of bacterial lineages over time in mice who were on the NPD at baseline and then after ten weeks on the LPD. Rows indicate bacterial lineages annotated at the class taxonomic level on the left. The color key on the right of the figure indicates relative abundance. Columns summarize the sequencing results from individual fecal specimens. Each column represents a different mouse. The columns are grouped by diet. (E) Bacterial lineages that change on the LPD. Four bacterial classes significantly differed between the NPD and LPD (FDR-corrected Wilcoxon test p-value < 0.05). Relative abundance of each class in both diet groups is shown. Box and whiskers show the interquartile range; black circles mark the outlier samples.

LPD has no effect on the initial colonization of ASF into the host microbiota.

We have previously shown that there is a reduction in gut bacterial biomass upon treatment of mice with oral antibiotics (vancomycin and neomycin) and PEG, thus

permitting the colonization of ASF upon inoculation by oral gavage [13]. However, the effect of a LPD on the colonization of ASF into the gut of a prepared host remains unknown. After preparation with antibiotics and PEG, we orally inoculated five of the LPD-fed mice with ASF (herein referred to as “ASF-transplanted”). As a control group, we transplanted the other five LPD-fed mice using feces from conventionally-reared donor mice (herein referred to “NF-transplanted,” for Normal Feces). Using 16S rRNA tagged sequencing, we tracked taxonomic alterations in the gut microbiota over time. We found that NF-transplanted mice exhibited minimal change in the composition of their gut microbiota (**Supplementary Fig. 3-2**). However, the gut microbiota of ASF-transplanted mice underwent a shift in composition in a similar fashion to that previously observed in NPD-fed mice transplanted with ASF [13], as shown in **Fig. 3-3A and 3-3B.** In particular, the shifts along PC1 in both cohorts of mice represent changes due to the initial ASF inoculum (compare days 0 to 14 in both groups), whereas differences between the two cohorts of mice along PC2 may represent the effect of diet. These findings suggest that a LPD does not affect the initial colonization of ASF into the host microbiota.

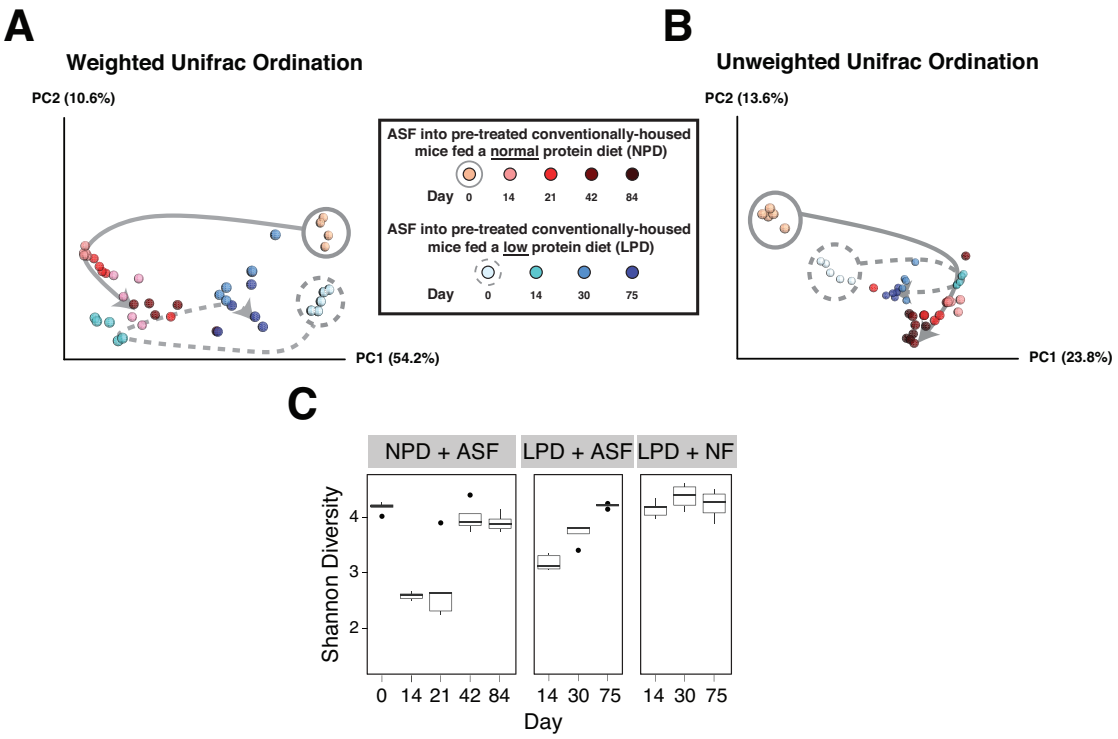


Figure 3-3. Effect of a LPD on the initial colonization of ASF and subsequent resilience over time. Principal coordinates analysis (PCoA) ordination of mice after transplantation with ASF. Changes in community membership were analyzed using (A) weighted and (B) unweighted Unifrac. Dietary groups are color coded as indicated with the shades of colors indicating progression in time. Day 0 samples have gray circles around them (solid for NPD, dashed for LPD). The arrows were added to help visualize the progression of time after ASF transplantation. (C) The interquartile range of Shannon diversity values are shown for mice on the NPD and LPD inoculated with either ASF or Normal Feces (NF). Black circles mark the outlier samples.

Diet affects the resilience of the gut microbiota engineered by inoculation with ASF.

By tracking the composition of mice inoculated with ASF, we determined the effect of a LPD on the ability of ASF to engineer a different microbiota composition. In the setting of a NPD, we previously observed that ASF transplantation led to the development of a new steady state community after one month composed of both ASF and the return of selected taxa of the Firmicutes phylum, but no non-ASF Bacteroidetes [13]. We proposed that *Parabacteroides* ASF519, the dominant taxon in ASF in feces, may

have prevented the return of other Bacteroidetes taxa by competitive niche exclusion. Tracking compositional changes in the gut microbiota over time, we found that in the setting of the LPD, the gut microbiota engineered by ASF inoculation developed into an alternative rich community with diversity similar to that on the NPD (**Fig. 3-3C**). However, ASF519 did not suppress the return of other Bacteroidetes. Instead, Bacteroidetes S24-7, a poorly classified yet common bacterial taxon in the commensal murine gut microbiota [31, 32], returned after ASF transplantation and reached an equilibrium state with ASF519 (**Fig. 3-4 and Supplementary Fig. 3-3**). We plotted the progression of the transplanted ASF community over time. We found that ASF reached a new steady state in the setting of the LPD at around 4 weeks after transplantation, similar to what we previously observed in the setting of the NPD [13] (**Fig. 3-3A and 3-3B**). However, this steady state more closely resembled the endogenous microbiota, likely as a result of the return of S24-7 on the LPD (best observed in **Fig. 3-3A** along PC1 – compare the solid to dotted grey line). Overall, these findings suggest that ability of ASF lineages to compete is reduced in the presence of a LPD.

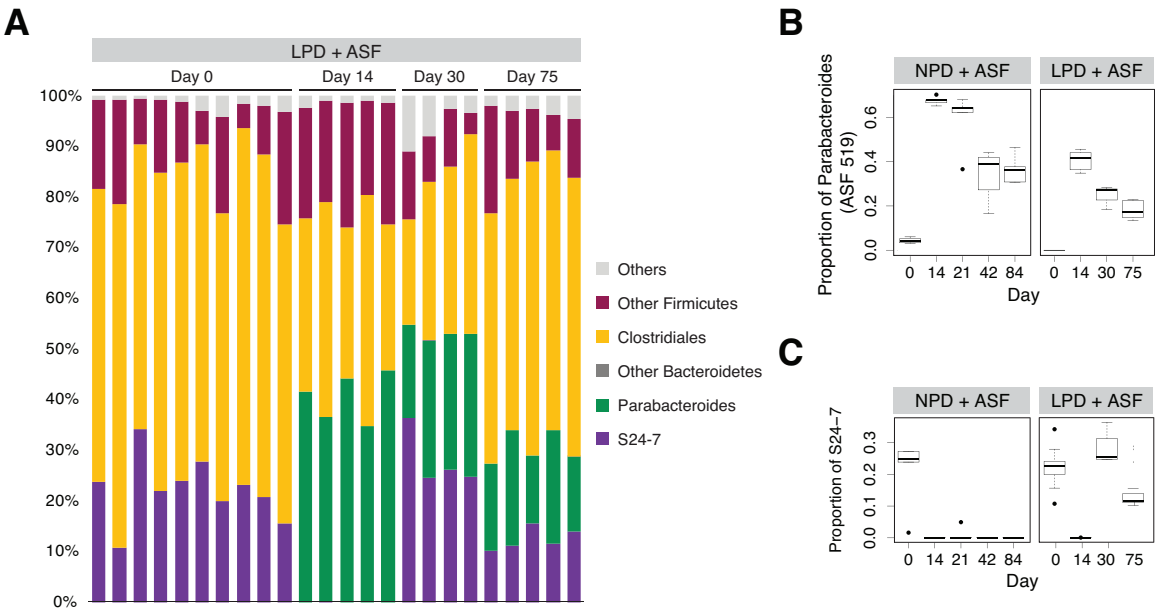


Figure 3-4. S24-7 returns after ASF transplantation into mice on a LPD but not on a NPD. (A) Relative abundance of bacterial taxa are shown. Each column represents a single sample of a pre-treated, ASF-inoculated mice on the LPD (LPD + ASF). Progression of inoculation is shown across multiple days post inoculation with ASF. Relative abundance of (B) Parabacteroides (including ASF 519) and (C) S24-7. Box and whiskers show the interquartile range; black circles mark the outlier samples.

The ASF-engineered gut microbiota lowers fecal ammonia more effectively than LPD alone.

We have previously shown that ASF transplantation durably reduces fecal ammonia by decreasing fecal urease activity [13]. Since a LPD itself mainly reduces fecal ammonia by decreasing the delivery of urea to the colon (**Fig. 3-1E and 3-1F**), we sought to determine whether the ASF-engineered microbiota would be able to reduce fecal ammonia levels below those achieved by a LPD alone. We measured fecal urea and fecal ammonia in mice at baseline on the NPD, after ten weeks on the LPD, and compared ASF and NF transplantation on the LPD. As shown in **Fig. 3-5A,** after the initial reduction in fecal ammonia levels induced by the LPD, ASF transplantation reduced fecal ammonia further than did NF transplantation. The ability of the ASF-

engineered microbiota to lower fecal ammonia levels below those achieved by the LPD alone is likely due to the reduction in fecal urease activity since there was no difference in fecal urea levels after NF and ASF transplantation (**Fig. 3-5B**). These results indicate that the functionality of the ASF-engineered gut microbiota is not significantly altered in the setting of a LPD despite alterations in its composition.

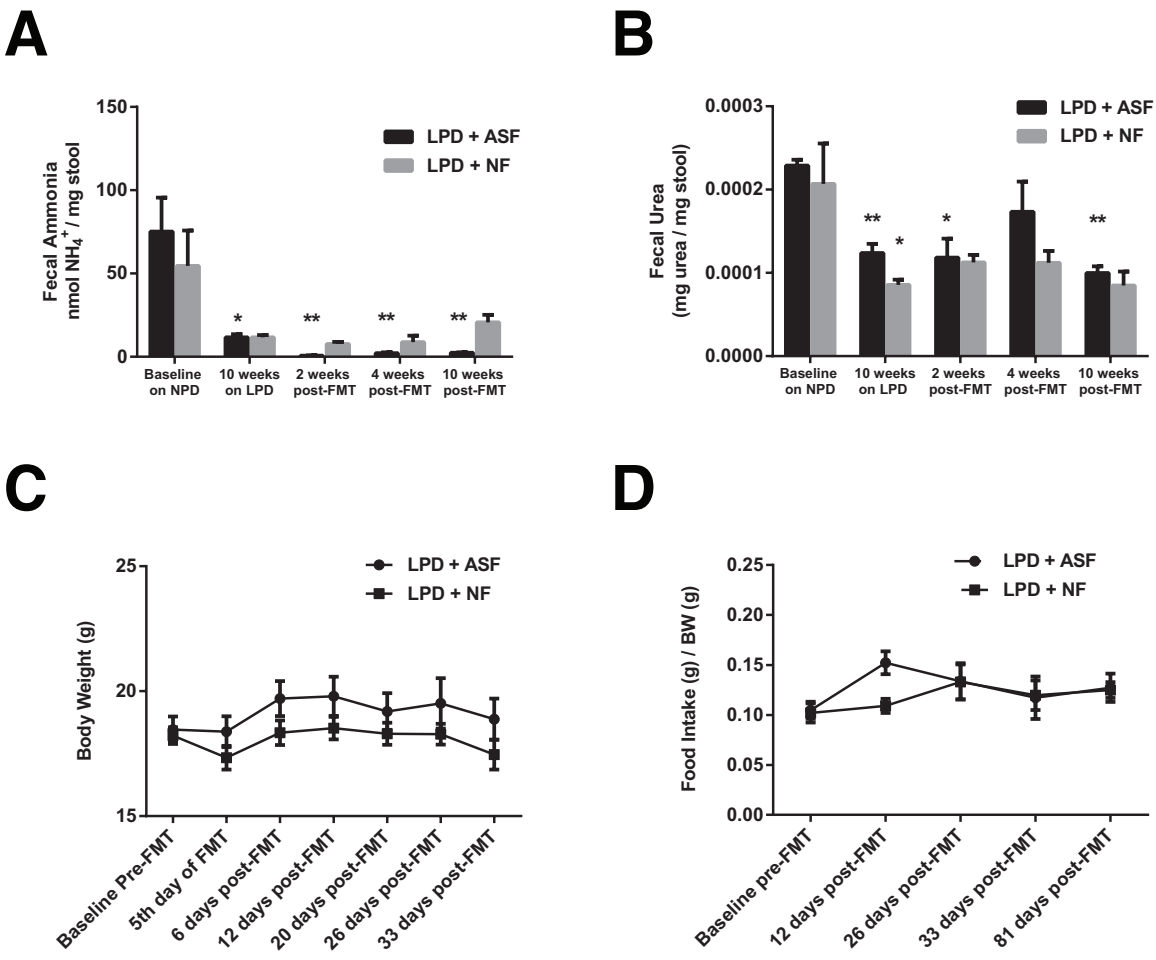


Figure 3-5. ASF transplantation alters colonic urea nitrogen recycling without significantly affecting host physiology. (A) ASF transplantation reduces fecal ammonia below the level achieved by the LPD alone (n=4-5 per group, *p<0.05 compared to baseline, **p<0.01 compared to 10 weeks on the LPD). (B) No difference in fecal urea level between ASF- and NF-transplanted mice (n=2-5 per group, *p<0.05 compared to baseline, **p<0.01 compared to baseline). No difference in (C) body weight or (D) food intake between ASF- and NF-transplanted mice (n=5 per group). Values represent mean ± SEM. Significance determined by two-tailed Student's t-test.

The low fecal urease and fecal ammonia-producing microbiota engineered by ASF inoculation does not exacerbate host metabolic dysfunction induced by LPD.

Urea is a nitrogenous waste product, but it is thought to contribute to host nutrition via urea nitrogen recycling by intestinal bacterial urease in both ruminants and non-ruminants, leading to microbial and/or host synthesis of peptides, amino acids, and other small molecules [15]. We asked whether this role of urea may become important for host physiology in the setting of a LPD, where systemic nitrogen is reduced. After transplanting the above cohort of mice with ASF or NF, we continued to monitor their body weight, food intake, and survival. Remarkably, despite the absence of colonic urea nitrogen recycling, ASF-transplanted mice did not differ significantly from NF-transplanted mice (**Fig. 3-5C, 3-5D, and Supplementary Fig. 3-4**). Thus, in the setting of a LPD, ASF transplantation does not lead to significant detrimental changes to host physiology and metabolism.

3.5 DISCUSSION

The success of FMT in the treatment of recurrent *Clostridium difficile* infections provides proof of concept that the gut microbiota can be a target for the treatment of disease in humans. The use of fecal transfer will likely be replaced by the use of defined microbial consortia with specific biological properties. As proof of concept, we have shown in murine models that a defined consortium of eight bacteria, known as Altered Schaedler Flora (ASF), can be used to engineer the gut microbiota with altered functionality, namely a reduction in fecal urease activity and ammonia production [13]. Critical to the success of this strategy is the substantial reduction in the biomass of the baseline microbiota to provide a niche into which the bacterial inoculum can colonize.

An important consideration for engineering the gut microbiota is resilience to environmental stress. An optimally engineered microbiota would be a rich community that stays intact in the presence of environmental stress. Diet is an important environmental stressor on the gut microbiota that should be considered when engineering gut microbial communities. As one example, the low ammonia-producing microbiota engineered by ASF, which has functional durability for several months in mice fed an irradiated diet, shows reduced resilience when the mice are fed a non-irradiated diet [13]. We chose to study the impact of dietary protein on the resilience of the ASF-engineered microbiota for several reasons: 1) Dietary protein has been shown to influence the composition of the gut microbiota in gnotobiotic mice [33]; 2) Protein consumption regulates the production of hepatic urea that may affect colonic urea delivery to the gut microbiota [15, 34]; 3) Protein-restricted diets are an important therapeutic modality for patients with hyperammonemic inborn errors of metabolism [21, 35].

Unlike the modulation of fat and fiber in mice, which have been shown to have a strong effect on the composition of the murine gut microbiota [1, 33], we show that severe restriction of dietary protein had a modest effect. Within the Firmicutes phylum the Clostridia and Erysipelotrichi classes decreased significantly on a LPD, consistent with the preference of taxa within Firmicutes, particularly Clostridia species, to metabolize amino acids and peptides [36, 37]. Alternatively, since we show that a LPD reduces serum urea concentrations with reduced delivery to the colon resulting in lower fecal ammonia levels, an alteration in nitrogen flux via ammonia into the gut microbiota [38, 39] may also have an effect on the composition of the bacterial microbiota.

Since we balanced protein with carbohydrate in the composition of the purified rodent diets, it is difficult to ascertain if the differences in the composition of the gut microbiota are due primarily to alterations in protein or in carbohydrate. We found that

two bacterial phyla present at low abundances increased significantly on a LPD. Specifically, the classes Mollicutes (Tenericutes phylum) and Coriobacteria (Actinobacteria phylum) increased on a LPD. Previous work has shown that Mollicutes proliferated on a typical Western diet characterized by high-fat/high-sugar content, likely because of their ability to import and process simple sugars [40]. Thus, an increase in the abundance of Mollicutes that we observed on a LPD could be due to the increase in carbohydrate content rather than the reduction in protein content. Another study also showed that gut colonization by Actinobacteria and Tenericutes was strongly correlated with decreased hepatic levels of glycogen and glucose [41], further suggesting the interplay between the host and these two phyla may be closely related to carbohydrate metabolism.

Despite the effect of a LPD on the composition of the murine gut microbiota at baseline, this did not have an effect on the initial colonization of ASF at 2 weeks, demonstrating that the use of antibiotics and PEG effectively prepared the environment of the gut for inoculation by a minimal defined bacterial consortium. Subsequently, development of the resultant engineered microbiota, determined by emergence of various bacterial taxa in addition to ASF, was distinctly different in mice fed a NPD versus a LPD. On a NPD, we previously showed that the dominant taxon *Parabacteroides* (ASF519) was able to exclude the entire Bacteroidetes phylum yet permit the reappearance of specific taxa belonging to the Firmicutes phylum. The observation that bacterial lineages with similar phylogeny exhibit competitive niche exclusion has been demonstrated in the *Bacteroides* genus where successful competition for carbohydrate substrates plays an important role [42]. By contrast, on a LPD, the resultant engineered microbiota appears to be more similar to baseline primarily due

to the reemergence of a single bacterial taxon belonging to the Bacteroidetes family, S24-7.

S24-7 has been previously recognized as a dominant taxonomic group in the murine microbiota. It was first characterized by Salzman *et al.*, who referred to the taxon as “mouse intestinal bacteria” [31]. The S24-7 taxon is phylogenetically distinct from other named genera in the order Bacteroidales. The taxon has been reported as altered in several recent mouse studies: it was increased in proportion following partial hepatectomy [43], associated with co-infection by *Hymenolepis* spp. [44], and decreased in proportion following antibiotic treatment for parenteral nutrition-associated liver injury [45]. However, to our knowledge, no study has previously characterized competition between S24-7 and other Bacteroidetes species in mice. The S24-7 taxon is typically encountered at very low (<1%) abundance in fecal samples from human populations. However, one study of a previously uncontacted Amerindian population reported the taxon to be enriched in isolated Yanomami Amerindians relative to Guahibo Amerindian, Malawian, and U.S. subjects [46]. The average abundance of the taxon in Yanomami Amerindians was reported to be nearly 5% of total bacteria, suggesting a potential role for S24-7 in the human gut.

Upon reduction of bacterial biomass through a combination of antibiotics and PEG, S24-7 is no longer detectable and shows no return over time after mice have been inoculated with ASF. Since both *Paracteroides* (ASF519) and S24-7 are closely related within the Bacteroidetes phylum, we speculate that S24-7 may be co-excluded from the luminal gut environment by ASF519 through competitive niche exclusion, a mechanism that has been hypothesized as the basis for the inversely-related proportions of Bacteroides and Prevotella in the human gut microbiota [2, 47], a predominant feature of “enterotypes” [48]. From a mechanistic standpoint, the basis of competitive niche exclusion

may involve the competition of metabolic substrates as has been demonstrated for Bacteroides species in a reductionist model system [42]. Since S24-7 reappears and co-exists at approximately equal levels with ASF519 in LPD-fed mice, the alteration of substrate availability via diet may have altered the luminal environment of the gut that reduces the need for competition between these two taxa. For example, a LPD may have altered the balance of nitrogen flux into the gut microbiota via the uptake of ammonia. Indeed, despite the return of S24-7 and the similarities between the composition of a gut microbiota of a conventionally-housed mouse and the ASF-engineered community established in LPD-fed mice, fecal ammonia levels remained much lower in ASF-transplanted mice that those transplanted with normal feces. This suggests that S24-7 may be urease negative. Further elucidation of such mechanism(s) will require genomic characterization of S24-7 along with an evaluation of its biological properties.

The quantification of fecal ammonia was used to determine the impact of microbiota composition on the function of the community. Despite the modest alterations in the gut microbiota induced by the consumption of a LPD, there was a significant reduction in fecal ammonia levels reflecting the reduced abundance of urea substrate available for hydrolysis by the gut microbiota. This observation emphasizes the notion that diet may have an indirect impact on the gut microbiota by alteration of the host similar to the outgrowth of a pathobiont due to the enhanced production of sulfated bile acids in mice fed milk fat [49]. Importantly, engineering of the gut microbiota using ASF led to a reduction in fecal ammonia levels significantly greater than that observed on a LPD.

Since ammonia, produced by the gut microbiota via urease activity, is absorbed by the host where it can be used for amino acid synthesis, it has been hypothesized that this form of nitrogen recycling may be important for host health especially under

conditions of limited protein intake [15, 50]. This might be a significant limitation of a strategy focused on reducing gut microbiota ammonia production for the treatment of hyperammonemia and hepatic encephalopathy [13]. Although LPD-fed mice did not exhibit growth, as would be expected, ASF transplantation with subsequent robust reduction of fecal ammonia levels did not lead to any effects on food intake, growth, or mortality relative to LPD-fed mice transplanted with normal feces who had much higher levels of fecal ammonia. Since patients with hyperammonemic inborn errors of metabolism are placed on a LPD to prevent metabolic crises, our observations provide preliminary evidence that the engineering of gut microbiota to reduce fecal ammonia production may be well tolerated in this patient population. However, additional safety studies are needed.

In summary, we show that diet has a significant effect on the ability of a defined microbial consortium to engineer the composition of the gut microbiota. Specifically, LPD alters the co-exclusion of two dominant taxa within the Bacteroidetes phylum. Given the alterations in the syntropic host-microbiota interactions in nitrogen flux that occur in the levels of urea delivery from the host to the gut microbiota, the reduced production of ammonia via bacterial urease, and the uptake of ammonia by both the host and the gut microbiota, a LPD may be a particularly important environmental stressor that will impact upon the composition of an engineered microbiota. Nevertheless, the functionality of the engineered gut microbiota, as quantified by a reduction in fecal ammonia levels, remained intact. Together with the absence of detrimental effects on host physiology in the setting of a LPD, the reduction in fecal ammonia levels via engineering of the gut microbiota may be an effective therapeutic strategy for patients with hyperammonemic inborn errors of metabolism.

3.6 ACKNOWLEDGEMENTS

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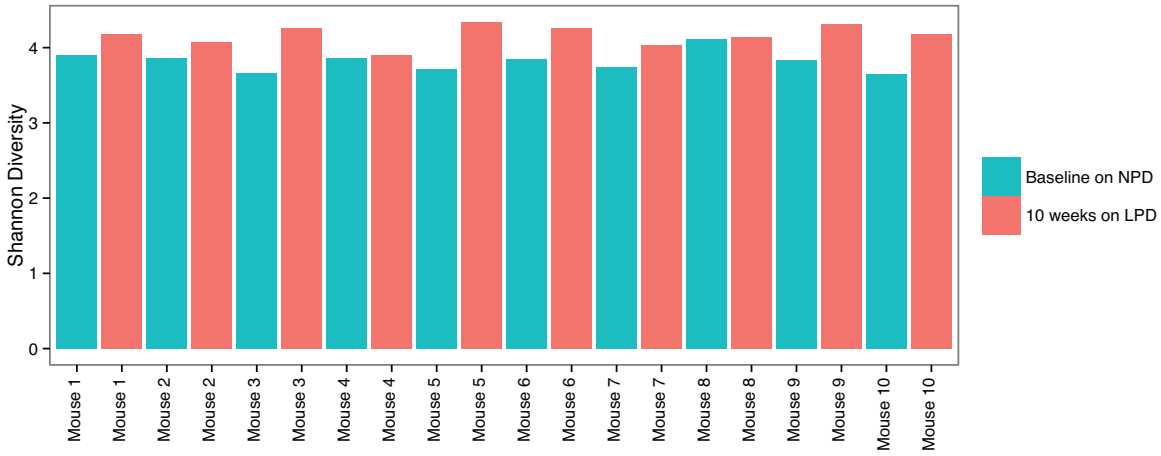
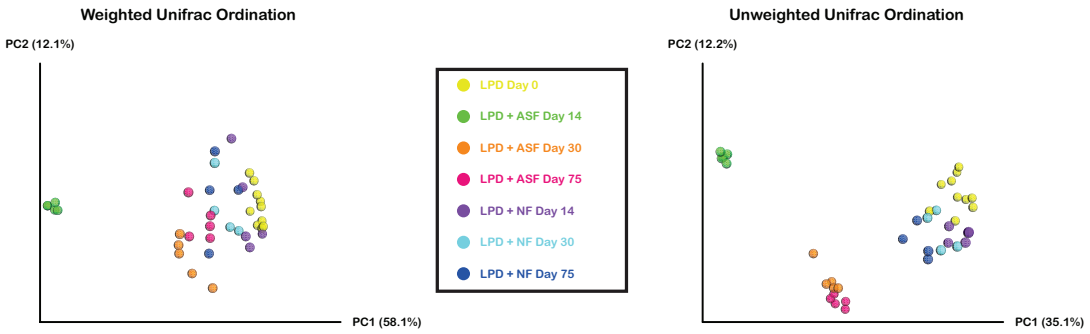
3.8 SUPPLEMENTARY INFORMATION

	NPD		LPD	
	gram%	kcal%	gram%	kcal%
Protein	20	21	3	3
Carbohydrate	66	68	83	85
Fat	5	12	5	12
Total		100		100
kcal/gram	3.9		3.9	
Ingredient	**gram**	**kcal**	**gram**	**kcal**
Casein	200	800	30	120
DL-Methionine	3	12	0.45	1.8
Corn Starch	150	600	190	760
Sucrose	500	2000	632.5	2530
Cellulose, BW200	50	0	50	0
Corn oil	50	450	50	450
Mineral Mix S10001	35	0	35	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	**1000**	**3902**	**1000**	**3902**

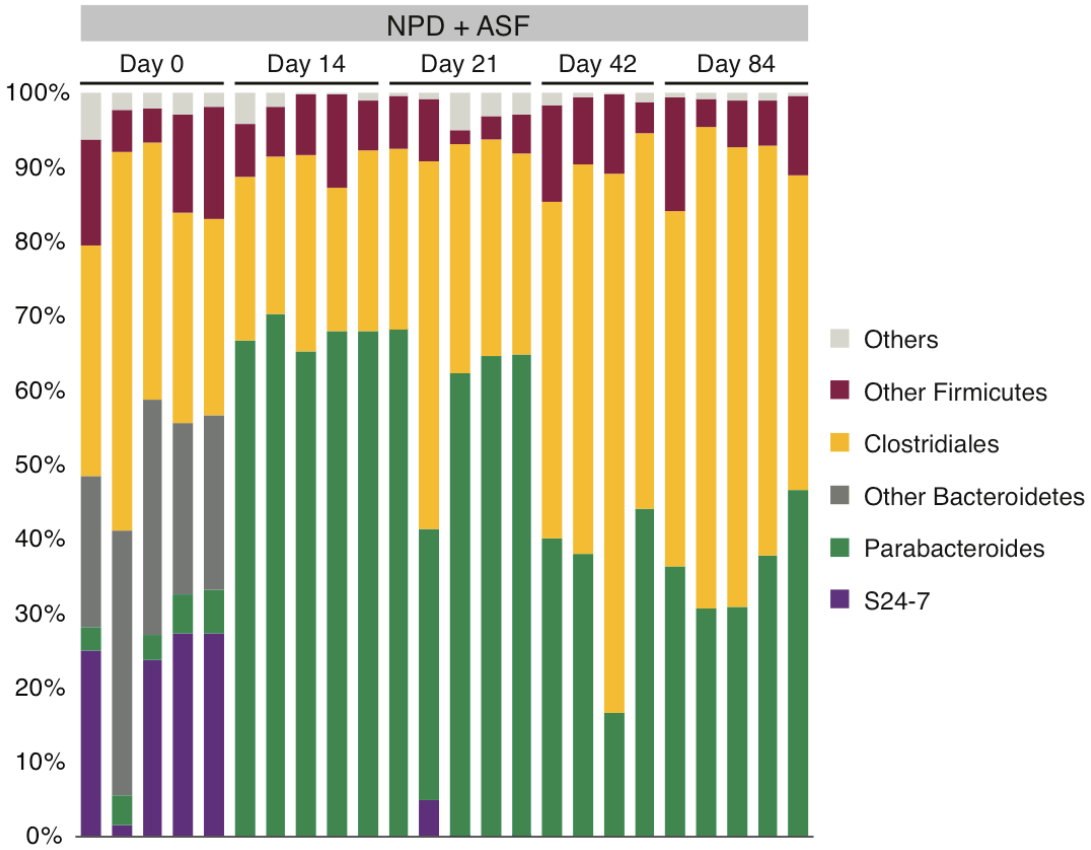
Supplementary Table 3-1. Components of normal protein and low protein diets.



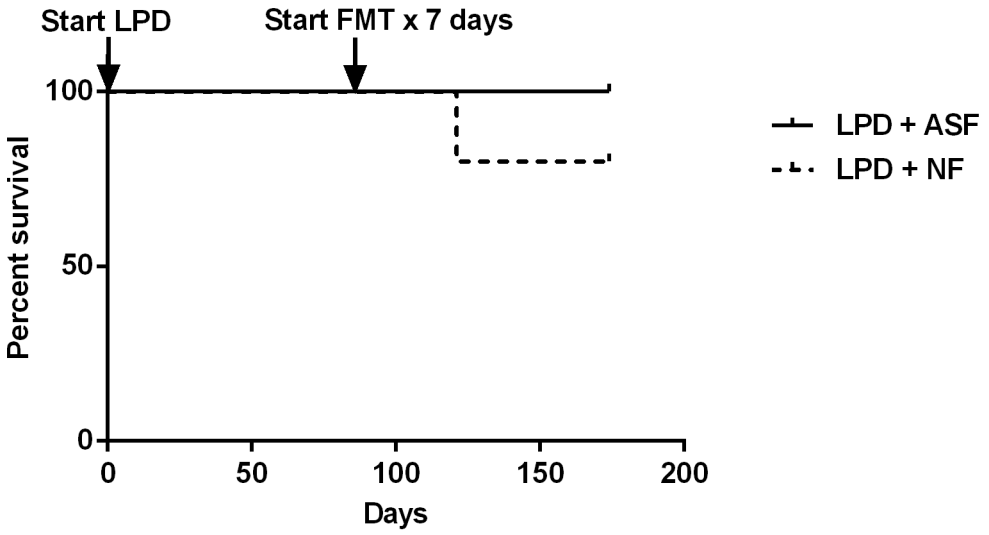
Supplementary Figure 3-1. Diversity in each mouse after a LPD. Shannon diversity is shown for all ten mice while on the NPD (blue) and after ten weeks on the LPD (salmon).



Supplementary Figure 3-2. Principal coordinates analysis ordination of mice on the LPD after transplantation with ASF or NF. Changes in community membership were analyzed using (A) weighted and (B) unweighted Unifrac.



Supplementary Figure 3-3. Relative abundance of bacterial taxa after ASF transplantation. Each bar represents a single sample. Samples represent pre-treated ASF-inoculated mice on the NPD (NPD + ASF). Progression is shown across multiple days post-inoculation with ASF.



Supplementary Figure 3-4. Murine mortality on a LPD. Kaplan Meier curve showing no significant difference in survival between ASF- and NF-transplanted mice on the LPD (n=5 per group at start of experiment).

CHAPTER 4. Conclusions and Future Directions

We can dramatically reduce the endogenous biomass of the murine gut through the use of antibiotics and polyethylene glycol and subsequently reconfigure the composition and function of the gut microbiota by transplanting a new defined bacterial consortium. In this dissertation, we reduced gut urea nitrogen recycling and colonic ammonia production via transplantation of ASF, a defined bacterial consortium with minimal urease gene content and activity. ASF transplantation was associated with decreased morbidity and mortality in a murine model of hepatic injury and fibrosis (**Chapter 2**). Given the impact of diet on the gut microbiota as well as the use of dietary protein restriction in the treatment of urea cycle disorders, we also investigated the effects of a low protein diet (LPD) on the structure and function of the transplanted ASF community. Although ASF demonstrated decreased resilience in response to dietary stress on a LPD and allowed the colonization and proliferation of the Bacteroidetes family S24-7, the ASF-engineered microbial community maintained low colonic ammonia production (**Chapter 3**). These findings point to the therapeutic potential of a humanized ASF for treating conditions of hyperammonemia, such as HE in cirrhosis and urea cycle disorders. Furthermore, this work provides proof of concept for the therapeutic modulation of host metabolic processes and informs future investigations into the management of disease processes through the transplantation of engineered gut microbiota with targeted physiologic properties.

A number of important questions and challenges remain. Gut ammonia production and subsequent absorption by the host are strongly implicated in the pathogenesis of HE. Urea hydrolysis to carbon dioxide and ammonia via bacterial urease activity in the gut represents a simple metabolic pathway for therapeutic

intervention. As such, we were able to use a defined bacterial consortium without urease activity to alter mammalian host nitrogen metabolism and ameliorate the clinical sequelae of hyperammonemia. However, in disease states with multifactorial and/or obscure pathogeneses, such as inflammatory bowel disease and obesity, the identification of pathologic pathways involving the gut microbiota remains unclear. This lack of clarity makes it difficult to design defined bacterial consortia capable of modulating these processes. Hippocrates famously said that "all disease begins in the gut." While he may not have been insinuating the role of the gut microbiota, the ongoing challenge is to elucidate the gut microbiota-dependent pathways linked to disease processes and modulate them to treat or even cure disease. Metabolomic studies may provide a viable approach. In one example, untargeted metabolomics studies were used to generate profiles of plasma small molecules predictive of cardiovascular disease. This led to the identification of the gut microbiota-dependent choline-TMA-TMAO pathway (see **Section 1.5**) that is associated with increased risk for cardiovascular disease[1, 2]. In order translate this finding into therapeutic utility, one would need to identify ways to modulate the pathway through either pharmacological interventions or engineering the gut microbiota. Indeed, one recent study showed that a choline analog, 3,3-dimethyl-1-butanol, non-lethally inhibited TMA formation by targeting microbial TMA lyases and attenuated choline diet-enhanced atherosclerosis in mice[3].

To translate our own findings into human biology, we would need to identify and isolate bacterial taxa from the human gut microbiota that lack urease. Although ASF is innocuous in mice[4, 5], it is part of the commensal murine gut microbiota and cannot be used for human inoculation. But the absence of urease gene/activity should not be the only criterion in the development of a humanized version of ASF. One important consideration is whether ASF contains certain metabolic properties that allow the

consortium members to coalesce as a functional microbial community – for example, the exchange of substrates and metabolites in the absence of urease-dependent ammonia production. At the same time, the factors that make ASF resilient as a microbial community and exclude the return of other Bacteroidetes in the setting of a normal protein diet (see **Chapter 2**) remain to be determined. We found that ASF exhibited decreased resilience in the setting of a LPD. As such, it is important to understand how dietary changes affect the stability and resiliency of a therapeutic humanized ASF. We have developed a pilot human Defined Consortium (hDC) composed of bacterial strains isolated from the feces of a healthy human subject (**Table 4-1)**. This consortium is distinct from ASF because it is derived from the human microbiota and contains taxa distinct from genera found in ASF. Consortium members were selected based on the lack of urease gene and activity (determined through whole genome analysis and urease assay testing) and represent Bacteroidetes, Firmicutes, and Actinobacteria, the major phyla in human microbiota[6, 7]. Preliminary data revealed that hDC inoculation into prepared mice corroborates the finding obtained with ASF, leading to a sustained reduction in fecal ammonia (**Fig. 4-1**). Future investigations will need to address the above considerations and examine the efficacy, resiliency, and safety of hDC in a murine model of hyperammonemia and hepatic injury before we can consider the use of a humanized ASF a viable therapeutic option in HE treatment.

Phylum	Genus
Bacteriodetes	*Bacteroides eggerthii*
Bacteriodetes	*Paraprevotella clara*
Bacteriodetes	*Bacteroides vulgatus*
Firmicutes	*Coprococcus comes*
Firmicutes	*Dorea longicatena*
Firmicutes	*Clostridium XI*
Actinobacteria	*Bifidobacterium longum*
Actinobacteria	*Collinsella aerofaciens*

Table 4-1. Composition of the human Defined Consortium (hDC). Phylum names are in left column, and genera represented are in right column.

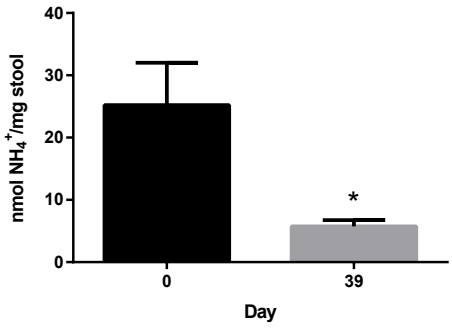


Figure 4-1. Fecal ammonia levels pre- and post-inoculation of hDC into prepared conventionally-colonized mice (n=10, *p<0.05)

From a basic scientific perspective, we also need to understand the overall significance of bacterial urease in nitrogen metabolism and protein homeostasis for the host-microbiota superorganism. Enteric bacteria utilize ammonia as the preferred source of nitrogen since ammonia provides the highest growth rate for bacteria of any nitrogen source[8]. Thus, the production of ammonia via urea hydrolysis by bacterial urease may be a critical component of bacterial nitrogen metabolism, including amino acid biosynthesis. Based on preliminary data, ASF colonization in mice led to significant reductions in plasma concentrations of essential amino acids (EAAs) and an increase in Fischer's Ratio (valine + leucine + isoleucine) / (phenylalanine + tyrosine) relative to control mice (**Table 4-2**). These results suggest that ASF colonization may protect against morbidity and mortality associated with hepatic injury by altering plasma EAA levels and the subsequent production of false neurotransmitters via urease-dependent mechanisms (see **Section 1.3**). Evidence from the literature supports the notion that the gut microbiota contributes to protein homeostasis of the mammalian host through microbial de novo biosynthesis of EAAs. This has been demonstrated in rats[9], pigs[10], rabbits[11], and humans[12]. The studies primarily involved the oral administration of an inorganic ^{15}N-labeled nitrogen source (e.g. $^{15}NH_4Cl$ or $^{15}N_2$-urea) to the host followed by the detection of ^{15}N-labelled amino acids in host tissues, specifically lysine and threonine. Since lysine and threonine are the only EAAs that do not undergo transamination in mammalian tissues[9], the presence of ^{15}N-labeled lysine and threonine indicates synthesis by the gut microbiota and subsequent intestinal absorption by the

host. The importance of coprophagia was demonstrated in rats, where it was shown that the utilization of microbial lysine occurred exclusively through coprophagia rather than direct absorption during initial passage through the GI tract[13]. In human studies, the administration of ^{15}N-compounds to both normal subjects and otherwise healthy subjects with ileostomies showed that microbial threonine and lysine contributions range from 8 to 17% and 5 to 21%, respectively[12, 14]. In rats, pigs, and humans, between 1 and 20% of circulating host plasma lysine, urinary lysine, and body protein lysine of the host are derived from intestinal microbial sources[15]. These results demonstrate the importance of microbial nitrogen flux to mammalian host physiology.

	Portal Blood		Arterial Blood	
	Control Mean ± SD	ASF Mean ± SD	Control Mean ± SD	ASF Mean ± SD
METH	58.35 ± 10.55	28.84 ± 12.12**	57.32 ± 17.42	30.63 ± 3.45**
THR	173.01 ± 33.87	72.88 ± 31.52**	169.43 ± 27.44	84.49 ± 5.49**
LYS	201.82 ± 29.65	105.16 ± 48.92**	206.66 ± 50.26	120.57 ± 21.52**
TRYP	81.44 ± 5.81	80.17 ± 33.48	58.13 ± 6.88	59.28 ± 14.22
VAL	217.04 ± 51.15	136.52 ± 60.12*	189.31 ± 45.25	135.56 ± 14.46*
LEU	186.46 ± 50.75	110.01 ± 49.52*	159.55 ± 47.15	108.21 ± 13.48*
ILE	143.39 ± 35.79	62.36 ± 27.54**	118.76 ± 32.63	59.64 ± 6.97**
PHE	57.88 ± 10.55	41.00 ± 17.75*	56.96 ± 17.67	44.30 ± 2.89
TYR	140.02 ± 29.99	36.10 ± 15.51**	116.46 ± 21.19	34.30 ± 4.84**

Table 4-2. Plasma concentrations (nmol/mL) of essential amino acids in control and ASF FMT mice (n = 5 per group, *p<0.05 compared to control, **p<0.01 compared to control). Values in grey were used to calculate Fischer's Ratio.

I have designed a series of experiments to examine the impact of bacterial urease on amino acid metabolism of the gut microbiota and the host through ^{15}N flux studies. The oral administration of $^{15}NH_4Cl$ or $^{15}N_2$-urea to 1) control mice, 2) antibiotics-treated mice, and 3) ASF-transplanted mice followed by quantification of labeled and unlabeled amino acids in the stool and plasma will help to address some fundamental questions. For example, how important is the presence of gut microbiota and/or urease to normal mammalian amino acid homeostasis? Is the reduction in plasma EAAs of

ASF-colonized mice secondary to decreased microbial synthesis or increased microbial uptake, namely competition for dietary amino acids with the host? Can urease-negative bacteria synthesize amino acids at the same capacity as urease-positive bacteria if given the proper substrate, i.e. ammonia? Preliminary analyses with oral administration of $^{15}N_2$-urea showed significantly higher ^{15}N enrichment in fecal lysine of control mice than ASF-transplanted and antibiotics-treated mice (**Fig. 4-2**). This finding is consistent with the notion that urea cannot be hydrolyzed and utilized for microbial AA biosynthesis in the absence of urease. I plan to comprehensively examine the stool and plasma amino acid profiles in control, ASF-transplanted, and antibiotics-treated mice to elucidate the role of bacterial urease in host and gut microbiota amino acid metabolism. Future investigations will assess the importance of bacterial urease in the metabolic pathways of other nitrogen compounds including peptides, proteins, neurotransmitters, hormones, purines, and pyrimidines. This approach will involve both targeted and untargeted proteomics as well as metabolomics studies, utilizing various approaches including ^{15}N tracer experiments and LC/GCMS technologies. Overall, this line of investigation will enable us to better understand the impact of bacterial urease on host and gut microbiota nitrogen metabolism, with translational implications for health and disease.

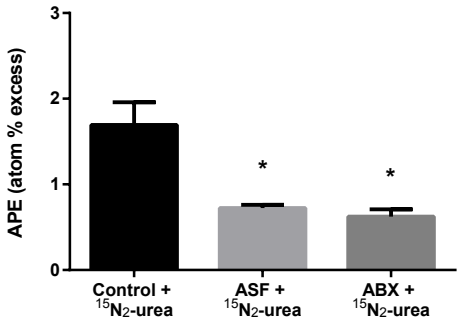


Figure 4-2. ^{15}N enrichment in fecal lysine after $^{15}N_2$-urea to control mice, ASF-transplanted mice, and ABX-treated mice (n=5 per group; *p<0.05 compared to control mice).

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