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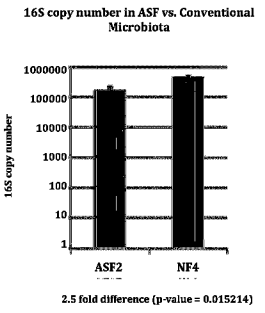

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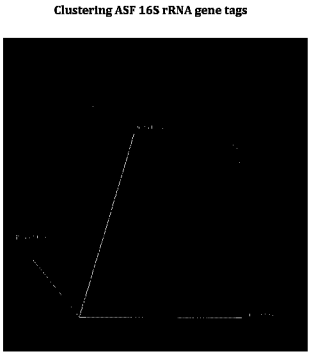

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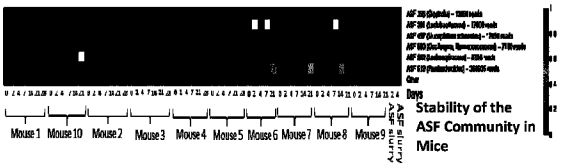

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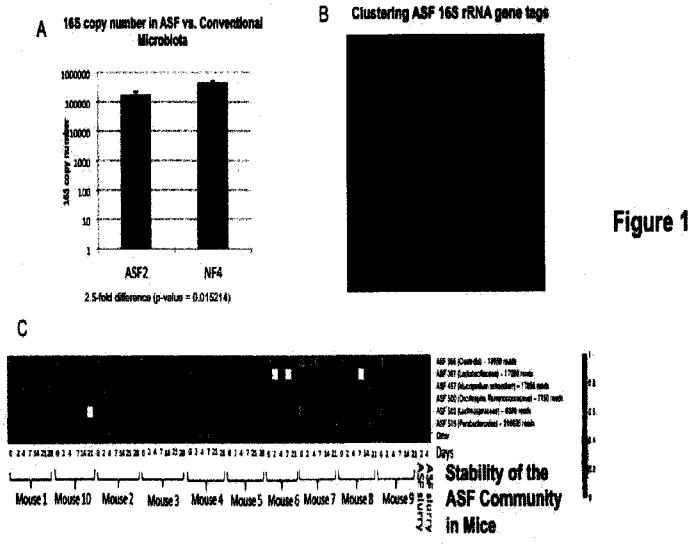

Figure 1

(57) Abstract: The invention features the use of a defined microbial consortia for the replacement of a gut microbiome associated with disease. In particular, the invention provides for the treatment of hyperammonemia, *Clostridium difficile* colitis, hepatic encephalopathy associated with cirrhosis, and inflammatory bowel disease.

COMPOSITIONS COMPRISING A DEFINED MICROBIOME AND METHODS OF USE THEREOF

PRIORITY CLAIM

This application claims priority to US Provisional Application Nos. 61/886,268 and 61/974,686 filed October 3, 2013 and April 3, 2014 respectively.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This work was supported by the following grants from the National Institutes of Health, Grant Nos: UH2/3 DK083981 and DK089472. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Growing evidence implicates the gut microbiome as a factor in the pathogenesis of a number of disease processes, including inflammatory bowel diseases, atherosclerosis, obesity, diabetes, and colon cancer. The association of these disease processes with an altered microbial community structure suggests that interventions that restore the normal resilient gut microbial community might be an innovative intervention for the treatment of *Clostridium difficile* colitis, hyperammonemia associated with inborn errors of metabolism, and inflammatory bowel disease.

Hyperammonemia occurs in many inherited metabolic diseases, most prominently the urea cycle disorders in which there is a failure to detoxify ammonia to urea. Current treatment includes a low-protein diet and drugs that relieve hyperammonemia, but these approaches are only partially effective in preventing/treating the devastating neurologic consequences caused by elevated levels of circulating ammonia. A significant amount of body ammonia forms in the gut from the hydrolysis of urea by intestinal bacteria. Oral antibiotic treatment may attenuate hyperammonemia, but the effectiveness of antibiotics wanes over time due to the development of antibiotic resistance.

There is a need in the art for novel methods of treating a subject having a disease

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associated with an undesirable gut microbiome. There is a further need in the art for novel methods of restoring normal gut flora in a subject or altering the gut microbiota associated with disease to alleviate symptoms caused by the disease. The present invention addresses these needs.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method for altering gut microbiota in a subject to reduce urease gene content or activity via reducing or eliminating urease producing bacteria from the gut in order to reduce ammonia production therefrom is disclosed. An exemplary method comprises administering to the subject an effective amount of one or more antibiotics sufficient to reduce the population of bacteria in the gut microbiota to a level suitable for repopulation of newly introduced bacteria, administering an effective amount of a purgative agent to the subject, thereby purging the intestines of said subject. Subsequently, a composition comprising a defined microbial consortia of bacter is administered the subject , the bacteria having minimal urease activity or no urease gene content, under conditions wherein said bacteria colonize the gut. The method results in the beneficial reduction of urease gene content, or activity or both in gut microbiota and ammonia production in the gut of the subject.

In certain embodiments, the subject has a disease associated with an undesirable gut microbiome, said treatment alleviating symptoms associated with said undesirable gut microbiome. The composition can be administered to the subject via several routes, which include, without limitation, endoscopy, by enteroscopy, by colonoscopy, a nasoduodenal catheter, an enema and orally in a consumable capsule or pill.

In one embodiment , the defined microbial consortia comprises one or more bacteria belonging to a genus selected from the group consisting of Bifidobacterium, Bacteroides, Tannerella, Parabacteroides, Bacillus, Lactobacillus, Anaerostipes, Anaerostipes, Blautia, Coprococcus, Dorea, Clostridium XI, Collinsella, and Paraprevotella. In a preferred embodiment, the consortia comprises Clostridium sp., Lactobacillus sp., Lactobacillus murinus, Mucispirillum schaedleri, Eubacterium plexicaudatum, Firmicutes bacterium, Clostridium sp. and Parabacteroides sp. In a particularly preferred embodiment, the consortia consists of Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii str., and Bacteroides vulgates.

The method of the invention can be used to advantage for the treatment of a variety of disorders, which include, without limitation, Carbamyl phosphate synthetase deficiency, Ornithine transcarbamylase deficiency, Arginosuccinate synthetase deficiency, Arginosuccinate lyase deficiency, Arginase deficiency, or N-acetylglutamate synthetase deficiency, Propionic acidemia, Methylmalonic acidemia, Isovaleric acidemia, Fatty Acid Oxidation Disorder, Short chain acyl-coA dehydrogenase deficiency, Medium-chain acyl-coA dehydrogenase deficiency, Long-chain acyl-coA dehydrogenase deficiency, a disorder of Amino Acid Transport, Lysinuric protein intolerance, Hyperammonemia, hyperornithinemia, homocitrullinemia syndrome, Transient Hyperammonemia of the Newborn, hyperinsulinemia, hypoglycemia syndrome, Clostridium difficile colitis, inflammatory bowel disease, atherosclerosis, obesity, metabolic syndrome, diabetes, colon cancer, cirrhosis, and hepatic encephalopathy.

In another embodiment, the method comprises determining the efficacy of treatment by assessing a parameter selected from the group consisting of 16S rRNA copy number, 16S rRNA gene sequencing, fecal urease levels, fecal or circulating amino acids, biogenic amines, fecal ammonia levels, and circulating ammonia levels.

In yet another aspect, a composition comprising an effective amount of a defined microbial consortia is provided. An exemplary consortia comprises a combination of one or more bacteria selected from the group consisting of Bifidobacterium, Collinsella, Bacteroides, Petrimonas, Tannerella, Parabacteroides, Bacillus, Isobaculum, Enterococcus, Lactobacillus, Anaerostipes, Blautia, Coprococcus, Dorea, Oribacterium, Syntrophococcus, Clostridium XI, Catenibacterium, Mitsuokella, Escherichia/Shigella, and Parapervotella, said consortia being effective to reduce urease levels in gut microbiota, said composition being present in a biological carrier suitable for administration to, and colonization of the gut. In yet another embodiment, the consortia consists of bacteria belonging to a genus selected from the group consisting of Bifidobacterium, Bacteroides, Tannerella, Parabacteroides, Bacillus, Lactobacillus, Anaerostipes, Anaerostipes, Blautia, Coprococcus, Dorea, and Clostridium XI. In a particularly preferred embodiment, the consortia comprises at least Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii str., and Bacteroides vulgates.

Also encompassed by the present invention is a process for preparing a defined microbial consortia composition for decreasing urease levels in the gut. The process comprising preparing a liquid culture of bacteria cells comprising said defined microbial consortia described above, harvesting the cells after a suitable period of growth and resuspending the cells in lyophilization medium, the medium optionally comprising cyroprotectorants, biological or chemical oxygen scavengers. The cells are then transferred into a lyophilizer under anaerobic conditions and lyophilized. Lyphilized cells are then packaged into a capsule or pill under oxygen free conditions and optionally further packaged into glass ampoules to maintain anaerobic conditions during shipment and storage. Also provide are capsules or pills containing the defined microbial consortia produced by the foregoing process.

In yet another aspect of the invention, the method entails administration of a single urease negative bacterial population for altering gut microbiota to reduce urease gene content or activity. The steps employed for this approach are comparable to those provided above. In this embodiment, the bacteria are selected from the group consisting of urease negative Lactobacillus acidophilus L. acidophilus, L. acidophilus, L. acidophilus, Lactobacillus casei, L. casei Immunitas, Lactobacillus fermentum, Lactobacillus johnsonii Lactobacillus paracasei Lactobacillus plantarum , Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus lactis, Bifidobacterium lactis, Bifidobacterium longum , Bifidobactenium breve.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

By "a defined microbial consortia" is meant a purified and/or isolated population of known microbes.

By "undesirable gut microbiome" is meant a community of microbes comprising a pathogen or having a biological activity associated with a pathogenic process. In one embodiment, an undesirable gut microbiome comprises microbes having urease activity. In another embodiment, an undesirable gut microbiome comprises an increased number or percentage of *Clostridium difficile* relative to the number or percentage of *C. difficile* and/or toxin production present in the gut of a healthy control subject.

By "normal gut microbiota" is meant a population of microbes that is substantially similar to the population of microbes present in the gut of a healthy control subject.

By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

In this disclosure, "comprises," "comprising," "containing" and "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean " includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Exemplary diseases include hyperammonemia, *Clostridium difficile* colitis, hepatic encephalopathy associated with cirrhosis, and inflammatory bowel disease.

By "effective amount" is meant the amount of a composition of the invention (e.g., defined microbial consortia) required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of a defined microbial consortia varies depending upon the manner of administration, the indication for administration, the age, body weight and height, sex, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

The terms "isolated," "purified," or "biologically pure" refers to material that is free to varying degrees from components which normally accompany it as found in its native state.

"Isolate" denotes a degree of separation from original source or surroundings. "Purify" denotes a degree of separation that is higher than isolation. Thus, a purified isolated bacterium that is part of a defined microbial consortia is at least about 90%, 95% or 100% free of bacteria, fungi, viruses, or other undefined microbes.

By "marker" is meant any analyte associated with a disease or disorder. In one embodiment, a marker of the invention is urease activity (e.g., fecal urease) or circulating ammonia.

By "reference" is meant a standard or control condition.

By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

By "instructional materials" is meant any information, resource and guidance that helps teaching and transferring skills to others.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C show that there the abundance of bacteria is relatively similar between ASF and the normal commensal microbiota (NF4). Nevertheless ASF composition is distinctive from the commensal microbiota in a 16S rRNA PCoA plot (Figure 1B, red=ASF, blue=conventional). The ASF consortium shows significant resilience when ASF colonized mice are housed in a SPF environment for approximately 1 month. Figure 1A is a graph showing 16S copy number in ASF bacteria relative to a conventional microbiota. Figure 1B shows results of a UniFrac-based PCoA analysis. Figure 1C shows results of 16S rRNA sequencing of sequential fecal pellets collected from ten mice.

Figures 2A-2C show the development of a method for FMT in mice. Figures 2A and 2B are graphs. Figure 2A shows that 16S rRNA copy number is reduced by approximately 4 logs 72 hours following oral antibiotic initiation. 16S rRNA copy number returns to baseline 5 days after discontinuing antibiotics (Figure 2A). Figure 2B shows that 16S copy number was restored to baseline levels after ASF transplantation. Figure 2C shows that ASF transfer was as effective in pretreated conventionally housed as germ-free recipients.

Figure 3A provides diagrams showing long-term taxonomic resiliency of the transplanted ASF community over four months evaluated by both unweighted Unifrac (presence-absence of taxa) and weighted Unifrac. Figure 3B shows that ASF sequence reads remained stable at six weeks relative to ASF levels observed in conventionally-housed mice. Essentially, a new steady state develops with ASF and Clostridial taxa (Figure 4B) that exhibits long-lasting reduction of urease activity and ammonia production (Figure 6).

Figure 4A provides four graphs showing that the resilience of the ASF community was determined by the persistence of Paracteroides in both germ-free and pretreated conventional hosts where this taxon remained stable after about one month. Figure 4B shows that resilience of the ASF community was determined by the persistence of Paracteroides in germ-free and pretreated conventional hosts. It also demonstrates that specific bacterial taxa, predominantly Clostridia, appear over time to develop a stable new steady state with ASF that lacks urease activity and ammonia production.

Figures 5A-5D relate to the presence of a urease gene and urease activity in ASF. Figure 5A show results of ASF genomic sequencing (Figure 5A). Figures 5B and 5D show results of

urease assays in feces and using breath testing (Figure 5B). Figure 5C shows results of an ammonia assay.

Figure 6 shows results of a fecal urease activity analysis.

Figure 7 shows ammonia levels in the stool of mice treated with antibiotics, polyethylene glycol (PEG), and ASF.

Figure 8 shows, through the course of time, the levels of butyrate, propionate and acetate in the stool of mice treated with antibiotics, polyethylene glycol (PEG), and ASF.

Figure 9 shows 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 11. (b) Kaplan-Meier survival curves and (c) fecal ammonia levels in the low dose TAA study associated with results shown in Figure 5 (NF FMT = Normal flora fecal microbiota transplant. (d) Photomicrographs of H&E stained hepatic sections from mice treated with high dose TAA as indicated (200X) with quantification of hepatocellular necrosis (Figure 11b).

Figure 10 shows ASF transplantation into prepared mice reduces mortality and neurobehavioural abnormalities after thioacetamide-induced hepatic injury. (a) Kaplan-Meier survival curves of high dose TAA-induced hepatic injury in conventional vs. Prepared mice transplanted with ASF (N=15). (b) Spontaneous alternations quantified by Y-maze testing in Prepared mice that have received a fecal microbiota transplant (FMT) of either normal feces (NF) or ASF before TAA-induced hepatic injury (N=11 in ASF, N= 10 in NF, N=5 in control, *$p<0.05$).

Figure 11 shows differential liver injury does not explain differences with or without ASF. (a) Plasma alanine amino transferase (ALT) levels in untransplanted, antibiotic-treated, or ASF transplanted mice after TAA induced hepatic injury (N=10 in untransplanted and ASF transplanted groups, N=5 in control and antibiotic treated groups). (b) Liver damage quantified histologically by a pathologist reported as percent hepatic cellular necrosis (N=5 in each group, 10 random 200x HPF per liver, no statistically significant difference amongst the three treatment groups; blinded analysis).

Figure 12 shows a schematic diagram of the gut microbiota.

DETAILED DESCRIPTION OF THE INVENTION

The invention features use of a defined microbial consortia for the replacement of a gut microbiome associated with disease.

The invention is based, at least in part, on the discovery that transplantation of a defined microbial consortia into a subject provides for the long term replacement of a gut microbiome associated with disease. While fecal transplantation has been used to treat subjects with diseases associated with undesirable changes in the gut microbiome, inoculating subjects with undefined complex microbial communities raise a number of safety concerns. In particular, the possibility that fecal transplant might inadvertently result in the transfer of pathogens into a susceptible host. Therefore, a need exists to identify defined microbial consortia with well characterized biological functions that can be used to target gut microbiome-associated diseases.

As reported herein below, Altered Schaedler's Flora (ASF), a defined consortia of eight bacteria, were transferred into previously colonized mice where the flora remained a robust and resilient community for at least four months under specific pathogen free (SPF) housing conditions. Functional resiliency of the ASF community was demonstrated by genomic sequencing along with biochemical analyses that revealed the sustained lack of fecal urease activity and ammonia production in ASF transplanted mice. Unexpectedly, metabolomic analyses revealed the ASF transplanted mice were deficient in essential amino acids, a consequence of reduced microbial amino acid synthesis due to a deficiency in urease dependent ammonia production and the resultant inability of these mice to obtain dietary essential amino acids through corprophagia. These results demonstrate important syntrophic interactions between the host and the gut microbiome that may be of fundamental importance in the development of defined microbial communities for the treatment of disease.

Dysbiosis and Disease

Growing evidence implicates the gut microbiome as a factor in the pathogenesis of a number of diseases. The association of certain disease processes with alterations in the gut microbiome suggests that interventions that restore the normal resilient gut microbial community or alter the gut microbiota to remove undesirable bacteria might be an innovative intervention for disease treatment. Indeed, the high level of efficacy of fecal microbiota transplantation (FMT) in the treatment of refractory *Clostridia difficile* infection (CDI) provides proof-of-principle that a

specific human disease can be treated by inoculation with a gut microbial community. Together with studies in animal models (antibiotics in the development of *C. difficile* and *Salmonella*), these results also reinforce that notion that the use of a resilient microbial community is important to prevent and/or treat disease. Indeed, current probiotics consisting of a single or few live bacterial strains are largely ineffective in the treatment of *Clostridia difficile* infection.

Ultimately, fecal microbiota transplantation (FMT) will be replaced by the use of laboratory generated resilient microbial consortia that can be used to target specific diseases based on well-characterized microbial biological properties. One such example is the deleterious effects of ammonia production by the gut microbiota on the central nervous system in patients with chronic liver disease and/or inborn errors of metabolism, an entity known as hepatic encephalopathy. Treatments for hepatic encephalopathy include strategies to reduce microbial production of ammonia and its absorption. Therefore, the development of a resilient microbial community with minimal urease gene content to prevent or inhibit the production of ammonia should reduce the development of hepatic encephalopathy when inoculated into a susceptible patient population. When eliminating urease activity of the gut microbiota, it's important to recognize that the recycling of nitrogen through the production of ammonia is an example of symbiosis between the mammalian host and its gut microbiota that involves a syntrophic interaction (or cross-feeding). Urea production by the liver as a waste product is not only excreted in urine but is also transported into the colon where it is hydrolyzed by bacterial urease into both carbon dioxide and ammonia. Ammonia is then utilized by the microbiota for protein synthesis, reabsorbed by the host where it is reincorporated into the host nitrogen pool by hepatic metabolism, or excreted in the feces.

Using Altered Schaedler's Flora (ASF), a model gut microbial community that as reported herein exhibits long term reduction in urease activity and ammonia production , both the taxonomic and functional resiliency upon transplantation was examined in a previously colonized host. The impact of ASF on host metabolism was also determined. Transplantation of ASF was highly efficient in the properly prepared host where it demonstrates a remarkable level of taxonomic resiliency over a period of 4 months under SPF housing conditions. Invasion of non-ASF taxa over time involved specific bacterial taxa of the *Fimicutes* phylum but not *Bacteroidetes*. Functionally, urease activity remained very low with minimal ammonia production over this same time period. ASF has been used for decades to colonize immune

deficient mice without evidence of deleterious effects. Nevertheless, metabolic profiling of both the host and the transplanted microbiota revealed a reduction in essential amino acids demonstrating the importance of microbial urease activity in maintenance of the syntrophic interaction between the gut microbiome and its host with respect to nitrogen balance.

Methods of the Invention

The present invention provides methods of treating diseases or symptoms thereof associated with the presence of one or more undesirable bacteria in the gut of a subject. Accordingly, the invention provides compositions and methods for treating a subject having or at risk of developing a disease associated with undesirable changes in the gut microbiome, the method involving administering a therapeutically effective amount of a composition comprising a defined microbial consortia to a subject (e.g., a mammal, such as a mouse or human). Thus, one embodiment is a method of treating a subject suffering from or susceptible to a metabolic disease (e.g., an inborn error of metabolism, such as urea carbamylase deficiency).

In particular embodiments, the subject is identified as having a Urea Cycle Defect (e.g., Carbamyl phosphate synthetase deficiency, Ornithine transcarbamylase deficiency, Arginosuccinate synthetase deficiency, Arginosuccinate lyase deficiency, Arginase deficiency, or N-acetylglutamate synthetase deficiency. In other embodiments, the subject has an Organic Acidemias (e.g., Propionic acidemia, Methylmalonic acidemia, Isovaleric acidemia). In still other embodiments, the subject has a Fatty Acid Oxidation Disorder (e.g., Short chain acyl-coA dehydrogenase deficiency, Medium-chain acyl-coA dehydrogenase deficiency, Long-chain acyl-coA dehydrogenase deficiency) or a disorder of Amino Acid Transport (e.g., Lysinuric protein intolerance, Hvperammonemia, hyperornithinemia, homocitrullinemia syndrome). In still other embodiments, the subject has Transient Hyperammonemia of the Newborn, Hyperammonemia, hyperinsulinemi, or hypoglycemia syndrome.

In still other embodiments, the subject has cirrhosis, hepatic encephalopathy, or another liver disorder characterized by hyperammonemia. The method includes the step of administering to the mammal a therapeutic amount of an amount of a composition comprising a defined microbial consortia (e.g., a community of microbes deficient in urease activity) sufficient to treat the disease or disorder or symptom thereof, under conditions such that the disease or disorder is treated.

In still other embodiments, the subject has *Clostridium difficile* colitis or inflammatory bowel disease.

Compositions comprising a defined microbial consortia preferably contain a combination of one or more (e.g.,1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50) bacterial strains listed in Tables 1, 2, or 3A, 3B, 3C, 7A or 7B.

Table 1: Bacterial Strains from the Guts of Healthy Humans

Phylum	Class	Order	Family	Genus
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium
Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Collinsella
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Tannerella
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Isobaculum
Firmicutes	Bacilli	Lactobacillales	Enterocaccaceae	Enterococcus
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Syntrophococcus
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Catenibacterium
Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Mitsuokella
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella

Table 2: Microbiota Therapeutics Version 1 (MTv1)

Phylum	Class	Order	Family	Genus	Reason for choice
Actinobacteria	**Actinobacteria**	**Bifidobacteriales**	**Bifidobacteriaceae**	**Bifidobacterium**	common in healthy gut. populate the nodes, probiotic line acre
Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Collinseiia	
Bacteroidetes	**Bacteroidia**	**Bacteroidales**	**Bacteroidaceae**	**Bacteroides**	hASF, common in healthy gut populate the nodes
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimortas	
Bacteroidetes	**Bacteroidia**	**Bacteroidales**	**Porphyromonadaceae**	**Tannerella**	common in healthy gut, populate the nodes
Bacteroidetes	**Bacteroidia**	**Bacteroidales**	**Porphyromonadaceae**	**Parabacteroides**	hASF, common in healthy gut. populate the nodes
Firmicutes	**Bacilli**	**Bacillales**	**Bacillaceae**	**Bacillus**	common in healthy gut, populate the nodes
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	isobaculum	
Firmicutes	Bacilli	Lactobacillales	Enterocaccaceae	Enterococcus	
Firmicutes	**Bacilli**	**Lactobacillales**	**Lactobacillaceae**	**Lactobacillus**	hASF, common in healthy gut. populate the nodes, probiotic lineage
Firmicutes	**Clostridia**	**Clostridiales**	**Lachnospiraceae**	**Anaerostipes**	common in healthy gut populate the nodes
Firmicutes	**Clostridia**	**Clostridiales**	**Lachnospiraceae**	**Blautia**	common in healthy gut, populate the nodes
Firmicutes	**Clostridia**	**Clostridiales**	**Lachnospiraceae**	**Coprococcus**	common in healthy gut, populate the nodes
Firmicutes	**Clostridia**	**Clostridiales**	**Lachnospiraceae**	**Dorea**	common in healthy gut, populate the nodes
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium	
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Syntrophococcus	
Firmicutes	**Clostridia**	**Clostridiales**	**Peptostreptococcaceae**	**Clostridium XI**	hASF, common in healthy gut, populate the nodes
Firmicutes	Erysipelotricftia	Erysipelotrichales	Ervsipelotrichaceae	Catenibacterium	
Firmicutes	Neqativicutes	Selenomonadales	Veillonellaceae	Mitsuokella	
Proteobacteria	Gamma proteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/ Shigella	

*An exemplary defined microbial consortia comprises the bacteria shown in bold type.

Table 3A: Engineered human community low in urease

Paraprevotella clara
Bifidobacterium longum
Collinsella aerofaciens
Coprococcus comes
Dorea longicatena
Bacteroides eggerthii str.
Bacteroides vulgatus

Table 3B Human orthologs of ASF

Clostridium sp.	356
Lactobacillus sp.	360
Lactobacillus murinus	361
Flexistipes group	457
Eubacterium plexicaudatum	492
Low G--C content Gram + group	500
Clostridium sp.	502
Bacteroides sp.	519

Table 3C Mouse ASF species

TABLE 3C								
Genome features and accession numbers of the ASF bacteria					PMID:	24723722		
ASF no.	**Taxonomy**	**Genome size (Mb)**	**GC (%)**	**Gene count**	**Contig count**	**N_{50} (kb)**	**Fold cover-age**	**GenBank accession no.**
ASF356	*Clostridium* sp.	2.91	30.91	2,799	31	209	143	AQFQ00000000.1
ASF360	*Lactobacillus* sp.	2.01	35.86	1,930	244	19	47	AQFR00000000.1
ASF361	*Lactobacillus murinus*	2.17	39.96	2,102	78	59.7	160	AQFS00000000.1
ASF457	*Mucispirillum schaedleri*	2.33	31.15	2,144	39	151	142	AYGZ00000000.1

ASF492	*Eubacterium plexicaudatum*	6.51	42.86	6,217	248	74.4	119	AQFT00000000.1
ASF500	*Firmicutes* bacterium	3.7	58.77	3,563	42	300	137	AYJP00000000.1
ASF502	*Clostridium* sp.	6.48	47.9	6,062	134	137	82	AQFU00000000.1
ASF519	*Parabacteroides* sp.	6.87	43.45	5,477	24	584	143	AQFV00000000.1

Identifying a subject in need of treatment for a disease associated with the gut microbiome can be in the judgment of a health care professional and can be subjective (e.g. opinion) or objective (e.g. measurable by a test or diagnostic method). As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated. As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of, or susceptible to, developing a disorder or condition.

The therapeutic methods of the invention (which include prophylactic treatment) in general comprise administration of a therapeutically effective amount of a composition comprising a defined microbial consortia to a subject (e.g., human) in need thereof. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for a disease, disorder, or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider. The compositions herein may be also used in the treatment of any other disorders in dysbiosis may be implicated.

A subject is effectively treated whenever a clinically beneficial result ensues. This may mean, for example, a complete resolution of the symptoms associated with an undesirable gut microbiota, a decrease in the severity of the symptoms associated with an undesirable gut microbiota, or a slowing of the progression of symptoms associated with an undesirable gut

microbiota. As described above, such symptoms can be the result of an inborn metabolic error, for example, a urease cycle deficiency; a liver injury or chronic liver disease. Regardless of the cause, such patients have an impaired ability to process the ammonia delivered to the liver from the intestinal tract. As a result, such patients suffer from high circulating ammonia levels (hyperammonemia) which are correlated with damage to the central nervous system and can result in hepatic encephalopathy.

Thus in general, the methods include administering a defined microbial consortia having reduced urease activity to a patient having an undesirable gut microbiota. These methods can further include the steps of a) identifying a subject (e.g., a patient and, more specifically, a human patient) who has an undesirable gut microbiota, and b) providing to the subject a composition comprising a defined microbial consortia described herein. An amount of such a composition provided to the subject that results in a complete resolution of the symptoms associated with an undesirable gut microbiota, a decrease in the severity of the symptoms associated with an undesirable gut microbiota, or a slowing of the progression of symptoms associated with an undesirable gut microbiota, is considered a therapeutically effective amount. The present methods may also include a monitoring step to help optimize dosing and scheduling as well as predict outcome.

The present methods can include the steps of administering a treatment to reduce the level of bacteria within the intestinal lumen of the subject prior to administering a composition comprising any of the defined microbial consortia described herein. Thus the subject, whom we may refer to as a recipient or a host, can be pretreated to reduce the level of endogenous bacteria prior to administration of compositions. Pretreatment regimens can include specific antimicrobial agents, for example one or more antibiotics, for example, rifaximin, metronidazole, trimethoprim-sulfamethoxazole, neomycin or vancomycin. Pretreatment regimens can include non-specific agents, for example, a purgative, for example, polyethylene glycol or a laxative, for example, bisacodyl. Pretreatment regimens can also include dietary modification. The pretreatment regimen can include a combination of any of the treatment modalities above, for example, administration of an antibiotic, a purgative, a laxative, along with dietary modification. Concurrent administration of two or more agents does not require that the agents be administered at the same time or by the same route, as long as there is an overlap in the time period during

which the agents are exerting their therapeutic effect. Simultaneous or sequential administration is contemplated, as is administration on different days or weeks.

The compositions can also include a pharmaceutically acceptable carrier. We use the terms "pharmaceutically or biologically acceptable" (or "pharmacologically acceptable") to refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal or a human, as appropriate. The term "pharmaceutically acceptable carrier," as used herein, includes any and all solvents, dispersion media, coatings, antibacterial, isotonic and absorption delaying agents, buffers, excipients, binders, lubricants, gels, surfactants and the like, that may be used as media for a pharmaceutically acceptable substance.

This methods of the invention include administration of a defined microbial consortia of bacteria having minimal urease activity formulated as pharmaceutical compositions which contain, as the active ingredient, the defined microbial consortia described herein, in combination with one or more pharmaceutically acceptable carriers. In some embodiments, the defined microbial consortia can be sterilized using conventional sterilization techniques before or after it is combined with the pharmaceutically acceptable carrier. In making the compositions of the invention, the defined microbial consortia are typically mixed with an excipient, diluted by an excipient or enclosed within such a carrier in the form of, for example, a capsule, tablet, sachet, paper, or other container. When the excipient serves as a diluent, it can be a solid, semisolid, or liquid material (e.g., normal saline), which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders. As is known in the art, the type of diluent can vary depending upon the intended route of administration. The resulting compositions can include additional agents, such as preservatives. The excipient or carrier is selected on the basis of the mode and route of administration. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in Remington's Pharmaceutical Sciences (E. W. Martin), a well-known reference text in this field, and in the USP/NF (United States Pharmacopeia and the National Formulary). Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates,

tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The pharmaceutical compositions can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

Pharmaceutically acceptable compositions for use in the present methods, including those in a defined microbial consortium having minimal urease activity, can be entrapped in a colloid for oral delivery. Pharmaceutically acceptable compositions can be prepared according to standard techniques. The defined microbial consortium having minimal urease activity can be dried and compacted by grinding or pulverizing and inserted into a capsule for oral administration. In some embodiments, the defined microbial consortium having minimal urease activity can be combined one or more excipients, for example, a disintegrant, a filler, a glidant, or a preservative. Suitable capsules include both hard shell capsules or soft-shelled capsules. Any lipid-based or polymer-based colloid may be used to form the capusule. Exemplary polymers useful for colloid preparations include gelatin, plant polysaccharides or their derivatives such as carrageenans and modified forms of starch and cellulose, e.g., hypromellose. Optionally, other ingredients may be added to the gelling agent solution, for example plasticizers such as glycerin and/or sorbitol to decrease the capsule's hardness, coloring agents, preservatives, disintegrants, lubricants and surface treatment. In some embodiments, the capsule does not include gelatin. In other embodiments, the capsule does not include plant polysaccharides or their derivatives.

Regardless of their original source or the manner in which they are obtained, the compositions comprising the defined microbial consortium having minimal urease activity can be formulated in accordance with their use. These compositions can be prepared in a manner well known in the pharmaceutical art, and can be administered by a variety of routes, depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be oral, gastrointestinal, or rectal. Administration can be in the form of a single bolus dose, or may be, for example, by a continuous perfusion pump.

The compositions can be formulated in a unit dosage form, each dosage containing, for example, from about 0.005 mg to about 2000 mg of a defined microbial consortium having minimal urease activity per dose. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. For preparing solid compositions such as tablets, the principal active ingredient is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, the active ingredient is typically dispersed evenly throughout the composition so that the composition can be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation is then subdivided into unit dosage forms of the type described above containing from, for example, 0.005 mg to about 1000 mg of the defined microbial consortium having minimal urease activity of the present invention.

The compositions can be formulated in a unit dosage form, each dosage containing, for example, from about 0.1 mg to about 50 mg, from about 0.1 mg to about 40 mg, from about 0.1 mg to about 20 mg, from about 0.1 mg to about 10 mg, from about 0.2 mg to about 20 mg, from about 0.3 mg to about 15 mg, from about 0.4 mg to about 10 mg, from about 0.5 mg to about 1 mg; from about 0.5 mg to about 100 mg, from about 0.5 mg to about 50 mg, from about 0.5 mg to about 30 mg,, from about 0.5 mg to about 20 mg, from about 0.5 mg to about 10 mg, from about 0.5 mg to about 5 mg; from about 1 mg from to about 50 mg, from about 1 mg to about 30 mg,, from about 1 mg to about 20 mg, from about 1 mg to about 10 mg, from about 1 mg to about 5 mg; from about 5 mg to about 50 mg, from about 5 mg to about 20 mg, from about 5 mg to about 10 mg; from about 10 mg to about 100 mg, from about 20 mg to about 200 mg, from about 30 mg to about 150 mg, from about 40 mg to about 100 mg, from about 50 mg to about 100 mg of the active ingredient.

In some embodiments, tablets or pills of the present invention can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner

component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the compositions of the present invention can be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

The proportion or concentration of the compositions of the invention in a pharmaceutical composition can vary depending upon a number of factors including dosage, chemical characteristics (e.g., hydrophobicity), and the route of administration. For example, the defined microbial consortium having minimal urease activity can be provided in a capsule containing from about 0.005 mg to about 1000 mg for oral administration. Alternatively or in addition, the dosage can be expressed as cfu/g of dry weight. The dosage may vary, but can range from the equivalent of about 10^2 to about 10^{12} cfu/g, e.g., 1 x10^2 cfu/g, 5 x10^2 cfu/g, 1 x10^3 cfu/g, 5 x10^3 cfu/g, 1 x10^4 cfu/g, 5 x104 cfu/g, 1 x105 cfu/g, 5 x10^5 cfu/g, 1 x10^6 cfu/g, 5 x10^6 cfu/g, 1 x10^7 cfu/g, 5 x10^7 cfu/g, 1 x10^8 cfu/g, 5 x10^8 cfu/g, 1 x10^9 cfu/g, 5 x10^9 cfu/g, 1 x10^{10} cfu/g, 5 x10^{10} cfu/g, 1 x10^{11} cfu/g, 5 x10^{11} cfu/g, 1 x10^{12} cfu/g.of dry weight.

Compositions comprising a defined microbial consortia can be administered to the gastrointestinal tract of a subject by nasoduodenal catheter, by enema, or by endoscopy, enteroscopy, or colonoscopy or orally in a consumable capsule or pill. In certain embodiments, the defined microbial consortia are diluted in a suitable excipient (e.g., saline solution). In a preferred embodiment, the bacteria are delivered in lyophilized form.

Regardless of how the compositions are formulated, the dosage required will depend on the route of administration, the nature of the formulation, the nature of the subject's condition, e.g., immaturity of the immune system or a gastrointestinal disorder, the subject's size, weight, surface area, age, and sex, other drugs being administered, and the judgment of the attending clinicians. In some embodiments, suitable dosages are in the range of 0.01-1,000 mg/kg. Some typical dose ranges are from about 1 µg/kg to about 1 g/kg of body weight per day. In some embodiments, the dose range is from about 0.01 mg/kg to about 100 mg/kg of body weight per day. In some embodiments, the dose can be, for example, 1 mg/kg, 2 mg/kg, 5 mg/kg, 10 mg/kg,

20 mg/kg, 50 mg/kg or 100 mg/kg. Alternatively or in addition, the dosage can be expressed as cfu/g of dry weight. The dosage may vary, but can range from the equivalent of about 10^2 to about 10^{12} cfu/g, *e.g.*, 1 x10^2 cfu/g, 5 x10^2 cfu/g, 1 x10^3 cfu/g, 5 x10^3 cfu/g, 1 x10^4 cfu/g, 5 x10^4 cfu/g, 1 x10^5 cfu/g, 5 x10^5 cfu/g, 1 x10^6 cfu/g, 5 x10^6 cfu/g, 1 x10^7 cfu/g, 5 x10^7 cfu/g, 1 x10^8 cfu/g, 5 x10^8 cfu/g, 1 x10^9 cfu/g, 5 x10^9 cfu/g, 1 x10^{10} cfu/g, 5 x10^{10} cfu/g, 1 x10^{11} cfu/g, 5 x10^{11} cfu/g, 1 x10^{12} cfu/g of dry weight. Preferably, at least 1 x 10^{10}cfu/g is used.

The dosage is likely to depend on such variables as the type and extent of progression of the disease or disorder, the overall health status of the particular patient, the relative biological efficacy of the compound selected, formulation of the excipient, and its route of administration. Effective doses can be extrapolated from dose-response curves derived from in vitro or animal model test systems. Useful assay systems include for example, analysis of the particular gut microbial community, fecal urease activity or ammonia levels. Wide variations in the needed dosage are to be expected in view of the variety of useful microbial consortia and the differing efficiencies of various routes of administration. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art. Administrations can be single or multiple (e.g., 2- or 3-, 4-, 6-, 8-, 10-, 20-, 50-, 100-, 150-, or more fold). The duration of treatment with any composition provided herein can be any length of time from as short as one day to as long as the life span of the host (e.g., many years). For example, a composition can be administered once a week (for, for example, 4 weeks to many months or years); once a month (for example, three to twelve months or for many years); or once a year for a period of 5 years, ten years, or longer. It is also noted that the frequency of treatment can be variable. For example, the present compositions can be administered once (or twice, three times, etc.) daily, weekly, monthly, or yearly.

Any method known to those in the art can be used to determine if a particular response is induced. Clinical methods that can assess the degree of a particular disease state can be used to determine if a response is induced. For example, a subject can be monitored for symptomatic relief, e.g., relief from lethargy, irritability, poor feeding, vomiting hyperventilation, seizures, ataxia. Alternatively or in addition, serum markers, for example, plasma concentration of ammonium, arginine, citrulline; urinary markers, for example, orotic acid can also be assayed

The compositions may also be administered in conjunction with other therapeutic modalities. Other therapeutic modalities will vary according to the particular disorder, but can

include, for example, dietary modification, hemodialysis, therapeutic agents such as sodium benzoate, phenylacetate, arginine, or surgical remedies, for example, liver transplantation. Concurrent administration of two or more therapeutic agents does not require that the agents be administered at the same time or by the same route, as long as there is an overlap in the time period during which the agents are exerting their therapeutic effect. Simultaneous or sequential administration is contemplated, as is administration on different days or weeks.

In one embodiment, the invention provides a method of monitoring treatment progress, for example, by measuring urease activity (e.g., fecal urease), fecal ammonia levels or circulating ammonia levels. In other embodiments, the monitoring involves detecting gut microbes (e.g., by 16S rRNA gene sequencing or 16S rRNA copy number). The method includes the step of determining a level of diagnostic marker (marker) or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to a disorder or symptoms thereof associated with dysbiosis, in which the subject has been administered a therapeutic amount of a composition herein sufficient to treat the disease or symptoms thereof. The level of marker determined in the method can be compared to known levels of marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of marker in the subject is determined prior to beginning treatment according to this invention; this pre-treatment level of marker can then be compared to the level of marker in the subject after the treatment commences, to determine the efficacy of the treatment. In certain preferred embodiments, a level of marker in the subject is determined serially after beginning treatment according to this invention; the level of marker can then be compared over time to determine the efficacy of the treatment and need for dose adjustment.

Formulation of Microbial Consortia and Methods of Delivery

Compositions comprising a defined microbial consortia are preferably administered to a subject by nasoduodenal catheter, by enema, or by endoscopy, enteroscopy, or colonoscopy or orally in a consumable capsule or pill. In certain embodiments, the defined microbial consortia are diluted in a suitable excipient (e.g., saline solution). In a preferred embodiment, the bacteria

are delivery in lyophilized form. An exemplary protocol for this approach is provided below to facilitate practice of the invention.

Lyophilization Procedure and Process Development:

In one aspect, the microbial consortium will be delivered as a lyophilized (freeze-dried) powder packaged in a consumable gelatin capsule. In brief, the liquid culture will be: centrifuged, resuspended in a lyophilization medium which will optionally include cryoprotectants and biological- and/or chemical-oxygen scavengers (See Table 5) transferred under anaerobic conditions to a shelf lyophilizer, lyophilized, encapsulated in a gelatin capsule under anaerobic conditions, and packaged in a glass ampoule to maintain oxygen free conditions during transport and storage. Details on each of these steps, as well as method development and quality control (QC) considerations are detailed below.

Preparation of Culture for Lyophilization.

Liquid cultures (10^{10} colony forming units, or CFU) of the strain(s) in the logarithmic or early stationary phase of growth are centrifuged to form a pellet of cells. The culture medium (supernatant) is then removed, and the pellet resuspended in a lyophilization medium; multiple lyophilization medium can be utilized and include without limitation, those set forth in **(Table 1)**.

Lyophilization Medium
10-20% (wt./vol) skim milk[1–3]
Reagent 18 (Tryticase Soy Broth, Sucrose, Bovine Serum Albumin Fraction V) [1,2]
Reagent 20 (Sucrose, Bovine Serum Albumin Fraction V) [1]
Microbial Freeze Drying Buffer[2,3]

Table 4. Different lyophilization medium for formulation of the microbial consortium.

Following lyophilization of the consortium, we will optionally include in the formulation oxygen-scavenging excipients for reducing O_2, thereby protecting the obligate anaerobic consortium (Tables 2 and 3A, 3B, 3C, 7A and 7B). Chemical excipients include, without limitation, (1) ferrous sulfide (125 μM)[1,4,5] and (2) L-cysteine HCl (10 mM) [6,7]; additionally, (3) *Lactobacillus acidophilus* (10^{10} CFU), a facultative anaerobe widely used in probiotics[8,9], will be used as a biological excipient both with and without the chemical excipients. To ensure quality control, we can utilize a freeze-drying indicator (OPS Diagnostics, Lebanon, NJ), which is a colorimetric indicator that turns from red to blue when the water content is below 2%, to confirm that the sample has been properly lyophilized. Additionally, during process development we will include a phosphorescence-based oxygen nanoprobe that will be used to determine the shelf life of the gelatin capsule and confirm anaerobic conditions within the lyophilized mixture. All preparation will be performed in an anaerobic chamber (Coy Labs, Grass Lake, MI) under sterile technique.

Excipient(s)	Concentration
Ferrous Sulfide	125 μM
L-Cysteine HCl	10 mM
L. acidophilus	10^{10} CFU
Ferrous Sulfide + L-Cysteine HCl	125 μM & 10 mM
Ferrous Sulfide + *L. acidophilus*	125 μM & 10^{10} CFU
L-Cysteine HCl + *L. acidophilus*	10 mM & 10^{10} CFU
Ferrous Sulfide + L-Cysteine HCl+*L. acidophilus*	125 μM, 10 mM, & 10^{10} CFU

Table 5. List of different excipients (single and combination) for use with lyophilized bacterial consortium.

Lyophilization, Encapsulation, and Packaging.

The solution containing the lyophilization medium, resuspended pellet, and excipient(s) will be transferred to a lyophilizer that has been flushed with nitrogen gas to maintain an

anaerobic headspace. Lyophilizers are commercially available and can be obtained from BenchTop Pro with Omnitronics, SP Scientific, Warminster, PA for example. Lyophilization will initially be perfomed using the steps set forth below and in the following references: (ATCC. Methods for freezing and freeze-dryingbacteria. 2014. Available on the world wide web at atcc.custhelp.com/app/answers/detail/a_id/140/~/methods-for-freezing-and-freeze-drying-bacteria; Diagnostics O. Bacteria Freeze Drying Protocol. 2014. Available at: on the world wide web at.opsdiagnostics.com/notes/ranpri/rpbacteriafdprotocol.htm; Diagnostics O. Bacteria Lyophilization Overview. 2014. Available at: on the world wide web at http://opsdiagnostics.com/ notes/ranpri/bacteria_lyophilization_overview.htm; Savini M, et al. Nutrients 2010;2(3):330-9;.Gitaitis RD. Refinement of Lyophilization Methodology for Storage of Large Numbers of Bacterial Strains. Plant Dis. 1987;July:615; Simon EM, et al. Maintaining Cultures for Biotechnology and Industry. Elsevier; 1996:133-159; Jörg Overmann. Principles of enrichment, isolation, cultivation, and preservation of prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, eds. The Prokaryotes. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013; Malik KA. J. Microbiol. Methods 12:117-124 (1990).

General Lyophilization Procedure:

Cells are grown to late log or early stationary phase under optimal conditions on the medium of choice, harvested by centrifugation and the culture broth removed. The pellet is then resuspended in an in equal volume of lyophilization medium. Cells are divided into aliquots and placed into sterile vials or tubes (approximately 250-500 µl representing ~ 10^8 bacteria). The tubes are then placed in the lyophilizer for a suitable time period to ensure full lyophilization of the sample. The samples are frozen down to -40°C.When using instruments that provide for control of rate of freezing, a drop of 1°C per minute is preferred. Once the samples reach temperature, they should be visibly frozen. The sample is then subjected to vacuum pressure for drying.

Primary drying is the longest phase of the freeze drying process. The time for primary drying will also depend upon the volume of the sample. For bacteria, samples rarely need to be large and typically are 0.25 to 0.5 ml. A limited number of samples (10-20) in a shelf dryer can be completely dried in just a couple of hours. A fully loaded dryer with several hundred samples will take longer. As a standard guide, freeze dry overnight.

Samples may still contain moisture following primary drying. The amount is variable but ranges between 2 and 4%. This moisture level can be reduced by pumping heat into the sample during the secondary drying phase. This phase is relatively short, lasting 1 to 2 hours, but important for long-term viability. With the vacuum in place, the vials are stoppered using the stoppering plate/mechanism. The vacuum is then released, the vials removed and the stoppers further secured with rubber bungs/stoppers with foil crimp seals. It is best to store the vials at 4°C in the dark.

Optionally, the freeze dried bacteria are tested for viability as compared to the original culture and can be monitored for stability/viability of the freeze dried cultures by testing at 30, 90, 180 and 365 days.

It should be noted that these steps can optimized if necessary to ensure maximum survival of the bacterial consortium. The resulting product will be then be transferred back to the anaerobic chamber for downstream processing. The lyophilized product will be weighed (to ensure 10^{10} CFU/capsule) and packaged in gelatin capsules (Capsugel, Morristown, NJ) under anaerobic conditions. These capsules will then be packaged in glass ampoules (Schott, Elmsford, NY) to maintain anaerobic conditions during shipment and storage.

Viability Testing.

Robustness of the product over time will be assessed using different configurations containing single and various factorial mixtures of excipients prepared via the same lyophilization, encapsulation, and packaging procedures. Products will then be stored in the laboratory on a shelf at room temperature and tested in triplicate for viability at 0, 30, 60, 180, and 360 days from the date of production. Oxygen content will be measured via phosphorescence of the oxygen nanoprobe on a bi-weekly basis (Albenberg L, Esipova T V, Judge CP, et al. Correlation Between Intraluminal Oxygen Gradient and Radial Partitioning of Intestinal Microbiota in Humans and Mice. Gastroenterology 2014). Validation will be performed by breaking the ampoule under aerobic conditions (as would be encountered when delivering the capsule to a subject in a medical setting) and then placing the gel capsule in pre-reduced culture medium (same medium as was used to grow the initial liquid cultures). Growth can be measured using optical density (OD_{600}) and colony counting (CFU/mL) at six hour time

points up to three days after inoculation. This will ensure viability of the consortium for delivery to a human subject via this method over a continuous five day dosage period.

Alternative Method Development. Concurrently with methods for production of gel capsules containing lyophilized cultures, we will also freeze large liquid volumes containing viable cells to be thawed and administered via colonoscopy, endoscopy, enteroscopy, enema or nasoduodenal tube. In brief, liquid cultures will be centrifuged, the pellets will be resuspended in a nutrient-rich broth(ATCC. ATCC ® Bacterial Culture Guide Tips and Techniques for Culturing Bacteria and Bacteriophages Table of Contents. Manassas, Virginia; :6. Available at: http://www.atcc.org/~/media/PDFs/Culture Guides/Previews/ATCC _Bacterial_Culture_Guide_Preview.ashx.)(containing 10% glucose and 10% skim milk as cryoprotectants(Hubálek Z. Protectants used in the cryopreservation of microorganisms. Cryobiology 2003;46(3):205-229. doi:10.1016/S0011-2240(03)00046-4; Moore LW, Carlson RV. Liqquid Nitrogen Storage of Phytopathogenic Bacteria. Phytopathology 1987;65:246-250), and 10 mM L-cysteine HCl as an oxygen scavenger), and stored at -80°C in sealed syringes within secondary containment in pre-measured doses. For administration to subjects, the samples will be thawed and the syringes will be attached directly to the delivery mechanism (i.e., enema or NG tube) to minimize oxygen contamination.

Exemplary regimen to reduce the concentration of gut bacteria prior to administration of microbial consortium in a patient in need thereof

Prior to initial administration of microbial consortium, it may be necessary to reduce the concentration of bacteria within the intestinal lumen. Prior research has demonstrated that a single antibiotic does not appreciably reduce total bacteria concentration within feces although some antibiotics (e.g. vancomycin) dramatically alter the composition of the gut microbiota (Vrieze J Hepatol 2014;60:824-31). Furthermore, not all combinations of orally administered antibiotics can reduce the concentration of bacteria within the gut lumen. For example, a combination of rifaximin 550mg orally twice daily plus metronidazole 500mg orally twice daily plus trimethoprim-sulfamethoxazole (160mg-800mg) orally twice daily for three days results in minimal change in total fecal bacterial concentration ($p>0.4$ paired t-test, $n=5$ human subjects).

Reduction in the concentration of bacteria within the gut lumen prior to the administration of microbial consortium is accomplished with a combination of antibiotics that are effective within the intestinal lumen, dietary modification and a bowel lavage to reduce the fecal biomass. During the first 3 days, adult patients will consume vancomycin 500mg orally every 6 hours and neomycin 1000mg orally every 6 hours. The dose for children must be reduced according to the child's weight (vancomycin 40 mg/kg/day in 4 divided doses, not to exceed 500 mg PO every 6 hours; neomycin 25 mg/kg/dose PO every 8 hours, not to exceed 1 gram PO every 8 hours). On the second day patients consume only a clear liquid diet followed by 2-4L of polyethylene glycol based bowel purgative (such as GoLytely®). Slight variation in the preparation regimen is possible, particularly with respect to the timing of administration of the polyethylene glycol based bowel purge. This can be administered on either the 2nd or the 3rd day of the regimen. Similarly, smaller volume polyethylene glycol bowel purgatives can be combined with oral laxatives such as bisacodyl.

Following the antibiotic and PEG bowel cleansing protocol, the microbial consortium will be inoculated either orally via a capsule, into the upper GI track via a nasoduodenal tube, endoscopy or enteroscopy or into the lower GI tract via a colonoscopy or enema. The inoculation will be a minimum of one day but may be repeated daily for a maximum of 7 days.

The preparation regimen is not required for chronic dosing of microbial consortium. However, following a lapse in therapy the bowel preparation regimen may be required prior to re-initiation of therapy.

Kits

The invention provides kits for promoting the expansion of a defined microbial consortia in the gut of a host. In particular embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic composition comprising the defined microbial consortia; such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments. The kit may also contain the gelatin capsules described above and instructions for methods of administration.

The kit preferably contains instructions that generally include information about the use of the composition for the expansion of the microbial consortia in the gut of the subject. The kit

further contains precautions; warnings; indications; counter-indications; overdosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook, 1989); "Oligonucleotide Synthesis" (Gait, 1984); "Animal Cell Culture" (Freshney, 1987); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1996); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Current Protocols in Molecular Biology" (Ausubel, 1987); "PCR: The Polymerase Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the microbial consortia, formulation methods, , screening assays, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: ASF is a distinctive gut microbial community that exhibited resiliency under SPF housing conditions.

ASF consists of 8 bacterial strains developed in the 1970s that are collectively known to induce immune tolerance, and are currently used commercially to colonize immune deficient mice to maintain health. To characterize potential donors containing the ASF community, 16S rRNA gene sequencing was performed on fecal DNA obtained from ASF colonized mice housed in an isolator. Although there was only a small reduction in the overall abundance of bacteria in

the ASF community relative to mice with conventional microbiota (Figure 1A), the ASF community could be visualized as a very distinctive community on a UniFrac-based PCoA analysis (Figure 1B). To determine the resiliency of the ASF in mice housed in SPF conditions, sequential fecal pellets were collected from ten mice for analysis by 16S rRNA sequencing (Figure 1C). Six taxa were resolved at the depth of sequencing performed with Parabacteroides being the dominant component of ASF accounting for roughly 90% of sequence reads at the time of transfer of mice from an isolator to SPF conditions. At 28 days, Parabacteroides remained the dominant taxon with the other 5 ASF members being present along with the appearance of "other" non-ASF taxa accounting for approximately 20-30% of reads indicating a moderate level of ASF resiliency to disruption by other gut bacteria.

Example 2: Preparation of mice with antibiotics and polyethylene glycol allows the transplantation of ASF into previously colonized mice.

Mixed results have been reported regarding the transfer of conventional microbiota between rodents with some sources reporting successful transfer with repetitive inoculation, while others report that the use of antibiotics prevents effective transfer. Reasoning that significant reduction of the endogenous conventional microbiota would be needed for effective transfer of ASF due to its reduced membership, studies were performed to determine the optimal timing of microbiota transfer based on the response of conventional microbiota to the oral delivery of two nonabsorbable antibiotics, neomycin and vancomycin. A maximal reduction of 16S rRNA copy number by approximately 4 logs was achieved within 72 hours of oral antibiotic initiation with the return of 16S rRNA copy number to baseline 5 days after discontinuing antibiotics (Figure 2A). Having defined a time window for the optimal introduction of ASF, fecal slurries of ASF obtained from ASF colonized mice (Taconic) were sequentially gavaged into conventionally housed recipients for seven days following a 72 hour treatment with oral antibiotics and a 24 hour intestinal purge using polyethene glycol (pretreated conventional). The results demonstrating the effectiveness of ASF transfer into these recipients and its resilience over the next 21 days, relative to conventionally housed mice without pretreatment and germ-free recipients, are shown in Figures 2B and 2C, where 16S copy number after transplantation has been restored to baseline levels after ASR transplantation (Figure 2B). While no transfer of ASF was observed in conventionally housed mice that did not receive the pretreatment, ASF

transfer was as effective in pretreated conventionally housed as germ-free recipients (Figure 2C). Interestingly, the ASF consortium was more resilient over 21 days in the pretreated conventional mice than in the germ-free recipients.

Example 3: Long-term taxonomic resilience of the transplanted ASF community established by the ability of *Parabacteroides* to inhibit invasion of *Bacteroides,* but not *Clostridia genera* into the gut microbiota niche.

Long-term taxonomic resiliency of the transplanted ASF community over four months evaluated by both unweighted Unifrac (presence-absence of taxa, Figure 3A) and weighted Unifrac (relative abundance, Figure 3A), revealed that the transplanted community rapidly established a new intermediate steady state in between the ASF inoculum and the conventional, pre-treatment community. The proportion of ASF sequence reads remained relatively stable after six weeks at approximately 40% in germ-free recipients and 50% in pretreatment conventional hosts both considerably greater than the 10% proportion of ASF community members normally observed in conventionally-housed mice (Figure 3B). Resilience of the ASF community was largely determined by the persistence of Paracteroides in both germ-free and pretreated conventional hosts where this taxon remained stable after about one month (Figure 4A). The temporal relationship between *Parabacteroides* and "others" was remarkably similar in both ASF colonized mice and pretreated conventional hosts showing that the new steady state of the ASF community in transplanted mice was due to the SPF environment in which the host resides and is not a manifestation of the transplantation procedure.

As is common in humans, the bacteria in the *Bacteroides* genus are the dominant taxa in conventionally housed mice. Upon transplantation of ASF into either germ-free or pretreated conventional hosts the ASF community persists, but specific organisms from the pretransplant community slowly increase in abundance (Figure 4B). Rather than being stochastic, the bacterial taxa capable of time-dependent displacement of the ASF community were specific taxa primarily from the *Clostridium* genus. Thus, *Parabacteroides* maintained its niche in the gut as the predominant representative of the *Bacteroidetes* phylum, but an equilibrium was established with the *Firmicutes* phylum primarily composed of the *Clostridium* genus.

Example 4: ASF has minimal urease gene representation and enzymatic activity.

The lack of a mammalian urease gene makes the hydrolysis of urea a microbiota-specific function whereby the subsequent production of ammonia is shared between the host and gut microbiota for utilization in protein synthesis. Ammonia is also largely responsible for the alkaline pH of the colonic luminal environment acting to buffer the short chain fatty acids produced by the microbiota fermentation of diet and host derived glycans. Genomic sequencing of the ASF community revealed minimal gene representation in the total community with no urease gene identified in the Parabacteroides genome (Figure 5A). To establish the lack of urease enzymatic activity as a functional characteristic of the ASF community that potential impact on both the gut microbiota and host metabolome, both *ex vivo* and *in vivo* urease activity assays were performed. Fecal urease activity (Figure 5B) was undetectable and ammonia levels were minimal (Figure 5C) in fecal pellets collected from mice colonized with ASF. Using intravenously delivered ^{13}C-urea to quantify urease activity *in vivo* through the production of $^{13}CO_2$ in a breath test, this directly demonstrated that urea delivered by the host undergoes minimal hydrolysis in ASF colonized mice (Figure 5D). Consistent with the long-term displacement of *Bacteroides* by *Parabacteroides* after transplantation, fecal urease activity remained dramatically reduced for at least four months in pretreated conventional transplanted mice demonstrating both the taxonomic and functional resilience of the ASF community (Figure 6).

Example 5: The ASF community and transplanted mice are both deficient in essential amino acids.

The question of whether the decreased urease activity and ammonia production of the ASF community would reduce the production of amino acids by the gut microbiota and their availability to the host was explored. The latter is particularly relevant in rodents where corprophagia has been shown to be important to maintain essential amino acid levels. Transplantation of ASF into pretreated conventional mice led to a significant reduction in fecal ammonia levels (Figure 7). A metabolomic analysis of fecal pellets prior to and after ASF transplantation revealed that ASF transplantation is not able to alter butyrate production in a durable fashion in contrast to ammonia (Figure 8). This result highlights the exclusive ability of

the invention herein to reprogram the metabolome of the gut microbiota by specifically the targeting nitrogen balance.

To further evaluate the effect of ASF transplantation on host nitrogen balance, plasma amino acid profiling was performed on both the portal and arterial circulation with the notion that the portal-systemic ratio of amino acid levels would be indicative of compensatory hepatic responses needed to maintain nitrogen homeostasis (Table 4).

In the portal circulation, the levels of nearly all essential amino acids were significantly reduced after ASF transplantation, the only exception being tryptophan which remained unchanged. This profile was nearly identical to that in the arterial circulation indicating a systemic deficiency of essential amino acid levels after ASF transplantation for which the liver was unable to compensate. By contrast, ASF transplantation led to a significant reduction of three nonessential amino acids in the portal circulation with only one, threonine, remaining significantly reduced in the arterial circulation. These results demonstrate that urease activity is critical to maintain host essential amino acid levels.

An alternative explanation for the beneficial effects of ASF transplantation in the treatment of hepatic encephalopathy would be the effects of this newly developed gut microbiota steady state on plasma amino acid levels. Several essential amino acids, those that cannot be synthesized de novo by the host and must be obtained through diet, have been hypothesized to play an important role in the pathogenesis of HE. Among the essential amino acids are the aromatic amino acids including phenylalanine, tyrosine (which is only conditionally essential as it is formed from phenylalanine), and tryptophan. These aromatic amino acids are precursors to biogenic amines that are neurotransmitters such as catecholamines and serotonin as well as false neurotransmitters including phenylethanolamine, octopamine, and synephrine. Normally aromatic amino acids are decarboxylated to their respective amines, which subsequently undergo additional metabolism in the liver by monoamine oxidase into aldehydes and ammonia. When hepatic function is impaired as in acute liver failure, the precursor aromatic amino acids and their amines are shunted away from the pathway of aldehyde formation and may enter the central nervous system to become locally B-hydroxylated into false neurotransmitters and replace normal neurotransmitters[20]. During hepatic failure, it is well known that plasma levels of aromatic amino acids (AAA) are increased whereas branch chain amino acids (BCAA) are decreased. Fischer et al. proposed that the ratio of BCAA to AAA (BCAA/AAA = (valine) +

(leucine) + (isoleucine) / (phenylalanine) + (tyrosine)) would be predictive of HE[21]. Indeed, numerous reports have verified that a decrease in this ratio is associated with hepatic dysfunction[22]. ASF colonization led to a remarkable alteration in plasma levels of essential amino acids with a significantly higher Fischer's Ratio (3.87) relative to mice with a conventional microbiota (2.68, $p<0.01$). In total, these results support the additional notion that ASF colonization may also protect against morbidity and mortality associated with liver failure by altering plasma essential amino acids and the subsequent production of false neurotransmitters.

Table 6. Concentrations (nmol/mL) of essential amino acids from sera in control and ASF FMT mice (n = 5 in each group, * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control).

TABLE 6	Portal Blood		Arterial Blood	
	Control Mean (± SD)	ASF Mean (± SD)	Control Mean (± SD)	ASF Mean (± SD)
THR	173.01 (± 33.87)	72.88 (± 31.52)**	169.43 (± 27.44)	84.49 (± 5.49)**
TRYP	81.44 (± 5.81)	80.17 (± 33.48)	58.13 (± 6.88)	59.28 (± 14.22)
METH	58.35 (± 10.55)	28.84 (± 12.12)**	57.32 (± 17.42)	30.63 (± 3.45)**
VAL	217.04 (± 51.15)	136.52 (± 60.12)*	189.31 (± 45.25)	135.56 (± 14.46)*
PHE	57.88 (± 10.55)	41.00 (± 17.75)*	56.96 (± 17.67)	44.30 (± 2.89)
ILE	143.39 (± 35.79)	62.36 (± 27.54)**	118.76 (± 32.63)	59.64 (± 6.97)**
LEU	186.46 (± 50.75)	110.01 (± 49.52)*	159.55 (± 47.15)	108.21 (± 13.48)*
ORN	83.05 (± 17.03)	47.01 (± 25.14)*	162.16 (± 14.37)	89.12 (± 7.29)**
LYS	201.82 (± 29.65)	105.16 (± 48.92)**	206.66 (± 50.26)	120.57 (± 21.52)**
TYR	140.02 (± 29.99)	36.10 (± 15.51)**	116.46 (± 21.19)	34.30 (± 4.84)**
TABLE 4	Portal Blood		Arterial Blood	
	Control Mean (± SD)	ASF Mean (± SD)	Control Mean (± SD)	ASF Mean (± SD)
THR	173.01 (± 33.87)	72.88 (± 31.52)**	169.43 (± 27.44)	84.49 (± 5.49)**
TRYP	81.44 (± 5.81)	80.17 (± 33.48)	58.13 (± 6.88)	59.28 (± 14.22)
METH	58.35 (± 10.55)	28.84 (± 12.12)**	57.32 (± 17.42)	30.63 (± 3.45)**
VAL	217.04 (± 51.15)	136.52 (± 60.12)*	189.31 (± 45.25)	135.56 (± 14.46)*
PHE	57.88 (± 10.55)	41.00 (± 17.75)*	56.96 (± 17.67)	44.30 (± 2.89)
ILE	143.39 (± 35.79)	62.36 (± 27.54)**	118.76 (± 32.63)	59.64 (± 6.97)**
LEU	186.46 (± 50.75)	110.01 (± 49.52)*	159.55 (± 47.15)	108.21 (± 13.48)*
ORN	83.05 (± 17.03)	47.01 (± 25.14)*	162.16 (± 14.37)	89.12 (± 7.29)**
LYS	201.82 (± 29.65)	105.16 (± 48.92)**	206.66 (± 50.26)	120.57 (± 21.52)**
TYR	140.02 (± 29.99)	36.10 (± 15.51)**	116.46 (± 21.19)	34.30 (± 4.84)**

Example 6: ASF transplantation reduced cognitive impairment and mortality in a murine model of acute liver injury.

A major cause of morbidity and mortality associated with acute liver injury is the development of hepatic encephalopathy (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)(23) treatment. Prepared ASF-transplanted mice showed both a reduction in fecal ammonia levels ((Figure 9a)) and reduced mortality in response to high dose TAA compared to mice with conventional microbiota (Figure 10a)).

In mice, the TAA model has also been associated neurobehavioral abnormalities resembling hepatic encephalopathy in humans(24). Using a lower dose of TAA to reduce mortality, we analyzed memory and spatial learning in a Y-maze test comparing ASF transplanted mice to mice transplanted with normal feces (NF) as a control(25). The survival rates were similar between the two groups at 80-90% at the lower TAA dose ((Fig.9b)). Fecal ammonia levels were reduced in mice transplanted with ASF compared to NF ((Fig. 9c)). TAA treated mice transplanted with NF showed a decrease in cognitive function, quantified as spontaneous alternations in a Y-maze test, whereas ASF treated mice were not different from untreated controls ((Fig. 10b)). A possible alternative could be that ASF transplantation directly reduced liver injury. We tested this by monitoring plasma alanine aminotransferase (ALT) and quantifying histologic evidence of hepatocyte necrosis ((Fig. 11a and Fig. 11b). Both revealed that liver damage induced by TAA was not reduced by either ASF transplantation or ABX treatment. Thus improved survival and behavioral performance were associated with reduced ammonia levels and not reduced liver injury.

The success of FMT in the treatment of Clostridial difficile infection supports the concept of a resilient microbial community that, when transferred into a host with disease, can be therapeutic by altering a dysbiotic microbiota. However, since feces contains not only bacteria but also a multitude of archaeal, fungi, and viruses that have been poorly characterized and can change in ways that cannot be predicted, there is significant concern for both short- and long-term risks of FMT in humans. Although it is reasonable to perform FMT for refractory *Clostridial difficile* infections, there are obvious advantages to ultimately developing defined microbial consortia with defined biological properties that respond to the gut environment in

predictable ways to prevent the transfer of infectious pathogens and reduce long-term risks. Using ASF as a well-characterized, innocuous microbial community with beneficial immunologic properties provided evidence of efficient transfer into previously a colonized host and model both its taxonomic and functional resiliency over time with the host in a SPF environment. Importantly, the highly distinctive clustering of the eight ASF organisms as a consortium relative to the conventional mouse microbiota, allowed us to track the effectiveness of ASF transfer as well as resiliency over time with a level of precision.

The composition of ASF has been recently described with greater precision based on 16S rRNA gene phylotyping. The ASF community remains relatively stable with Parabacteroides remaining the dominant taxon over one month in SPF house mice.

Although transfer of an entire gut microbiota can be achieved through repetitive inoculation of a previously colonized host without pre-treatment, and another study reports the deleterious effect of antibiotics in the transfer of the gut microbiota, reduction of the endogenous gut microbiota is preferably reduced by oral antibiotic treatment with polyethylene glycol (PEG) for efficient transfer of a defined community of only eight organisms. The transfer of ASF into conventionally housed mice (pre-treated conventional) was as effective as into germ-free recipients. This mirrors the results of FMT in humans where the use of antibiotics and PEG is used, especially where human feces has been delivered into the upper gastro-intestinal (GI) tract via a nasoduodenal tube. The succession of ASF engraftment can be evaluated by the examination of the early time points during and after the inoculation of ASF. The lactobacilli genus is present in most mice prior to transfer but a number of the low abundance ASF taxa, *Mucispirilum*, *Oscilospria*, and *Lachnospariceae*, appear with repetitive gavage in both germ free and pre-treated conventional hosts. The last to appear is the Clostridial genus at day 14 in the latter hosts, one week after gavaging was complete perhaps indicating the need for the establishment of a specific niche that permits the establishment of more fastidious taxa.

By monitoring the composition of the transplanted ASF community over four months by 16S rRNA gene sequencing, its longitudinal resiliency to the environmental stress of the host being hosted in a SPF environment was assessed. In microbial ecology terms, resilience can be defined as the amount of stress or pertubation that a system can tolerate before its trajectory changes towards a different equilibrium. The use of antibiotics in the pre-treated conventional hosts is an extreme example of an environmental stress that not only disrupts the relative

abundance of taxa in the gut microbiota as previously described, but also dramatically reduces the total abundance of bacteria by at least four logs thus permitting the engraftment of the ASF community. Housing of the host in an SPF environment is much less extreme but, nevertheless, has an impact on the resilience of the implanted ASF community. Although able to engraft into both germ-free and pre-treated conventional hosts, short-term resiliency of the pure ASF community is limited as "other" taxa begin to appear within four weeks of inoculation even as some low abundance ASF members, like Clostridia, begin to appear. This lead to rapid establishment of a new steady state, whereby both types of transplanted hosts resemble the gut microbiota of ASF colonized mice housed long-term in an SPF environment largely driven by the ability of Parabacteroides to remain a dominant taxon. Possibly due to the well-described inflammatory response associated with the maturation of the mucosal immune system upon colonization, ASF is least resilient long-term when inoculated into a germ-free host.

Species rich communities tend to exhibit enhanced resiliency that are less prone to invasion by new species possibly because limiting resources are used more efficiently with different species specialized to each potentially limiting resource (Yatsunenko T et al., 2012, Nature, 486.7402: 222-227). In part, this may be due to the functional redundancy of a rich microbial community whereby multiple rare species are capable to filling a niche when an abundant species is compromised by an environmental disturbance (Yatsunenko T et al., 2012, Nature, 486.7402: 222-227). The remarkable genomic similarity of the gut microbiome amongst different individuals despite significant taxonomic inter-subject variability supports this notion (Ridaura VK et al., 2013, Science 6;341(6150)). A close examination of the bacterial taxa are excluded by ASF compared to those capable of invading the gut niche over time provide two observations supportive of these concepts. First, bacterial taxa of the *Bacteroides* genus, dominant taxa in the human gut microbiota (Koren O et al., 2013, PLoS Comput Biol. 9(1):e1002863) and conventionally-housed mice, were largely excluded from the gut microbiota in ASF colonized mice suggesting a remarkable ability of *Parabacteroides* to exclude *Bacteroides* within the gut niche. The high level of taxonomic and functional similarity between these two genera is reminiscent of the "trade-off" between *Bacteroides* and *Prevotella* in humans that co-exclude each other within the gut niche (Faust K et al., 2012, PLoS Comput Biol 8(7): e1002606) and form the basis of previously described "enterotypes" (Koren O et al., 2013, PLoS Comput Biol. 9(1):e1002863, and Wu GD, et al., 2011, Science 334(6052):105-8). The

biological basis by which *Bacteroides* and *Parabacteroides* may co-exclude each other is unknown but may be due to competition for limiting resources and/or the production of bacteroicins.

Second, rather than being stochastic, the bacterial taxa that invade the gut niche of ASF colonized mice are time dependent, consistent between all three models, and almost exclusively involve the *Clostridium* genus collectively reducing the proportional abundance of *Parabacteroides* by about 50%. The relative proportion of *Parabacteroides* and *Clostridial* genera, representatives of the *Bacteroidetes* and *Firmicutes* phyla, establish a new steady state after about one month approximating the composition of the conventional microbiota in both humans and mice where about 90% of the bacteria taxa reside within these two phyla. Rather than being considered a disruption of the ASF community, the rise in *Clostridia* may be due to a syntrophic synergism between these two genera that increases the richness of the gut microbiota and helps to both stabilize and enhance resiliency of the ASF community long-term as observed over the course of a four months study. The impact of the transplanted ASF community on host metabolism was also evaluated. The gut microbiota and its host co-existed in a state of mutualism that, in part, involves interactions involving nutrition and metabolism. The gut microbiota, whose collective genome exceeded its mammalian host by about 150-fold, exhibited functionalities that serve as an example of collective "superorganism". One example would be the robust representation of glycan degrading genes in the microbiome responsible for the fermentation of complex carbohydrates with the production of short chain fatty acids, compounds that have important functions in host immunity and metabolism. Similarly, these results demonstrated a syntrophic co-metabolic interaction between a mammalian host and its microbiota to maintain nitrogen balance dependent upon the microbial urease gene, a functionality not present in the mammalian genome. A waste product of the mammalian host, urea is delivered to the colon where the gut microbiota scavenges nitrogen by hydrolyzing urea into carbon dioxide and ammonia, the latter of which is utilized by the gut microbiota for protein synthesis, reabsorbed by the host, or excreted in the feces.

Although the recirculation of ammonia via the portal circulation might be one mechanism by which the host may be able to preserve nitrogen stores, these data suggested that an alternative pathway may be more important. Indeed, in the absence of portally delivered ammonia from the gut microbiota, a disproportional reduction in portal glutamine levels would

have been anticipated. The significant decrease of nearly all essential amino acids in both the portal and arterial circulation of mice transplanted with ASF is consistent with the consumption of a diet deficient in essential amino acids rather than a reduction in the delivery of ammonia through the portal circulation for protein synthesis in the liver. In addition to urease genes, the genome of the microbiota also permitted the synthesis of essential amino acids using ammonia as an essential substrate. Subsequent corprophagia is a well-established method by which rodents are able to obtain essential nutrients such as essential amino acids where ^{15}N isotope studies suggested that one quarter of leucine consumption occurs through corprophagia in rabbits.

Despite the limited role that the delivery of ammonia via the portal circulation may play in maintaining host nitrogen balance, it is of considerable consequence to patients with chronic liver disease and/or in born errors of metabolism who are predisposed to the development of hyperammonemia. In diseases such as cirrhosis or ornithine transcarbamylase deficiency the inability of the liver to utilize ammonia delivered by the portal circulation for amino acid and/or urea synthesis results in elevated systemic circulation of ammonia with development of neurotoxicity manifested clinically as hepatic encephalopathy. The use of antibiotics to reduce the production of ammonia by the gut microbiota is one of the modalities utilized for the treatment of this disorder. Given both the taxonomic and functional resilience of the transplanted ASF community with minimal production of ammonia one year after inoculation into previously colonized hosts, we propose that a humanized version of ASF will have utility in the treatment of these disorders.

Example 7: A single bacterial species lacking a urease gene can significantly reduce fecal ammonia levels when inoculated into a properly prepared host.

A murine commensal E. coli strain (MP1) tagged with GFP known to colonize mice (Lasaro, M., et al. J. Bacteriol. 196:1723-32 (2014)that lacks a urease gene and shows no urease activity, was inoculated by daily oral gavage for 5 days (1×10^9 cfu) into mice pretreated with vancomycin, neomycin, and PEG. Colony counts revealed long-term colonization of this bacterium at a level of approximately 1×10^6 cfu/gram of feces. Quantification of fecal ammonia levels revealed that fecal ammonia was reduced significantly by approximately 6-fold after 50 days. By contrast, the MP1 strain genetically engineered to express urease did not reduce fecal ammonia levels at 50 days post-inoculation. Therefore, a single bacterial species

lacking urease activity is sufficient to induce a long-term reduction in fecal ammonia levels when inoculated into a properly prepared host.

Table 7A

Previously characterized pro-biotic strains

Strain
Lactobacillus acidophilus
L. acidophilus
L. acidophilus
L. acidophilus
Lactobacillus casei
L. casei Immunitas
Lactobacillus fermentum
Lactobacillus johnsonii
Lactobacillus paracasei
Lactobacillus plantarum
Lactobacillus reuteri
Lactobacillus rhamnosus
Lactobacillus salivarius
Lactobacillus lactis
Bifidobacterium lactis
Bifidobacterium longum
B. longum
Bifidobactenium breve

Table 7B

Probiotic strains	Urease content	Genomes queried	
E. coli Nissle	no		1
Bifidobacterium breve	no		1
Bifidobacterium longum	no		3
Bifidobacterium infantis	yes	1 out of 2	
Lactobacillus acidophilus	no		1
Lactobacillus plantarum	no		3
Lactobacillus paracasei	no		1
Lactobacillus dubrueckii bulgaricus	no		2
Streptococcus	yes	3 out of 3	

thermopiles		
Lactobacillus paracasei	no	2
Lactobacillus reuteri	no	2
Lactobacillus casei	no	2
Lactobacillus rhamnosus	no	2

Above is a list of the urease gene content for the indicated strains. Those lacking urease genes have utility in the compositions and methods of the present invention, used alone or in combination with other suitable strains identified herein. Strains containing urease genes are excluded from use.

Example 8: Procedure for designing human bacterial communities for treating hyperammonemia and other forms of dysbiosis.

While the altered gut micriobiota described herein is useful for reducing urease production from the gut, additional novel defined microbial consortia can be obtained using the following exemplary steps. Stool is collected from adults in robust health who meet the following criteria and none of the following.

a) Consumption of prebiotics or probiotics in the 4 weeks prior to sample collection. This will include products specifically marketed as prebiotics or probiotics, including yogurt with live bacteria.
b) Diagnosis with inflammatory bowel disease, celiac disease, or other chronic intestinal disorders or any other chronic medical conditions.
c) Baseline bowel frequency less than every 2 days or greater than 3 times daily. Normal bowel frequency is every 3rd day to 3 times per day.[15-18] Although unknown, stool frequency could be related to the microbiome composition.[19-21]
d) Prior bowel resection surgery. It is unknown how prior bowel resection surgery may influence the microbiome composition, hence we will exclude these subjects.
e) Use of antibiotics in the prior 6 months. A small proportion of bacteria may require 6 months to recover after treatment with antibiotics.[22]

f) Use of antacids or NSAIDs in the preceding 2 weeks. NSAIDs have been associated with C. difficile colitis, although whether this is causative and whether this is mediated through changing the fecal microbiota composition is unknown.[23] Antacids could potentially alter the gut microbiota by changing the acid milieu or by altering fecal transit time.

g) Use of any of the following medications in the 4 weeks prior to randomization: proton pump inhibitors (PPI), histamine receptor antagonists, narcotics, tricyclic antidepressants, anticholinergics (e.g., hyoscamine), metoclopramide, anti-diarrhea medications, laxatives. PPIs and other acid suppression medications are thought to potentially alter the intestinal microbiota.[23-26] Antacids could potentially alter the gut microbiota by changing the acid milieu or by altering fecal transit time. Other medications (such as narcotics) that could alter fecal transit time will be prohibited. Within 2-4 weeks of discontinuing these medications, the composition of the gut microbiota is expected to return to normal.[27, 28]

h) HIV infection, AIDS, or other known conditions resulting in immunosuppression.

i) Participant has experienced diarrhea within the two weeks prior to the stool collection. Diarrhea is defined as a change in bowel habits with an increased frequency or loose stools such that the stool could not be lifted with a fork.

j) Conditions or medications that interfere with the potential participant's completion of the study protocol or that may negatively alter the potential participant's samples.

Once obtained, bacterial strains are purified by repeated rounds of streaking and limiting dilution under anaerobic conditions on multiple types of media. They are then tested for survival at after freezing at -80°C and subsequent thawing. After phylogenetically characterizing the isolated strains by sequencing of 16S rRNA gene sequences, information on Genus and species attribution is used to ascertain the probability of containing urease genes by comparison to databases of complete bacterial genome sequences. Suitable strains are then formulated into communities with structures resembling resilient human communities from healthy individuals (containing multiple Firmicutes, Bacteriodetes, and other taxa), but using only strains identified as lacking urease genes. Human strains are also evaluated by comparison to murine ASF. Specifically, because Parabacteriodes was predominant in ASF in colon, this approach will

ensure that members of the Parabacteriodes/Bacteriodes group are represented in engineered communties.

Following identification of suitable consortia, these are tested in mice for ability to persist in a suitably prepared animal. The newly identified consortia can then be assess for alteration of the disease parameters targeted for alleviation. For example, in the present case, the consortia are tested for ability to lower fecal ammonia level and/or in rescue in models of ammonia toxicity.

Selected bacterial strains are characterized by near-complete genome sequencing and the sequences are assessed for the presence or absence certain types of genes, e.g., urease genes, antibiotic resistance genes, and or virulence gene representation. Once satisfied that the strains meet these criteria, strains can be tested functionally for ureases activity and/or for antibiotic resistance to identify antibiotic classes to which all strains are sensitive. Should any adverse gene content be identified, the consortia will be edited to remove any members with adverse gene content or activities as set forth above, then the final community retested.

Using the steps outlined above, we have identified a seven member community that passes these criteria and includes:
Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii, and Bacteroides vulgatus.

Accordingly, this community can be used to advantage in human subjects in the methods of treatment described herein.

Using the methodology described herein to characterize isolated strains, it is clear that any bacteria lacking urease, antibiotic resistance genes and virulence genes is a candidate for use in the compositions and methods described herein.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed

elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

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What is claimed is:

1. A composition comprising a consortium of bacteria having no urease gene content or urease activity, for use in treating hyperammonemia, hepatic encephalopathy, or another liver disorder characterized by hyperammonemia in a subject in need thereof, wherein:

a) prior to use of the composition, the subject has received an effective amount of one or more antibiotics sufficient to reduce the population of bacteria in the gut microbiota by at least 2-4 logs, when compared to the population of bacteria in the gut microbiota prior to receipt of the one or more antibiotics;

b) prior to use of the composition, the subject has received an effective amount of a purgative agent, and

c) use of the composition of the consortium of bacteria having no urease gene content or activity, under conditions wherein said bacteria colonize the gut, and wherein said consortium of bacteria comprises *Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii str.,* and *Bacteroides vulgates* bacteria.

2. The composition for use according to claim 1, wherein said purgative agent is selected from the group consisting of polyethene glycol (PEG), magnesium citrate, sodium picosulphate plus magnesium citrate, PEG plus ascorbic acid, and sodium phosphate.

3. The composition for use according to claim 1, wherein said use of the composition is via a route selected from the group consisting of endoscopy, enteroscopy, colonoscopy, a nasoduodenal catheter, an enema and orally in a consumable capsule or pill.

4. The composition for use according to any one of claims 1 to 3, wherein in step a) the one or more antibiotics is for use for at least 48, 72, or 96 hours prior to the use of the composition; and wherein the antibiotic is neomycin and vancomycin.

5. The composition for use according to any one of claims 1 to 4, wherein in step b) the purgative agent is for use at least 12-36 hours prior to the use of the composition.

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6. The composition for use according to any one of claims 1 to 5, wherein the consortium of bacteria further comprises one or more bacteria belonging to a genus selected from the group consisting of Bifidobacterium, Bacteroides, Tannerella, Parabacteroides, Bacillus, Lactobacillus, Anaerostipes, Blautia, Coprococcus, Dorea, Clostridium XI, Collinsella, and Paraprevotella; or wherein the consortium consists of Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii str., and Bacteroides vulgates; or wherein the consortium further comprises Clostridium sp. Lactobacillus sp., Lactobacillus murinus, Mucispirillum schaedleri, Eubacterium plexicaudatum Firmicutes bacterium, Clostridium sp. and Parabacteroides sp.

7. The composition for use according to any one of claims 1 to 6, wherein the hyperammonemia, hepatic encephalopathy, or another liver disorder characterized by hyperammonemia is a metabolic disorder selected from the group consisting of Hyperammonemia, Transient Hyperammonemia of the Newborn, and hepatic encephalopathy.

8. The composition for use according to any one of claims 1 to 7 further comprising monitoring the treatment efficacy by assessing the subject for alterations of at least one hyperammonemia parameter selected from the group consisting of 16S rRNA copy number, l6S rRNA gene sequencing, fecal urease levels; fecal or circulating amino acids, biogenic amines, fecal ammonia levels, and circulating ammonia levels.

9. A composition comprising a consortium of bacteria having no urease gene content or activity, said consortium of bacteria comprising *Paraprevotella clara, Bifidobacterium longum, Collinsella aerofaciens, Coprococcus comes, Dorea longicatena, Bacteroides eggerthii str.,* and *Bacteroides vulgates* bacteria, and being present in a biological carrier suitable for administration to, and colonization, of the gut.

10. The composition of claim 9, wherein the consortium of bacteria comprises at least one further bacterium having no urease gene content or activity selected from Bifidobacterium, Bacteroides, Tannerella, Parabacteroides, Bacillus, Lactobacillus, Anaerostipes, Blautia,

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Coprococcus, Dorea, and Clostridium XI.

11. The composition for use according to claim 1, wherein said composition comprises the bacteria species diluted in a biologically-compatible solution suitable for administration to said subject.

12. The composition for use according to claim 11, wherein said biologically-compatible solution contains excipients.

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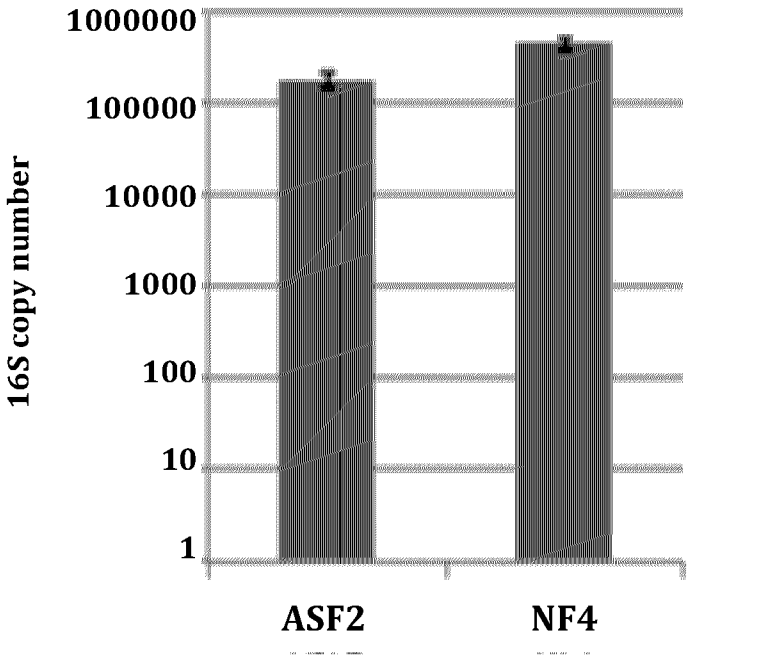

Figure 1A

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Clustering ASF 16S rRNA gene tags

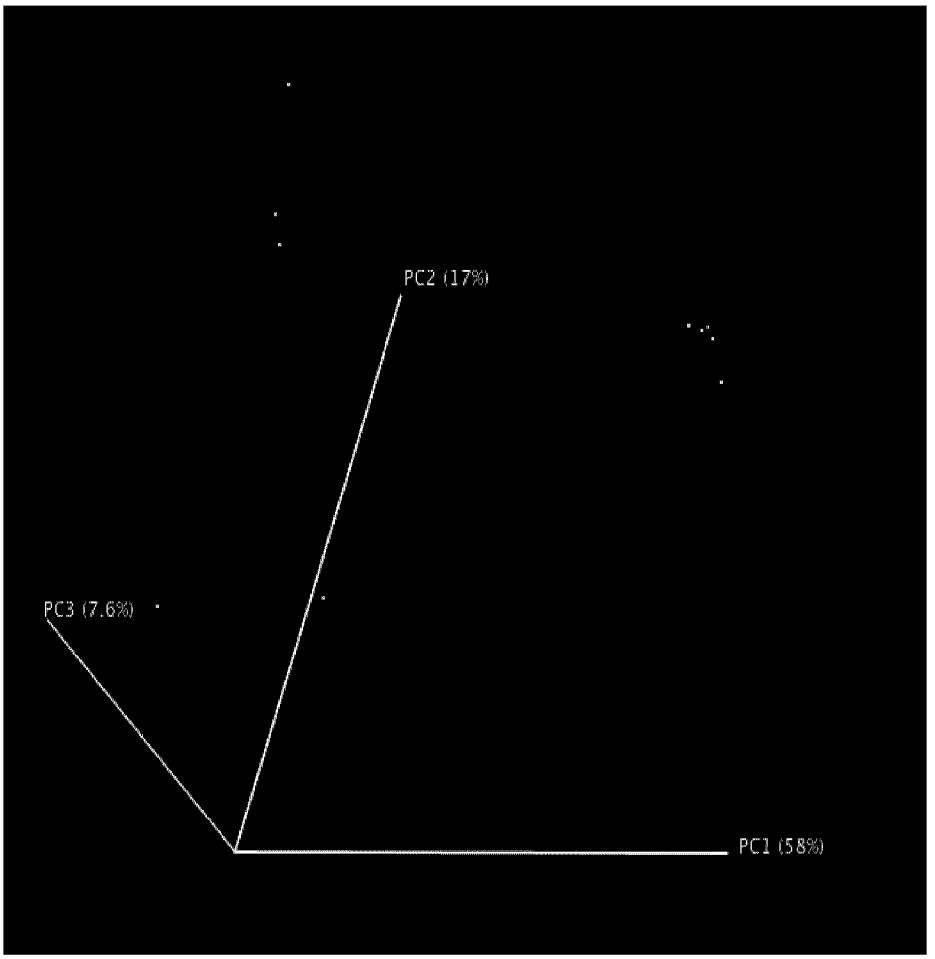

Figure 1B

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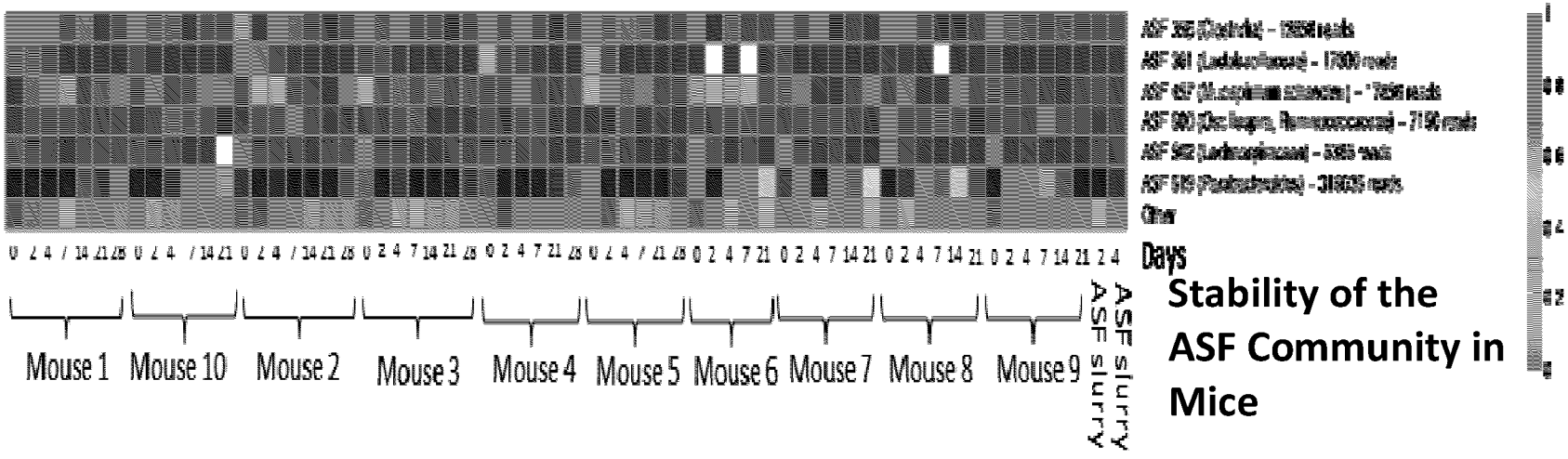

Figure 1C

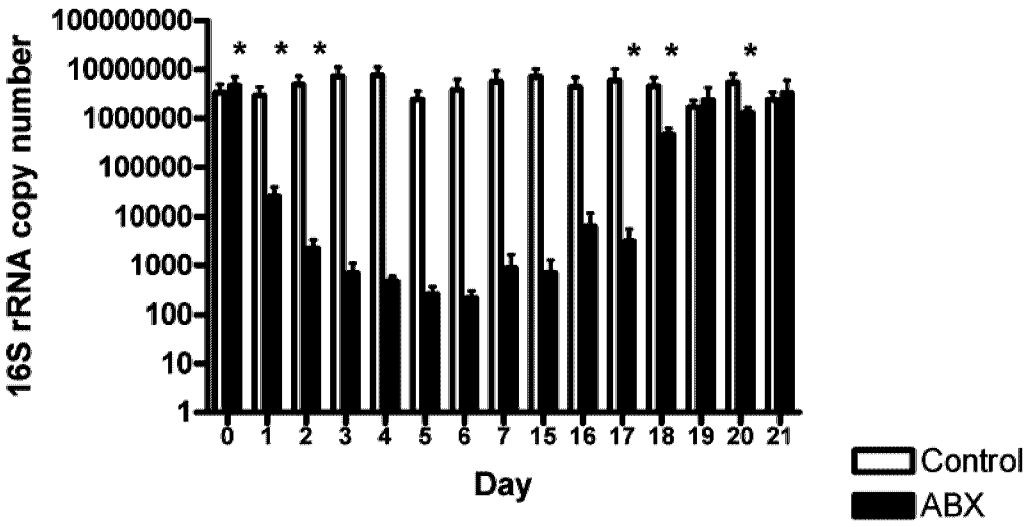

Figure 2A

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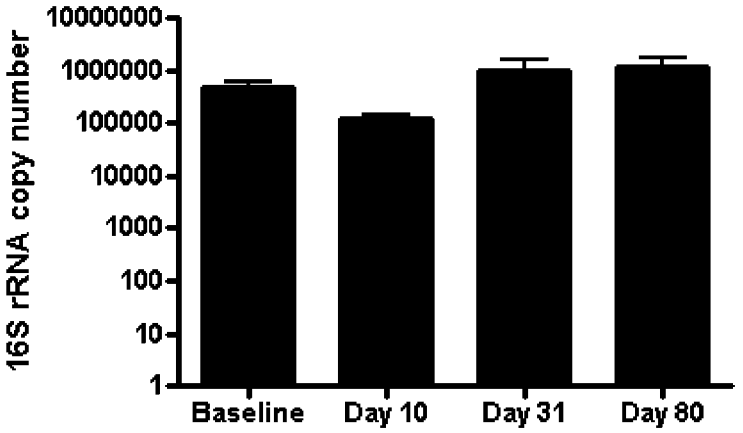

Figure 2B

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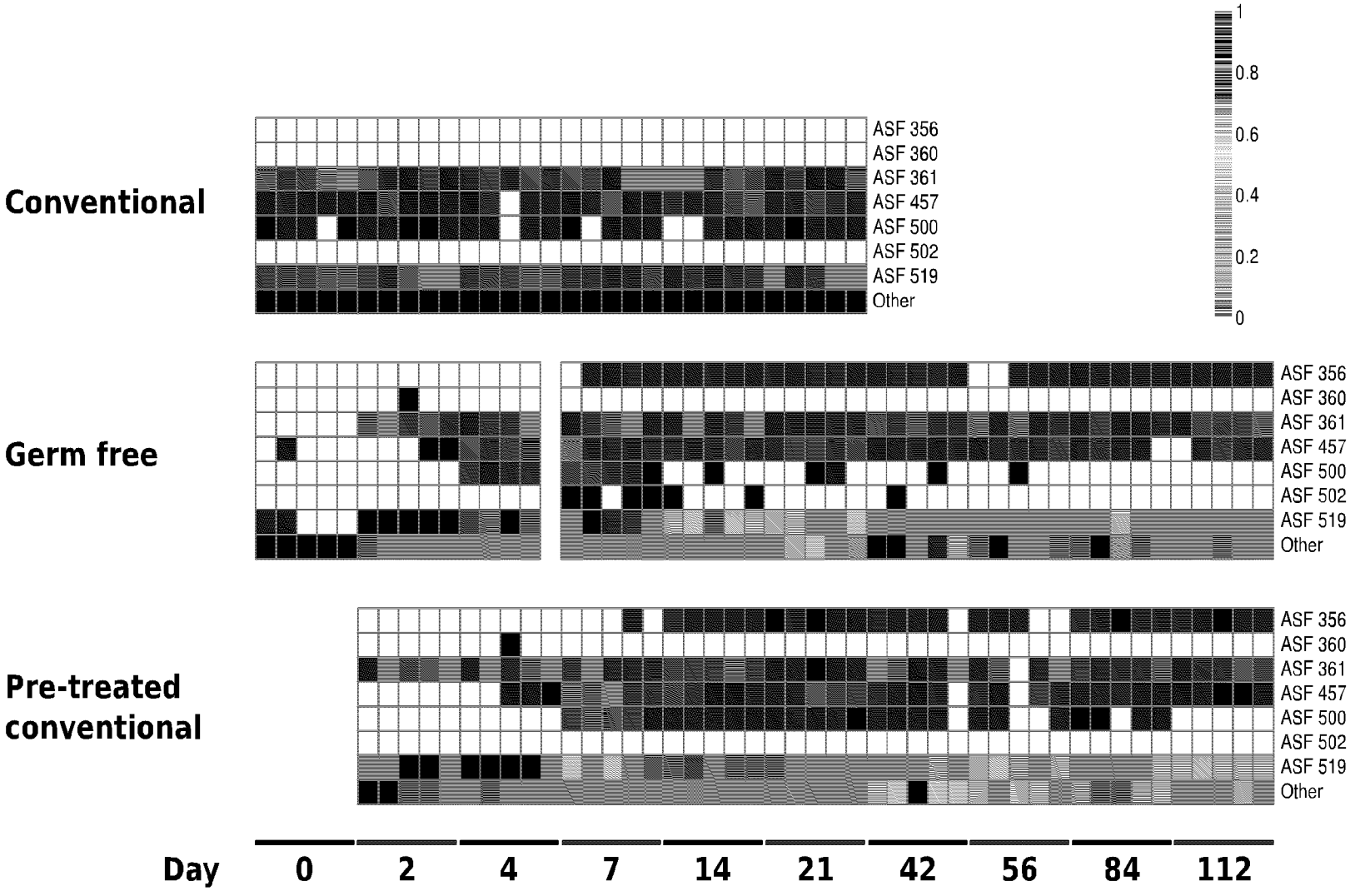

Figure 2C

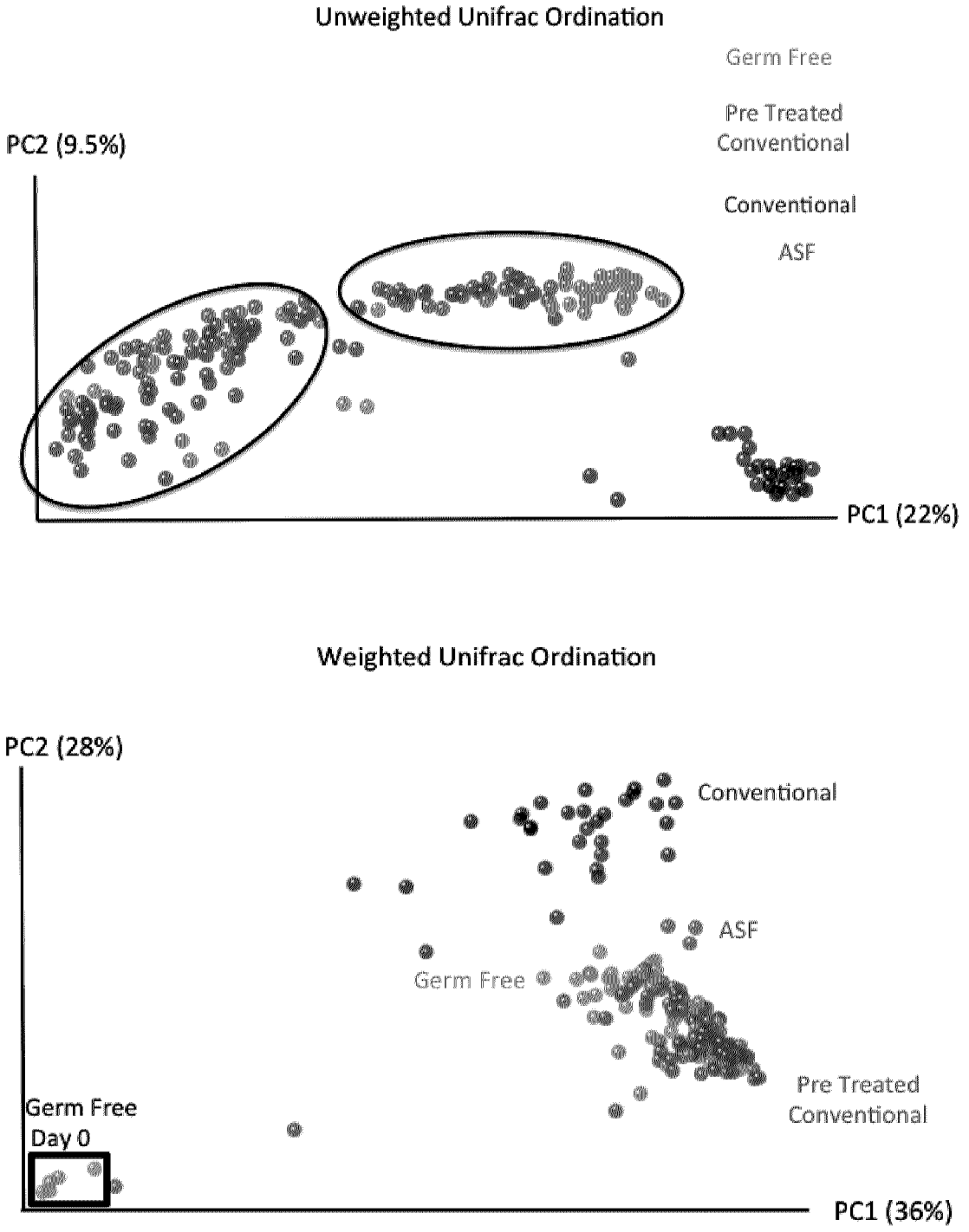

Figure 3A

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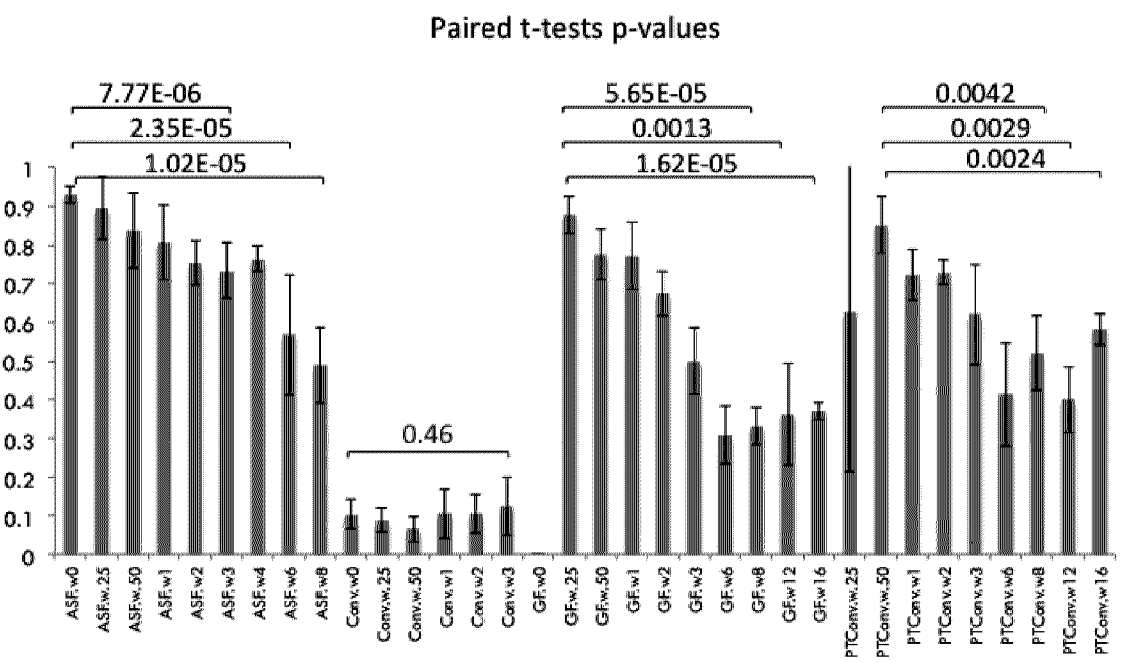

Figure 3B

Date Reçue/Date Received 2021-03-04

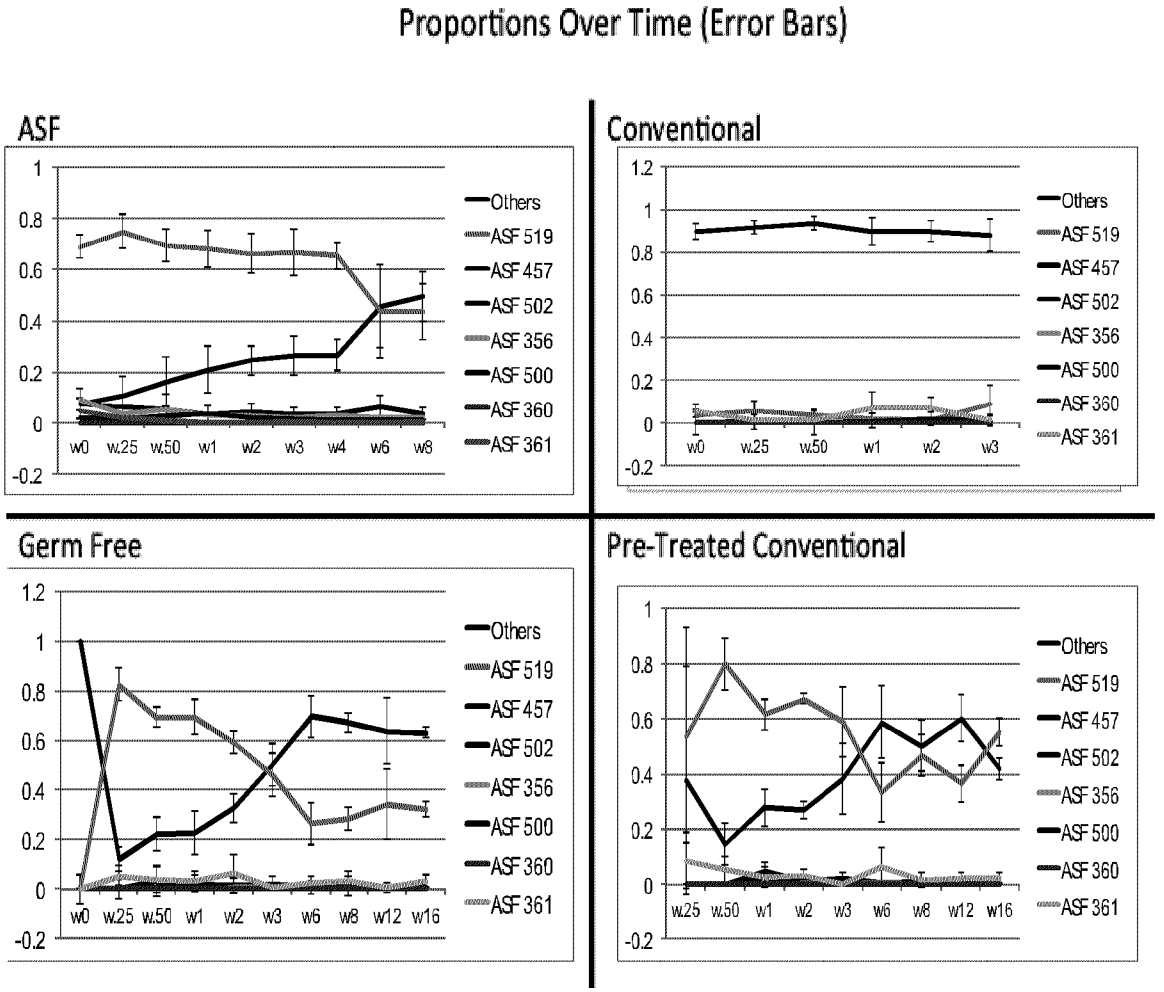

Figure 4A

Date Reçue/Date Received 2021-03-04

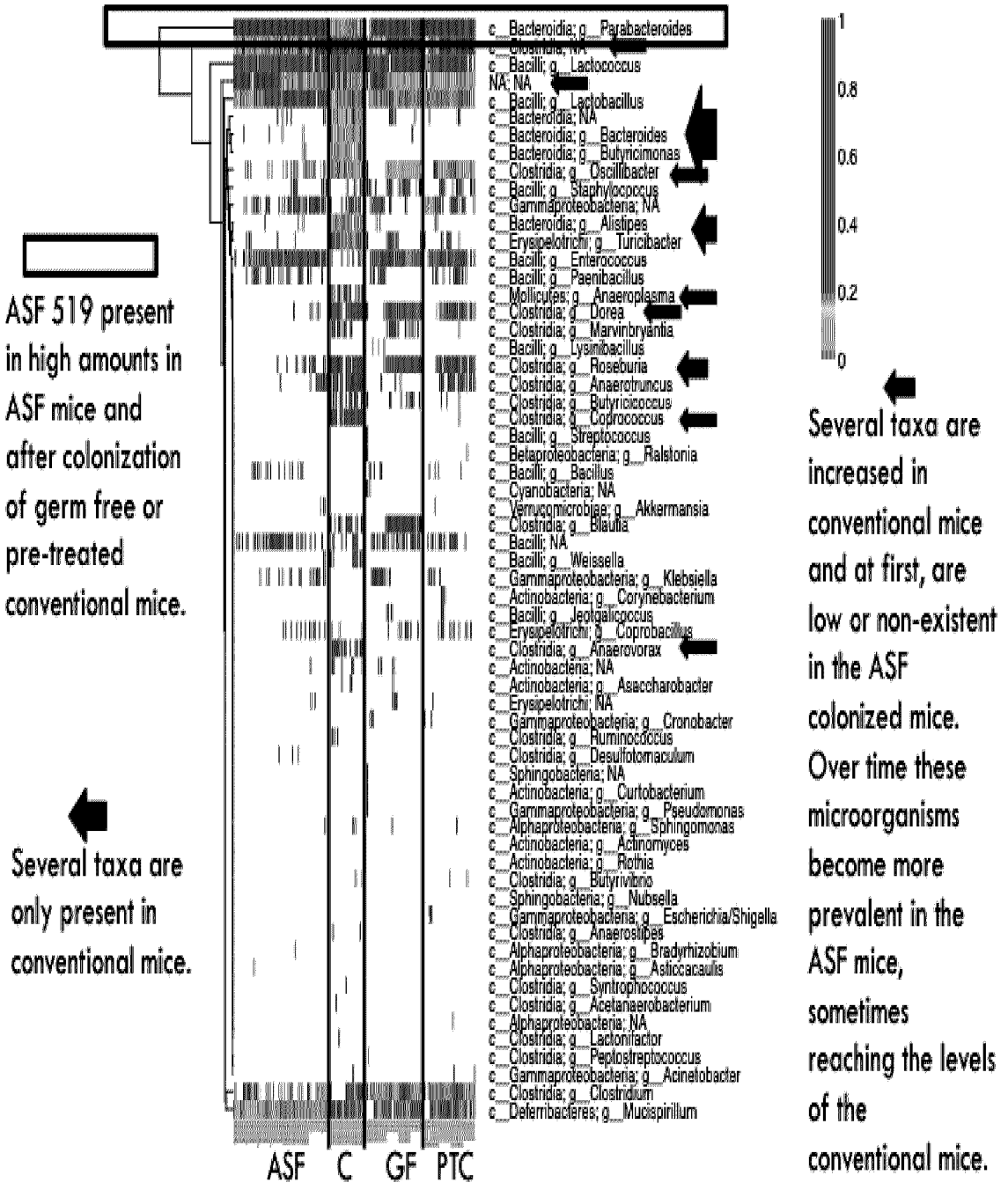

Figure 4B

Date Reçue/Date Received 2021-03-04

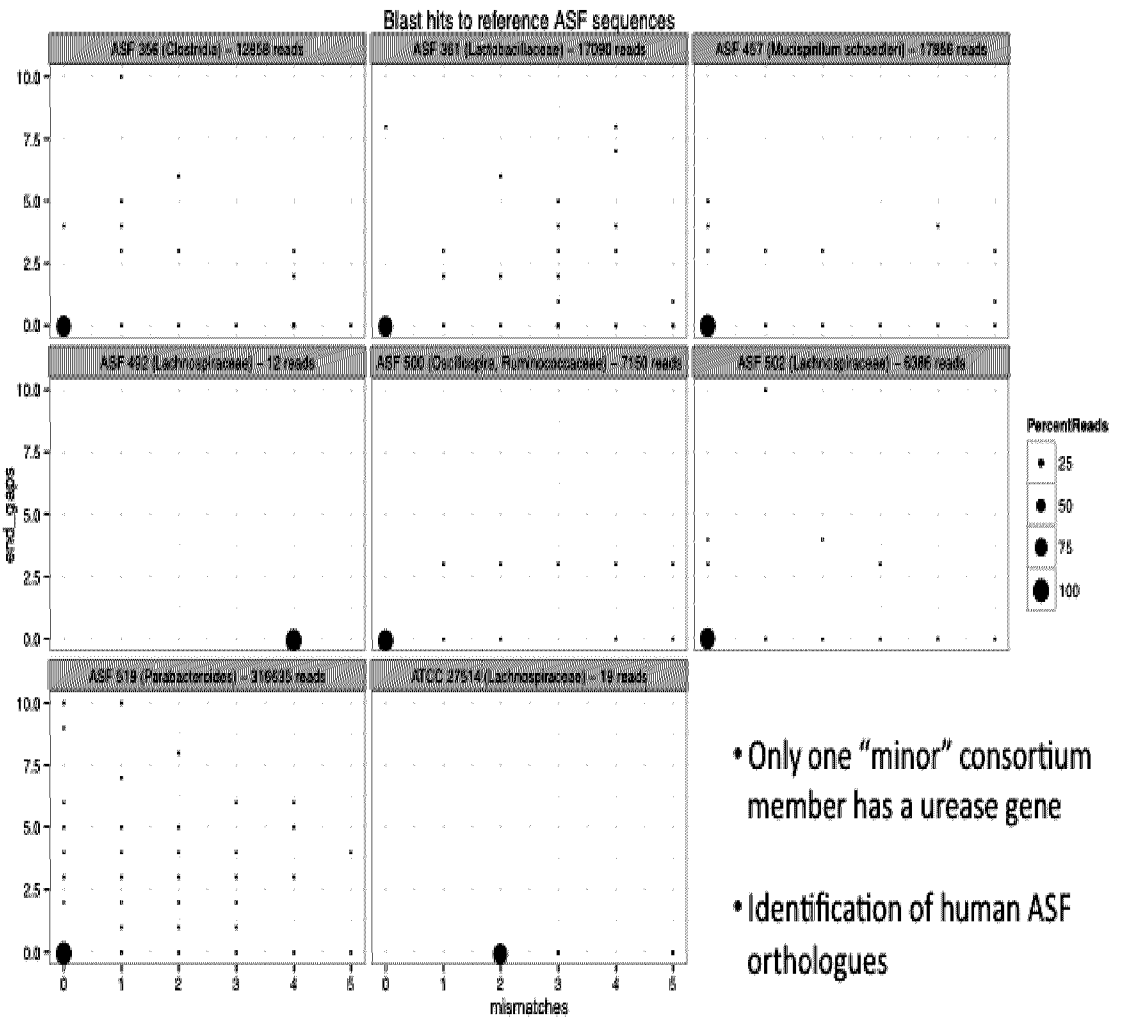

Figure 5A

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Assessing Urease Function of the ASF Community

$$^{13\text{-}14}\text{C-Urea} + H_2O \xrightarrow[\text{gut bacteria}]{\text{urease}} {}^{13\text{-}14}CO_2 + 2NH_3$$

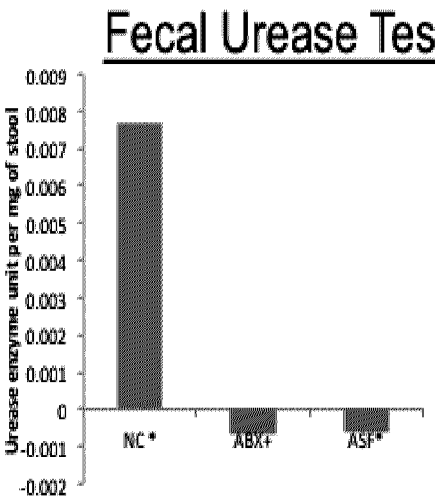

Figure 5B

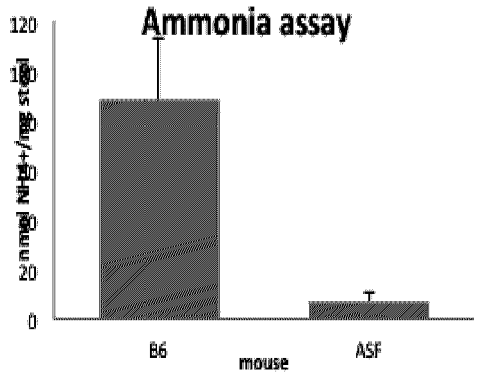

Figure 5C

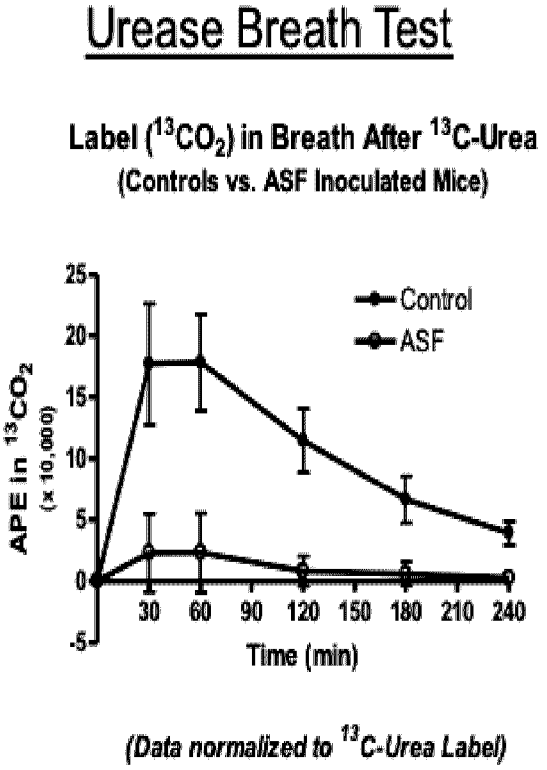

Figure 5D

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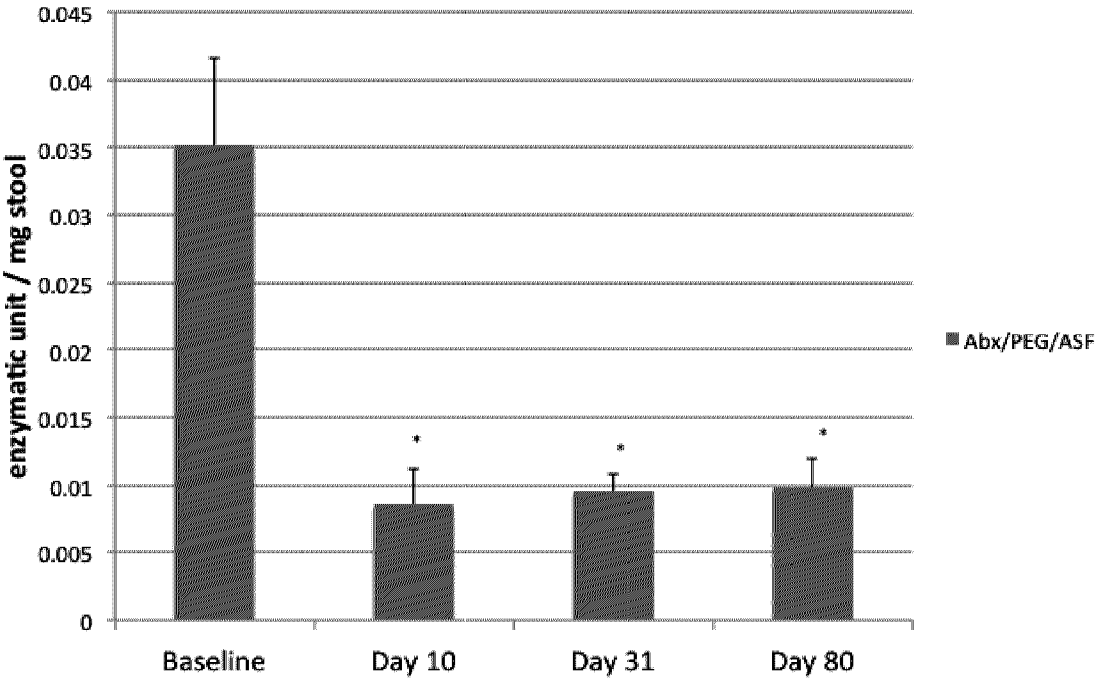
Figure 6
Fecal Urease Activity



* p<0.05 relative to respective baseline
** p<0.01 relative to respective baseline

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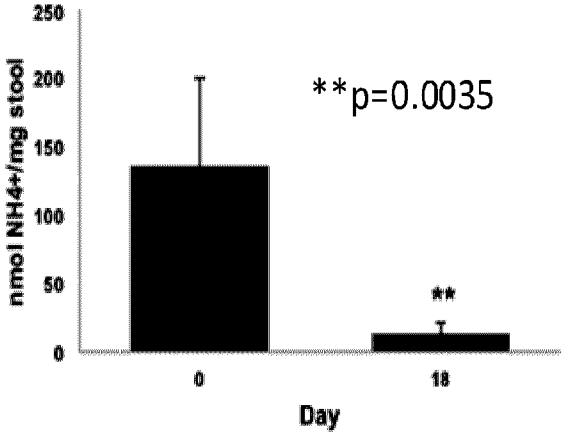
Figure 7

Ammonia results for Lillian/Mike's samples continued



ASF fecal amino acid analysis

ASF Fecal SCFA Analysis

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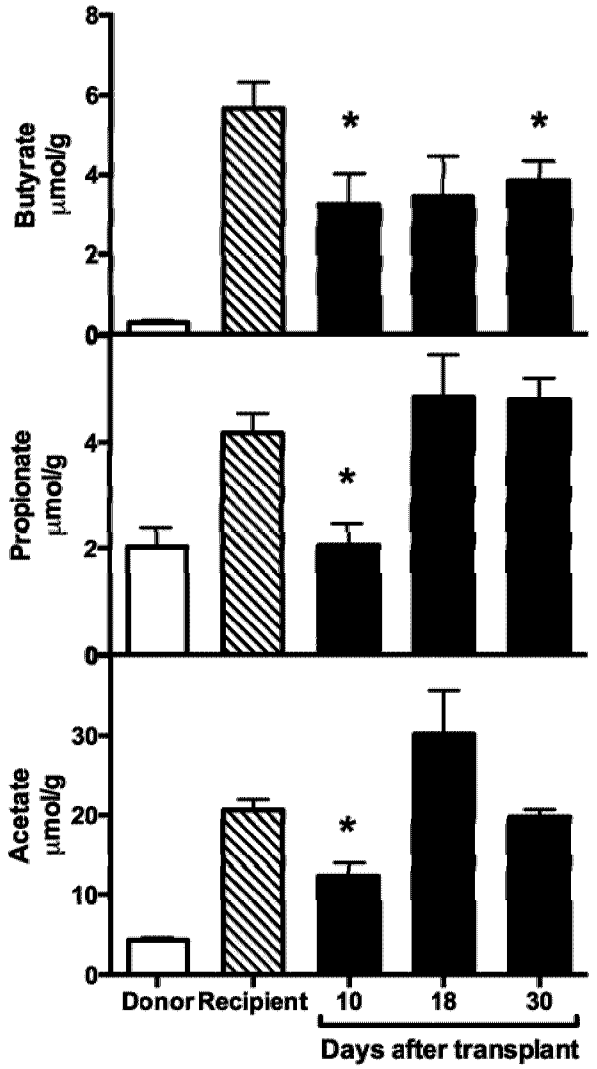
Figure 8



Butyrate pValues: Recipient to Day 10 = 0.04549; Recipient to Day 30 = 0.0504

Propionate pValues: Recipient to Day 10 = 0.0039

Acetate pValues: Recipient to Day 10 = 0.00447352

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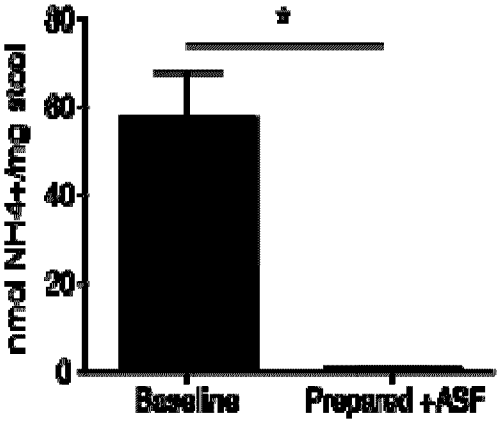

Figure 9A

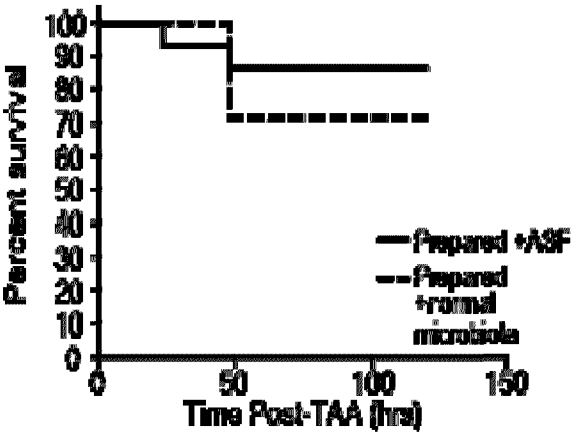

Figure 9B

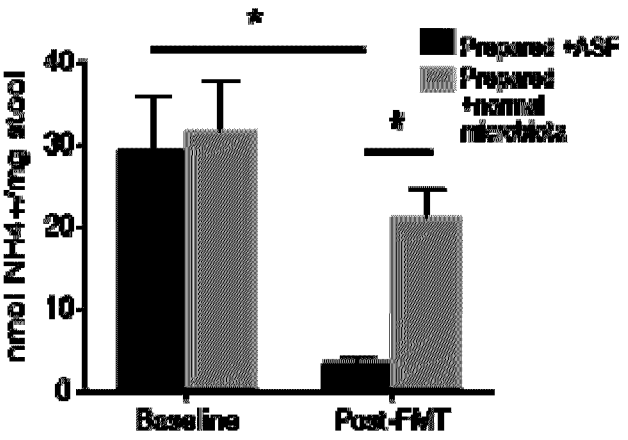

Figure 9C

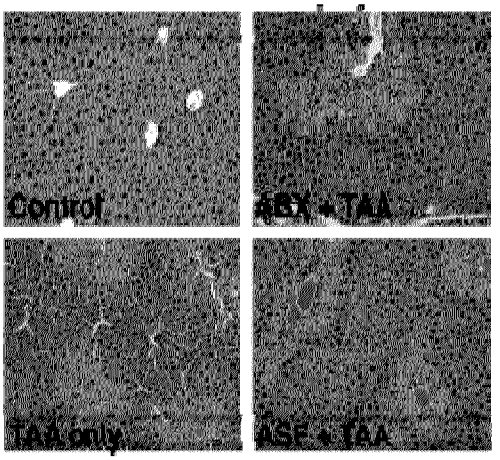

Figure 9D

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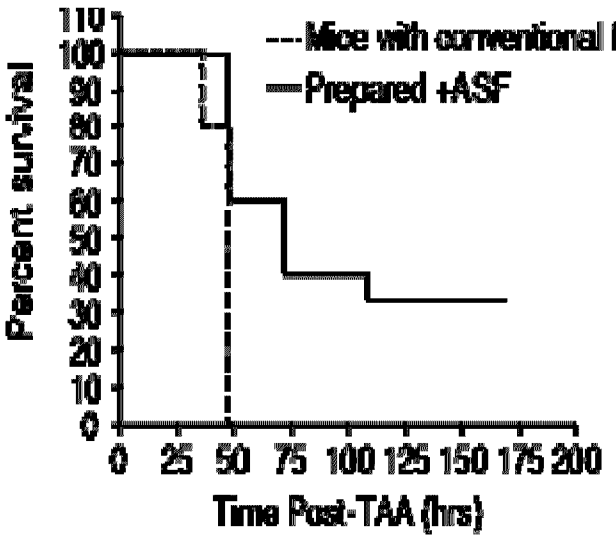

Figure 10A

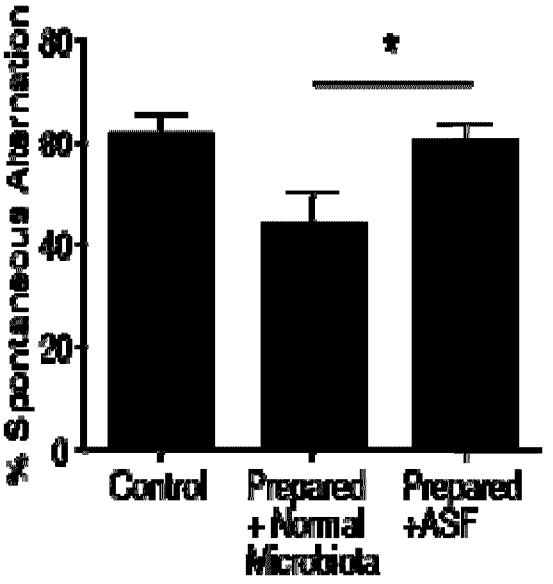

Figure 10B

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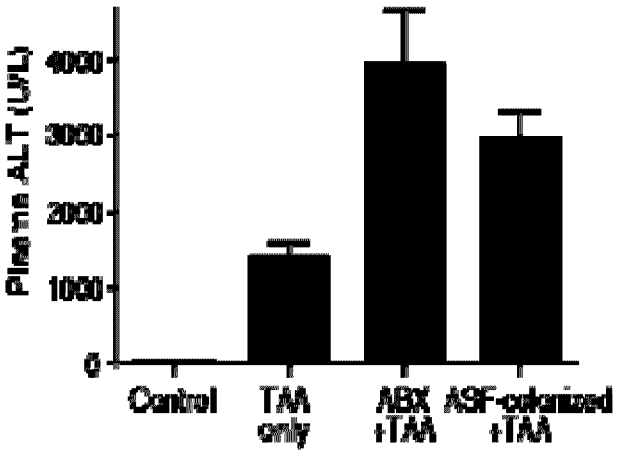

Figure 11A

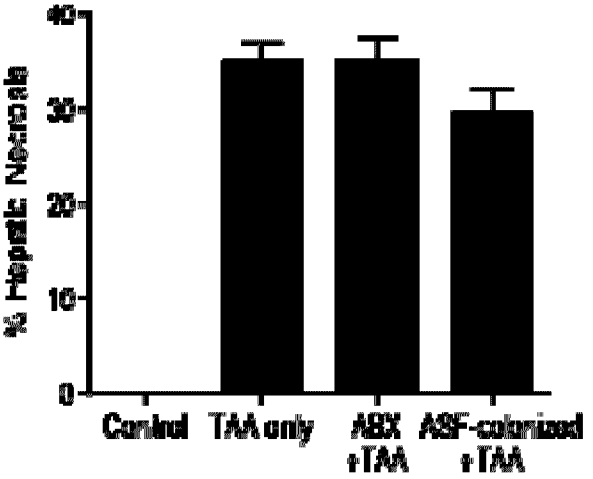

Figure 11B

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The gut microbiota

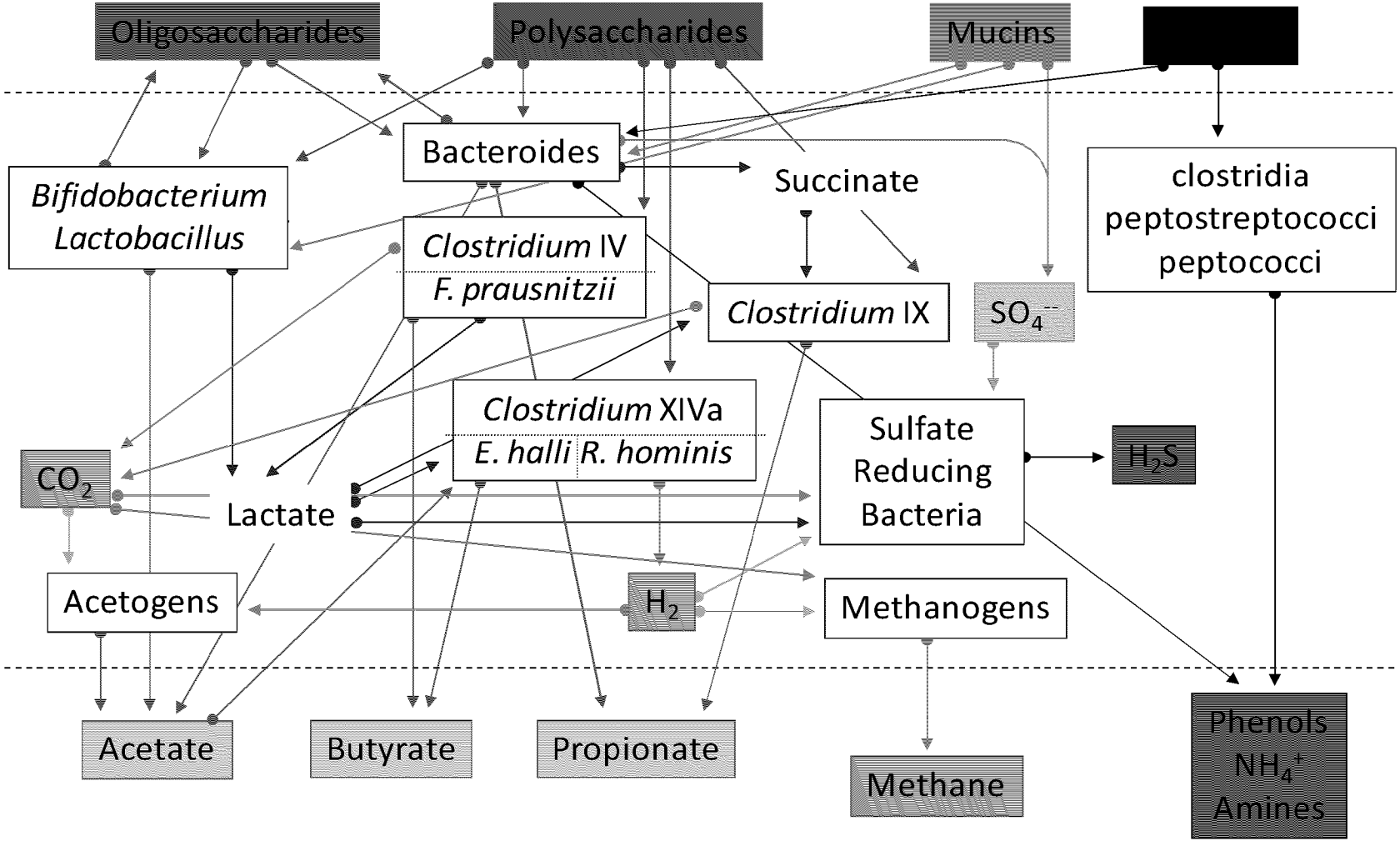
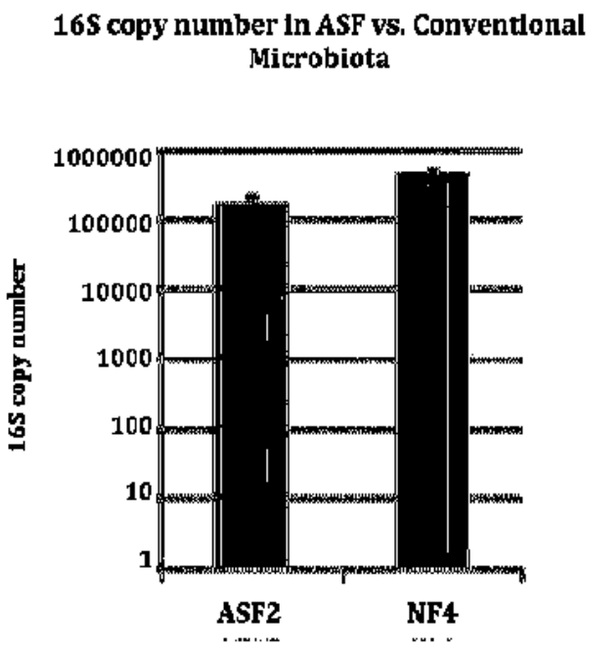
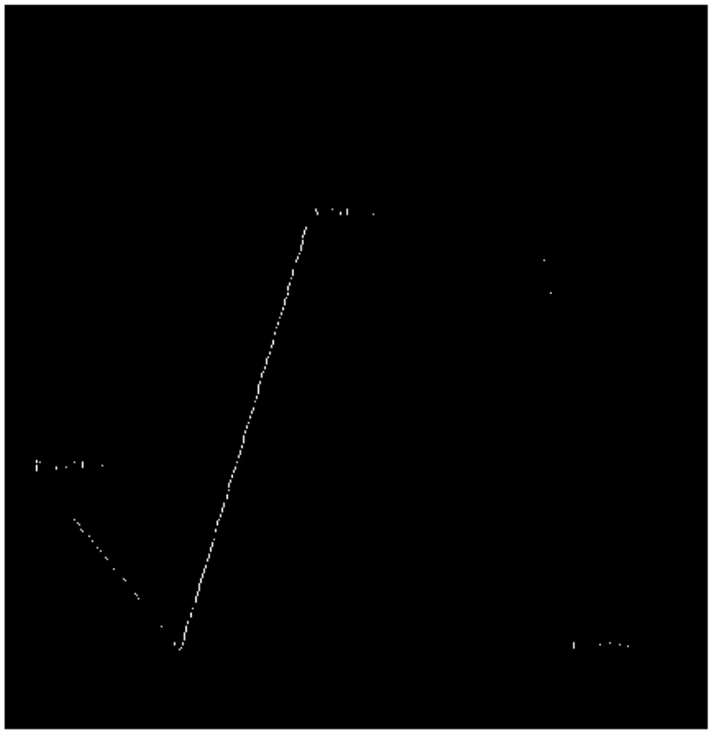


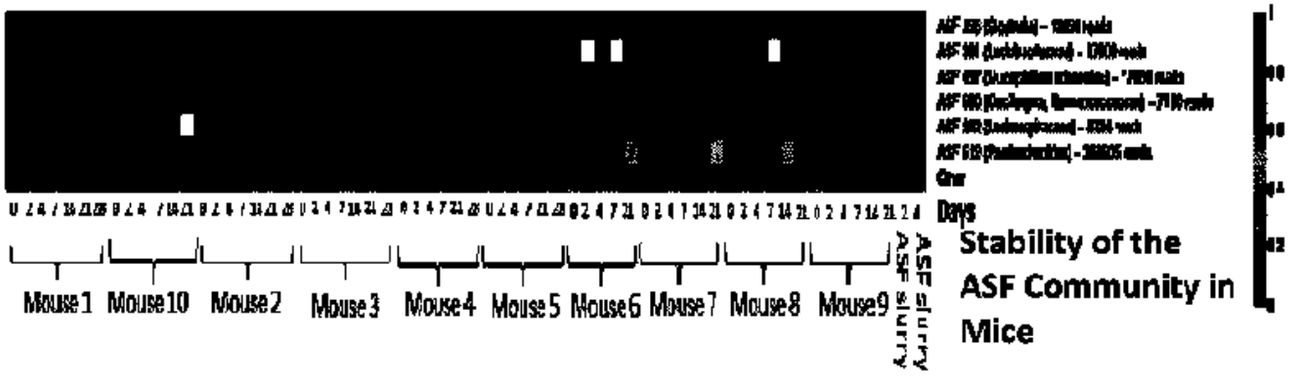
Figure 12


16S copy number in ASF vs. Conventional Microbiota
16S copy number
1000000
100000
10000
1000
100
10
1
ASF2
NF4
2.5 fold difference (p-value = 0.015214)

A


Clustering ASF 16S rRNA gene tags

B


Days
Mouse1
Mouse10
Mouse2
Mouse3
Mouse4
Mouse5
Mouse6
Mouse7
Mouse8
Mouse9
ASF slurry
Stability of the ASF Community in Mice

C