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**TAILORING GUT MICROBIOTA FOR ENHANCED
RESILIENCE AND PERFORMANCE UNDER SLEEP-
DEPRIVED CONDITIONS**

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PREFACE

This study was conducted at the Molecular Mechanisms branch (RHXJ), Human Centered ISR Division, Airman Systems Directorate of the 711th Human Performance Wing of the Air Force Research Laboratory (AFRL/711 HPW/RHXJ), Wright-Patterson AFB, OH. This final technical report covers the performance period June 1, 2014 – December 31, 2018 for AFRL Work Unit H0AP.

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This study was conducted under the approved Air Force Research Laboratory Institutional Animal Care and Use Committee, Animal Protocol Number F-WA-2015-0156-A. All experiments involving animals were approved by the Wright-Patterson Air Force Base Institute of Research, Institutional Animal Care and Use Committee (IACUC), and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Research, National Research Council, National Academies Press, 2011, and in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

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1.0 SUMMARY

Sleep deprivation has been associated with an increase in overall mortality and morbidity in a number of organisms including humans. Mechanistically, a loss of brain mass, due to neuronal death, has been observed after chronic sleep deprivation. Sleep deprivation also has enormous impacts on metabolic homeostasis, with increased prevalence of weight gain and obesity. The perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those that are induced by sleep deprivation. The importance of the gut microbiome in metabolic disorders such as obesity and diabetes has been reported and certain mixes of gut microbiota may protect or predispose the host to obesity. For example, a shift towards the genus of Firmicutes from Bacteroidetes has been observed in obese individuals. In contrast, if an obese individual loses weight, a shift towards Bacteroidetes occurs. Despite the similar patterns of metabolic disorders induced by perturbed gut microbiota and sleep deprivation, it is not clear if there is any interaction between these two conditions. To address this issue, changes in the gut microbiota and host functions following sleep deprivation were determined in this study, using a humanized rat model derived from transplanting native human microbiota into pseudo germ-free rats. The hypothesis to be tested in this study is that sleep deprivation initially causes degradation in the functional and structural integrity of the gastrointestinal tract, resulting in the leakage of pathogenic microbial cells and factors through the intestine, which in turn induces mucosal and systemic inflammation, dysregulated hypothalamic–pituitary–adrenal (HPA) axis and ultimately the onset of metabolic disorders. Data generated in this study can be used to develop a predictive metabolic network model of host-microbiota interaction, which can be validated under a variety of conditions of gut microbiota modulations using prebiotic, probiotic and synbiotic approaches. The validated model will be an extremely useful tool for identifying targets that can effectively tailor the gut microbiota and counter the adverse effects of sleep deprivation, thereby enhancing host resilience and performance. Therefore, knowledge gained from this study will significantly facilitate future development of novel strategies for resilience and performance enhancement and optimization in warfighters under sleep deprived or other stressful operational conditions. This final report will describe all the accomplishments of this 4-year project, including establishment of the rodent germ-free facilities, generation of the pseudo germ-free (PGF) rat model, development of the de-individualized humanized rat (DHR) model and identification of changes in gut microbiota and host functions following acute and chronic sleep deprivation.

2.0 INTRODUCTION

Sleep deprivation, sleep restriction, and circadian desynchrony are among the most commonly encountered stressors in military operations. Insufficient sleep leads to fatigue, sleepiness, impaired mood regulation, and cognitive and physical performance degradation (Turner et al. 2007; Mograss et al. 2009; Alhaider et al. 2011; Aleisa et al. 2011; Alhaider et al. 2010; McDermott et al. 2003; Guan et al. 2004). Previous studies using animal models showed a loss of brain mass occurring after chronic sleep deprivation, resulting from neuronal cell death, even in the presence of environmental enrichments (Mirmiran et al. 1986; Morrissey et al. 2004). These changes are likely due to a mechanism(s) involving the accumulation of misfolded proteins and subsequently activation of the apoptotic pathway (Cirelli et al. 2004; Mackiewicz et al. 2007; Cirelli and Tononi, 2008; Cirelli and Bushey, 2008; Thompson et al. 2010).

Sleep deprivation has enormous impacts on metabolic homeostasis, leading to increased risk of a range of metabolic disorders, including cardiovascular and gastrointestinal morbidity, peptic ulcers, obesity, insulin resistance, and type 2 diabetes. A relationship between sleep deprivation and prevalence of weight gain and obesity has been shown (Bollinger et al. 2009). Interestingly, the perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those that are associated with sleep deprivation. The importance of the gut microbiome in metabolic disorders such as obesity and diabetes has been demonstrated and certain mixes of gut microbiota may protect or predispose the host to obesity. A shift in gut microbiota populations towards the genus of Firmicutes from Bacteroidetes has been observed in obese individuals. In contrast, if an obese individual loses weight, a shift towards Bacteroidetes occurs (Cryan & Dinan, 2012). Despite the similar patterns of metabolic disorders induced by the perturbation of gut microbiota and sleep deprivation, it is not clear if there is any interaction between these two conditions.

Studies in obese populations provide important clues to the connection between sleep deprivation and the perturbation of gut microbiota. Obesity, in part, is characterized by systemic inflammation, in which the gut-brain axis is likely to be involved (Tahergorabi and Khazaei, 2013). While it is not clear how this inflammatory response is activated, sleep deprivation could compromise the functional and structural integrity of the gastrointestinal tract. On one hand, changes in intestinal functions can cause changes in the gut microbiota composition and metabolism. On the other hand, degradation in the structural integrity of the intestinal tract can result in increased intestinal permeability and subsequently the leakage of gut microbial cells and factors both externally and interstitially, causing exposure to degrading factors, including lipopolysaccharides (LPS). This degraded epithelial state in turn triggers mucosal and systemic inflammation. Jazwinska (1986) reported that the proliferation of jejunum cells, which function to restore cell loss in this region, was down-regulated after sleep deprivation. If this is proven to be frequently occurred throughout the intestine following sleep deprivation, it would provide a mechanistic basis for the observation that bacteria migrated from the intestine and translocated to extra-intestinal sites, resulting in systemic bacterial invasion and infection following sleep deprivation (Everson and Toth, 2000).

While sleep deprivation results in a dysregulated HPA axis and increased cortisol levels (Aldabal & Bahammam, 2011), stress is known to increase intestinal permeability, allowing gut microbiota an opportunity to translocate across the intestinal mucosa and directly interact with immune cells (Gareau et al., 2008; Teitelbaum et al. 2008). An exaggerated HPA response to restraint stress in germ-free (GF) mice, compared with specific pathogen-free (SPF) mice, further confirmed that gut microbiota plays an important role in HPA regulation (Sudo et al. 2004). Consistently, probiotics have been shown to prevent the increase in the intestinal permeability and HPA axis hyper-reactivity that were induced by maternal separation and restraint stress (Gareau et al. 2007; Ait-Belgnaoui et al. 2012). A recent study showed that sleep deprivation and high calorie, high fat Western diet act additively on the central nervous system (CNS) (Alzoubi et al. 2013).

After reviewing the literature on sleep, sleep deprivation, gut microbiota, metabolic diseases and psychological disorders, a hypothesis was developed that sleep deprivation initially degrades the functional and structural integrity of the gastrointestinal tract (and other organs including the brain), resulting in an altered metabolic homeostasis and intestinal permeability. While the former induces a perturbation of the gut microbiota, the latter results in the leakage of microbial cells and factors, including endotoxin Lipopolysaccharides (LPS). The combined effects of these events can trigger mucosal and systemic inflammation, increased levels of cortisol and the dysregulation of the HPA axis. A simplified version of this hypothesis, for the ease of visualization, is depicted in Figure 1.

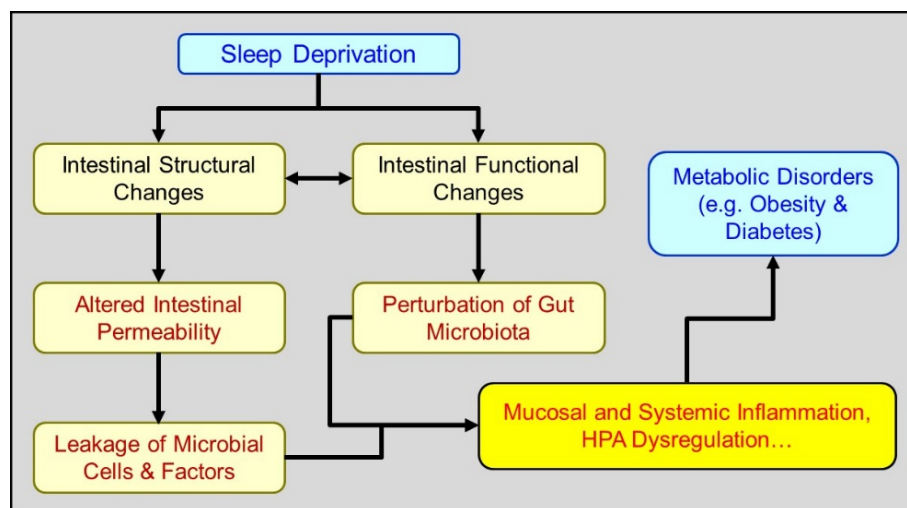


Figure 1. Hypothesis of Metabolic Disorders Induced by Sleep Deprivation

Graphical representation of the hypothesis shows the effects of sleep deprivation on the structural and functional changes in the intestinal tract and leakage of microbial cells and factors through the gut walls, resulting in mucosal and systemic inflammation, HPA axis dysregulation, and ultimately the onset of metabolic disorders.

To test this hypothesis, the effects of sleep deprivation on gut microbiota and host functions were investigated, using a gnotobiotic rat model that harbors the human gut microbiota (i.e. a “humanized” rat model). We anticipate that the results of these experiments can be used to generate a host-microbiota metabolic interaction model, which will allow the evaluation, *in-silico*, of the effect of gut microbiota modulation on host resilience. Subsequently, the most effective strategy can be selected for experimental validation. Therefore, the model, once available, will be an extremely valuable tool for the identification of targets and strategies for gut microbiota modulation to counter the adverse effects of sleep deprivation, thereby enhancing host resilience and performance.

To achieve these objectives, we first established a rodent Germ free (GF) facility for the generation and maintenance of the pseudo germ-free rats. After optimization of the procedures for generating PGF rats and fecal transplant, they were used in the development of a novel humanized rat model that will have a better representation and coverage of the entire spectrum of the human microbiota. Using this new animal model, we investigated the effects of acute and chronic sleep deprivation on gut microbiota population and functions, as well as the effects on host intestinal and systemic functions.

3.0 MATERIALS AND METHODS

3.1 Establishment of Germ-Free (GF) Facility

The Germ-Free (GF) Facility for generating and maintaining pseudo germ-free (PGF) and humanized rats, as well as for performing sleep deprivation experiments, was established in the vivarium at Wright-Patterson Air Force Base (WPAFB). There are five semi-rigid germ-free isolators in this facility, which provide a maximum capacity of housing a total of 180 rats. This laboratory is fully functional and ready to be used to generate and house PGF and/or humanized rats. The picture of the germ-free laboratory and a germ-free isolators are shown in Figures 2 and 3.



Figure 2. Rodent Germ-Free (GF) Facility Established under This Project at Wright-Patterson Air Force Base, Ohio



Figure 3. Close-up View of Semi-Rigid Germ-Free (GF) Isolator

3.2 Animal Husbandry

This study protocol was reviewed and approved by the Wright-Patterson Air Force Base Institute of Research Intuitional Animal Care and Use Committee (IACUC) and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments in this report were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experiments were performed in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals" (National Research Council of the National Academies, Guide for the Care and Use of Laboratory Animals).

The animals used in this study were 6-weeks old Specific Pathogen Free (SPF) Sprague Dawley Rats (from Charles River Laboratories, Wilmington, MA). They were socially housed, with 2 rats/cage, in clear plastic cages and provided tunnels, nesting material, and nylon bones for enrichment. Normal *naïve* and cage control animals were provided with conventional bedding (CellZorb, Cincinnati Lab Supply, Cincinnati, OH). Food (LabDiet Formulab Diet 5008, Cincinnati Lab Supply) and water were freely available. Animals that were maintained in a sterile environment inside the germ-free isolators were housed in the same manner, except that they were provided with sterilized cages and enrichment toys. They were also supplied with sterile food (Picolab Rodent Irradiated Chow, Charles River), sterile bedding (Alpha Dri Irradiated Bedding, Charles River) and sterilized (autoclaved) water. The animal rooms were climate controlled (20 – 26 degrees Celsius (°C), 30 – 50 % humidity) and maintained at a 12-h light/dark cycle (on at 0600).

3.3 Determination of Microbial Titers of Fecal materials, Intestinal Contents and Tissue Homogenates

Fecal materials (from human donors or rats) or intestinal contents (from rats) were homogenized using a sterile, mini pestle and dissolved in 0.75 mL of sterile, 1x phosphate buffered saline, pH 7.3 (PBS). Tissue samples, including liver, spleen, mesentery layer, mesenteric lymph nodes, distal ileum, cecum, proximal colon and intestinal contents (from the same regions) were collected were collected post-mortem and flash frozen in liquid nitrogen and stored at -80 °C. Tissues samples were later homogenized in 0.5 ml of sterile, 1x PBS using the Bullet Blender Gold (2 minutes on speed 6), RhinoVials, and 2.0 millimeter (mm) Zirconium Oxide Beads (3/tube) (all from Next Advance, Troy, NY). A portion (0.25 mL) of the homogenate (diluted when necessary) was spread evenly onto blood-agar plates (tryptone soya agar containing 5% sheep blood, from Thermo Fisher Scientific, Carlsbad, CA) in duplicates. Plates were incubated at 37 °C overnight, aerobically in a standard incubator and anaerobically using the BD GasPak EZ Gas Generating System (Thermo Fisher Scientific). The colonies were enumerated using the automated Scan 300 Colony Counter (Interscience Laboratories, Woburn, MA) and expressed as total (aerobic + anaerobic) colony forming units per gram of material (CFU/g).

3.4 Development of Antibiotic Cocktail for Generating Pseudo Germ-Free (PGF) Rats

Five antibiotics, Enrofloxacin, Neomycin, Vancomycin, Amphotericin B, and Ampicillin (all from Patterson Vet Inc.) were selected as the initial candidate components of the antibiotic cocktail. Some of these antibiotics are poorly absorbed through the wall of the gastrointestinal tract and thus not likely to result in significant systemic exposure. The effectiveness of each antibiotic was independently tested *in vitro* against rat gut microbiota by including the respective antibiotic in the blood-agar plates and following the same procedure described above for determining microbial titer, using cecal content from *naïve* SPF Sprague Dawley rats. The following initial concentrations of antibiotic were tested: Enrofloxacin (65 mg/L); Neomycin (150 mg/L); Vancomycin (350 mg/L); Amphotericin B (1000 mg/L); and Ampicillin (150 mg/L). Concentrations were subsequently increased or decreased in 2x serial dilutions (or increments) to determine the minimum effective concentration for each antibiotic. The most effective antibiotics were combined in a cocktail at the lowest concentrations that produced the maximum inhibitory effect on microbial growth. The effectiveness of the cocktail was tested *in vitro* using the same blood-agar plate culture method, then tested *in vivo* for its ability to generate PGF rats as described below.

3.5 Development of Pseudo Germ-Free (PGF) Rats

Fecal samples were collected prior to the placement of the animals in the GF isolators in order to obtain the baseline titer of the gut microbiota in these animals. The animals were then transferred to semi-rigid isolators, which were maintained in a GF room following the recommendations of the manufacturer. Admittance to the room was highly controlled and its GF status monitored throughout the study using culture swabs and PocketSwab Plus (Charm Sciences, Lawrence, MA). After 2 days of acclimation to the isolator environment, antibiotic treatment began by adding a cocktail containing Enrofloxacin (65 mg/L), Neomycin (1000 mg/L), and Vancomycin (500 mg/L) to the drinking water for 12 days. A fresh lot of drinking water containing the antibiotic cocktail prepared daily were used. The effectiveness of the cocktail was evaluated by euthanizing two animals at each one of the six time points (i.e., days 3, 6, 10, 14, 19, and 26) for the collection of the cecal contents. The microbial titer of the cecal content was determined using the procedure as described above. Cecal content was used because it provides a more reliable indicator than fecal samples for the depletion of the gut microbiota. Based on the result, the antibiotic cocktail was further refined until no culturable microbial cells could be detected in the cecal content after 3 days of antibiotics treatment.

3.6 Procurement of Human Fecal Microbiota

Fecal samples from normal, healthy, human donors were supplied by OpenBiome (Somerville, MA) through a contractual agreement. The subjects were Caucasian males aged 20 – 45, BMI 19 – 25, no drug abuse, no medications including antibiotics for at least 12 months, no infectious and other disorders including gastrointestinal, immunosuppressive, autoimmune, psychological, neurological, and sleep disorders.

OpenBiome also screened donors for 20 different stool collections and serological abnormalities. The fecal materials from six different donors were used in this study. The bacterial titer of each donor sample was determined using the blood-agar plate method described above, and their community structures analyzed by metagenomic (as described below).

3.7 Development of De-Individualized Humanized Rat (DHR) Model

The development of the DHR model involves four (4) steps: 1.) antibiotic treatment; 2.) fecal transplant; 3.) establishment of human microbiota in rat hosts; and 4.) de-Individualization of donor-specific microbiota. The first three steps were six (6) weeks long total (2 weeks long for each step), and the final step was 15 weeks in order to evaluate the long-term stability of the human microbiota in rats. Antibiotic treatment was as described above under the section of “Development of PGF”. During antibiotic treatment, fecal samples were collected daily, and 2 rats were euthanized at days 5 and 10. The samples were used to evaluate the depletion of the native rat gut microbiota using the plate culture method as described above.

Fecal transplant was accomplished by intra-gastric gavage with 8.5×10^8 CFU of human fecal microbiota suspended in 0.5 mL of 1x lysogeny broth (LB) (Thermo Fisher Scientific). The inoculum was either derived from a single donor (donor #74, #80 or #88) or a 3-donor mix composed of equal contributions from donors #37, #60 and #65, resulting in four humanized rat lines. Each PGF rat received a total of five inoculations at days 15, 17, 19, 22, and 25. The rats were allowed to recover for 2 weeks. This recovery period also allowed the human microbiota to colonize the intestinal tract of the recipients (i.e., establishment). In addition to daily fecal sample collection, two humanized rats were euthanized at days 26 and 40. The samples were used to confirm successful colonization of the human microbiota in the rat hosts, using the method described above.

De-Individualization of humanized rats began by forming two communities. De-individualized Community 1 was comprised of half the animals from each of the four humanized lines (donor #74-, #80-, #88- and 3-donor-mix-associated lines). De-individualized Community 2 was comprised of the remaining half of the #74- and #80-associated lines. The other half of the #88- and the 3-donor-mix-associated animals were maintained as independent lines. This design thus resulted in two de-individualized communities and two independent humanized lines. De-individualization was intended to generate a homogenous profile of human microbiota across the animals belonging to the assigned community by taking advantage of the coprophagic nature of rodents – the natural behavior of consuming fecal materials. This was achieved by periodically pooling the bedding (containing fecal pellets) from all community members, mixing it thoroughly, and redistributing it equally back to the cages of the members of that community. The mixing was performed in the GF isolators every second day for 15 weeks. The de-individualized communities and the independent lines were maintained in separated isolators under GF conditions, and all experimental procedures were performed in the isolators in order to prevent the introduction of other microbes to the humanized

communities or the independent lines. In addition, newly generated PGF rats were introduced into the de-individualized communities in the second week of the De-Individualization period, and supplied with a portion of the community bedding in order to test the acquisition of human microbiota through coprophagia alone (without direct fecal transplant). Groups of animals were euthanized at 5 time points (weeks 1, 2, 5, 10 and 15 of the de-Individualization period), and samples collected for microbial titer determination and metagenomic analysis to monitor the progress and stability of humanization and de-individualization, as well as the acquisition of human microbiota through coprophagia.

3.8 Metagenomics

Targeted metagenomic analysis was conducted using fecal materials or intestinal contents. The samples were flash frozen in liquid nitrogen and stored at -80 °C. Total deoxyribonucleic acid (DNA) was isolated from 250 mg of each sample using QIAamp DNA Stool Mini Kit (Qiagen, Carlsbad, CA), following the manufacturer's instructions. DNA was eluted in 150 µL of AE elution buffer (Qiagen), quantified using the ND-1000 NanoDrop Spectrophotometer (Thermo Fisher Scientific), and stored at -20 °C until use.

Hypervariable regions of 16S ribosomal deoxynucleic acid (rDNA) (including negative and positive controls) were amplified using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific) per the manufacturer's directions. This kit contains two wide-ranging primer sets (V2-4-8 and V3-6, 7-9) for coverage of multiple hypervariable regions, allowing the detection of a broad range of microbial species. DNA enrichment was confirmed after PCR amplification using 5 µL of the product on agarose gel (2%) electrophoresis. The DNA was purified using Agencourt AMPure XP magnetic beads (Thermo Fisher Scientific) and quantified on the 4200 TapeStation using D1000 screen tape and reagents (Agilent Technologies, Santa Clara, CA). Enzymatic fragmentation of the PCR products was accomplished with the Ion Plus Fragment Library Kit, according to manufacturer's directions (Life Technologies, Waltham, MA). Each sample was individually labeled to allow for multiplexing of the samples using Ion Xpress Barcode Adapters 1-96 Kit (Thermo Fisher Scientific).

For metagenomic sequencing and taxonomic analysis, each DNA library was diluted to 60 picomoles (pM) and pooled. Colony amplification by emulsion PCR, template preparation and chip loading were automated by the Ion Torrent Ion Chef System (Life Technologies) utilizing Ion 530 chips/kits (Thermo Fisher Scientific). DNA were sequenced using the Ion Torrent S5 Semiconductor Sequencer v5.2 (Thermo Fisher Scientific) and Ion S5 Sequencing Kit (Thermo Fisher Scientific). The metagenomic library was constructed with 400-bp inserts for each sample.

Taxonomic analysis of the raw sequences was accomplished using the Quantitative Insights into Microbial Ecology (QIIME) software, v1.9.1 (Caporaso et al. 2010). Operational taxonomic units (OTUs) were assigned based on a 97% sequence identity to the GreenGenes reference, v13.5 (DeSantis et al. 2016). Beta-diversity was

calculated using UniFrac methodology (Lozupone and Knight, 2005; Lozupone et al. 2011), and three-dimensional principal coordinate analysis (PCoA) plots visualized with the EMPeror (Vazquez-Baeza et al. 2013).

3.9 Acute and Chronic Sleep Deprivation Treatments

Sleep deprivation treatments of *naïve* Sprague Dawley SPF and DHR rats were achieved by placing the animals in the automated Sleep Fragmentation Chambers (Model 80391 from Lafayette Instrument Company, Lafayette, IN) inside the GF isolators. The animals were housed individually in their own chambers containing a thin layer of bedding. Food and water were freely available during the entire treatment period. Following 1 day of habituation, the animals were subjected to sleep deprivation treatment by activating the swipe bar of the Sleep Fragmentation Chambers. For acute sleep deprivation treatment, the swipe bar was set to operate for 6 days, 24 hours per day. Chronic sleep deprivation treatment lasted for 42 days, and the animals were allowed to have uninterrupted sleep for 3 hours per day starting at 08:00 until 11:00. Otherwise, the swipe bar was set to operate for the remaining time of each day. Upon the completion of the sleep deprivation treatment, the animals were euthanized and microbiota and tissue samples collected for metagenomic analysis and bioassays, as described above (or below).

3.10 Analysis of Biomarkers of Host Functions

Serum and tissue samples were collected post mortem as described above, at the time points indicated in Section 3.7 (for the development of DHR Model) or in Section 3.9 (for acute or chronic sleep deprivation), and stored at -80 °C until use. The intestine homogenate prepared as described in Section 3.3 was also used for the analysis of the myeloperoxidase (MPO) activity using the Myeloperoxidase Activity Assay Kit (Abcam, Cambridge, MA), according to the manufacturer's instructions. Other analytes of interest, including lipopolysaccharide binding protein (LBP), corticotrophin releasing factor (CRF), adrenocorticotrophic hormone (ACTH), corticosterone and a panel of pro-inflammatory cytokines were determined in the serum, using specific assay kits, per the manufacturer's instructions. Assay kits used in this study included Lipopolysaccharide Binding Protein Kit (from MyBioSource, San Diego, CA), ACTH ELISA Kit (from MyBioSource), Corticosterone ELISA Kit (from Arbor Assays, Ann Arbor, MI), Corticotrophin Releasing Factor ELISA Kit (from Kamiya Biomedical, Seattle WA), and SECTOR Imager 2400 with VPLEX Plus Pro-inflammatory Panel 2 Rat Kit (from Meso Scale Diagnostics, Rockville, MD). Statistical analysis was accomplished using Prism 7 for Windows, version 7.05 (GraphPad Software, La Jolla, CA).

3.11 Novel Object Recognition (NOR) Test

Learning and memory performance was determined using the novel object recognition (NOR) test. This task measures the encoding, formation and retrieval of episodic memory. Performance in this task relies on prefrontal cortex and hippocampus

(Vogel-Ciernia and Wood, 2014). The advantage of this test is that it is less stressful than other methods (such as the Morris water maze). Since the NOR test relies on the natural preference for novel objects displayed by rodents, no positive or negative reinforcement is needed. The test procedure consists of three phases: habituation, familiarization, and test. In the habituation phase, animals were allowed to freely explore the open-field arena for 3 – 4 days (30 – 60 minutes per day) in the absence of objects. During the familiarization phase, an animal was placed in the open-field arena containing two identical sample objects (A + A) for 5 minutes, and the time that the animal spent exploring each object was recorded. After 24 hours (i.e. the retention period), the animals were returned to the open-field arena with two objects, one identical to the sample object used in the familiarization phase, while the other with a larger size of the same shape (A + B), in a counter-balanced manner (Ennaceur 2010; Ennaceur and Delacour, 1988; Gaskin et al., 2010; Hammond et al., 2004; Taglialatela et al., 2009). The total times that the animal spent exploring the familiar and novel objects were recorded. The test was repeated 72 hours later using a novel object with a different shape (A + C). As above, the times that the animal spent exploring the familiar and novel objects were recorded. During the test phase, animals with normal learning and memory functions would naturally spend more time exploring the novel object. In general, the strongest novel object preference scores tend to occur early in the test phase while the novel object is still relatively novel (Broadbent et al., 2009).

4.0 RESULTS

4.1 Development of Antibiotic Cocktail for Generating Pseudo Germ-Free (PGF) Rats

To develop an antibiotic cocktail that can effectively and reproducibly deplete the native rats gut microbiota, five candidate antibiotics were selected and tested individually or in combination *in vitro*, using the blood-agar plate culture method. The four-drug cocktail containing 65 mg/L Enrofloxacin, 150 mg/L Neomycin, 350 mg/L Vancomycin and 1000 mg/L Amphotericin B, was capable of reducing the microbial titer of the cecal content by 90% after overnight incubation. The addition of Ampicillin (150 mg/L) to the cocktail did not significantly improve its effectiveness. Thus, Ampicillin was excluded from the final formulation of the cocktail. When tested alone, Amphotericin B (1000 mg/L) did not reduce the number of colonies significantly. Since this agent is an antifungal agent, this result suggests that the fungal population might be relatively minor in the rat cecal content, or the culture method used here might not be optimal for fungal growth. Based on these results and that of subsequent tests, the final antibiotic cocktail carried forward for *in vivo* testing contained Enrofloxacin (65 mg/L), Neomycin (1000 mg/L), and Vancomycin (500 mg/L).

4.2 Development of Pseudo Germ-Free (PGF) Rats

Despite the bacteriostatic/ bactericidal effectiveness of the antibiotic cocktail *in vitro*, it failed to eliminate all culturable microbial cells in the cecal content of the treated animals. Approximately 5% residual, culturable cells persisted after 12 days of antibiotic treatment. The concentrations of the antibiotics were subsequently increased 150–300% in the cocktail to ensure that all culturable microbes in the cecal content would be depleted completely. After achieving complete depletion of culturable cells *in vivo*, the concentration of each individual antibiotic were decreased gradually to determine the minimal concentrations needed to effectively generate a PGF status in rats. After several rounds of testing and optimization, it was found that a cocktail containing 200 mg/L Enrofloxacin, 800 mg/L Neomycin, and 850 mg/L Vancomycin was able to eliminate all culturable microbial cells in the cecal content after 3 days of treatment, and thus this cocktail was used in all subsequent experiments.

4.3 Metagenomic Analysis of Fecal Microbiota from Human Donors and *Naïve* Sprague Dawley Rats

During the period of developing the antibiotic cocktail and PGF rats, we have obtained the fecal materials from six human donors through a contract with OpenBiome (Somerville, MA). The titer of these samples ranged from $1.1 - 8.1 \times 10^8$ CFU/g. As expected, the titer of anaerobes is significantly higher than that of aerobes in all of these samples. The fecal microbiota from the human donors was analyzed using a high throughput sequencing method to determine their overall diversity and the relative abundance of bacterial taxa in the samples. As shown in Table 1, the relative abundance of *Bacteroidetes* and *Firmicutes*, the most abundant phyla in Western populations (Human Microbiome Project Consortium Report 2012), are quite diverse among the

donors recruited for this study. *Bacteroidetes* ranged from 51.9% - 71.8% and *Firmicutes* from 24.7% - 44.0%, which is consistent with other reports in the literature (Lozupone and Knight, 2005; Lozupone et al. 2011). The sequencing result also showed that the native rat microbiota is different from the human microbiota. Other phyla that were detected in the samples included *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and several minor phyla such as *Cyanobacteria*, *Deferribacteres*, *Synergistetes*, *TM7*, *Tenericutes* and *Verrucomicrobia* (see Figure 4). While the samples from five human donors appeared to have normal patterns of human gut microbiota, Donor #60 had a significantly higher level of *Fusobacteria* than all other donors (and the naïve rats as well). Although some species of *Fusobacteria* can cause human diseases (Afra et al. 2013), the rigorous testing conducted during donor screening did not indicate any signs of infection, nor other health issues, in Donor #60. Therefore, the sample from this donor was retained in the study and used for fecal transplant into PGF rats.

To explore the differences in the population structure between the fecal microbiota from the human donors and that from the naïve Sprague Dawley rats, Principal Coordinate Analysis (PCoA) of the UniFrac distance metric between these samples was performed using Emperor as described in the “Materials and Methods” section. As shown in Figure 5, the baseline samples from the rats and the samples from the human donors formed two well-separated clusters. Interestingly, the baseline rat fecal microbiota appeared to be heterogeneous, which were separated into two smaller clusters, although both of them still distinct from the cluster of human fecal microbiota. This result suggests that the rats used in this experiment might be derived from two independent colonies. However, technical issues of the high throughput sequencing experiment causing this clustering pattern could not be ruled out completely. The rats, which eventually became members of the De-Individualized Community 1, were represented in both clusters, while those that became members of De-Individualized Community 2 were only found in the leftmost cluster. Since antibiotic treatment depleted the gut microbiota, the gut microbiota of the PGF rats would be set to a near-zero state. Because of this reason, such variation in the baseline microbiota might only minimally affect the formation of the DHR model.

Table 1. Relative Abundance of Bacteroidetes and Firmicutes in the Fecal Microbiota from Human Donors and *Naïve* Sprague Dawley Rats

Sample	Bacteroidetes	Firmicutes
Donor #37	71.80%	24.70%
Donor #60	53.80%	35.10%
Donor #65	61.90%	33.50%
Donor #74	51.90%	44.00%
Donor #80	56.00%	40.00%
Donor #88-1*	71.30%	25.20%
Donor #88-2*	69.80%	27.40%
Mixed (#37, #60 & #65)**	59.10%	36.40%
Human Donors (Average)	61.00%	33.90%
<i>Naïve</i> Rat (Average)	46.90%	47.80%

* Results of two independent sequencing experiments for Donor #88;

** Mixed sample comprised of fecal materials from Donors #37, #60 & #65.

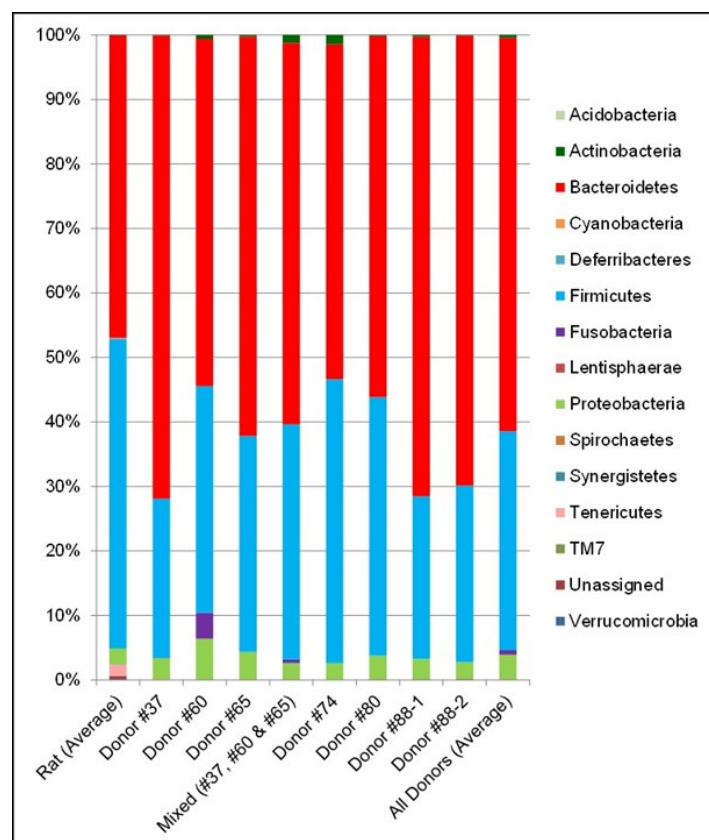


Figure 4. Alpha Diversity of Fecal Microbiota from *Naïve* Sprague Dawley Rats and Human Donors

Detectable bacterial phyla in the human and rat samples are shown. The abundance of *Fusobacteria* is much higher in Donor #60 than other human donors and the naive rats.

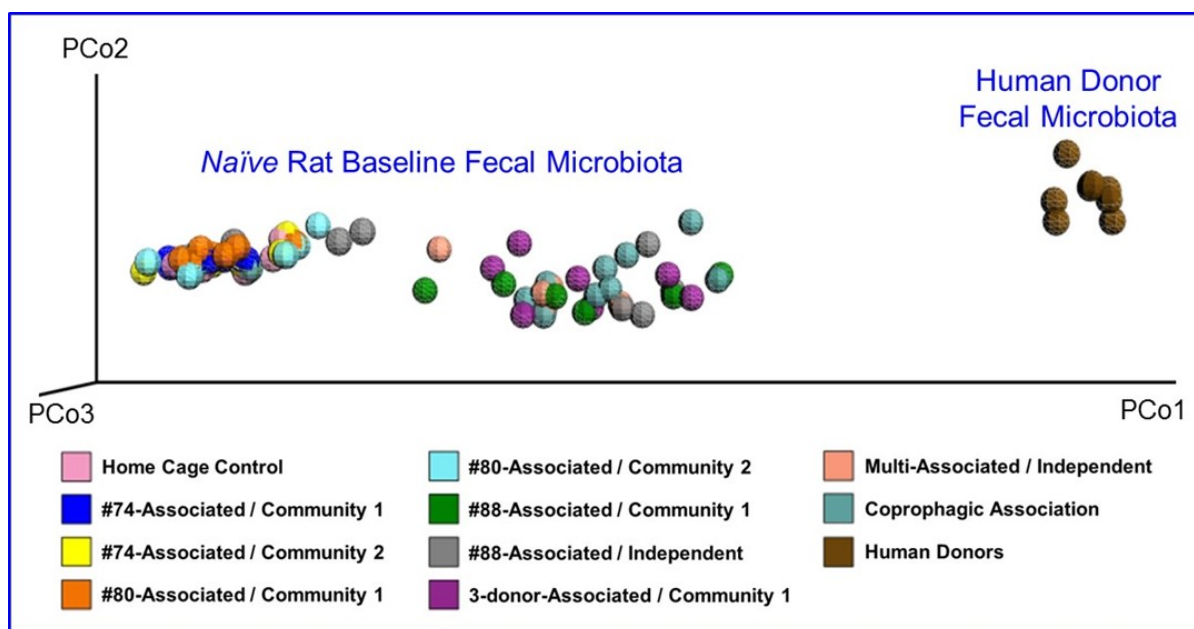


Figure 5. Principal Coordinate Analysis (PCoA) of Microbial Diversity in Fecal Samples from Human Donors and *Naïve* Sprague Dawley Rats at Baseline

The naïve rats are color-coded based upon which human donor's material it received, and its subsequent allocation for De-Individualization. The Home Cage Control rats were not treated with antibiotics, nor transplanted with human fecal samples. "Community 1" and "Community 2" refer to the two de-individualized communities. "Independent" refers to humanized lines that were not de-individualized. "Multi-Associated" is the same as Mix-Associated (using a mixture of the fecal materials from 3 donors). "Coprophagic Association" refers to the PGF rats that had no prior inoculation of the human fecal materials and were directly introduced to the de-individualized communities.

4.4 Development of De-Individualized Humanized Rat (DHR) Model

The experimental design of the DHR Model development is shown in Figure 6, using a 4-step design as described in Section 3.7. To ensure that the entire procedure would work successfully, several pilot experiments were performed before conducting the full-scale experiment of DHR Model development. Antibiotic treatment was as described in Section 3.5. During antibiotic treatment, fecal samples were collected daily, and 2 rats were euthanized at days 5 and 10. The samples were used to evaluate the depletion of the rat native gut microbiota using the plate culture method as described above. The results of these pilot experiments confirmed that the procedure used to produce PGF rats was highly reliable and reproducible. Additional pilot experiments were also performed to optimize the inoculum titer and the fecal transplant procedure. Based on the results of these experiments, we found that fecal transplant should be conducted immediately after the completion of the antibiotic treatment and that a minimum of three inoculations were needed to establish stably humanized rats. Therefore, the finalized procedure employed five inoculations of human microbiota, performed at days 1, 3, 5, 8 and 11, during the 2-week period of fecal transplant.

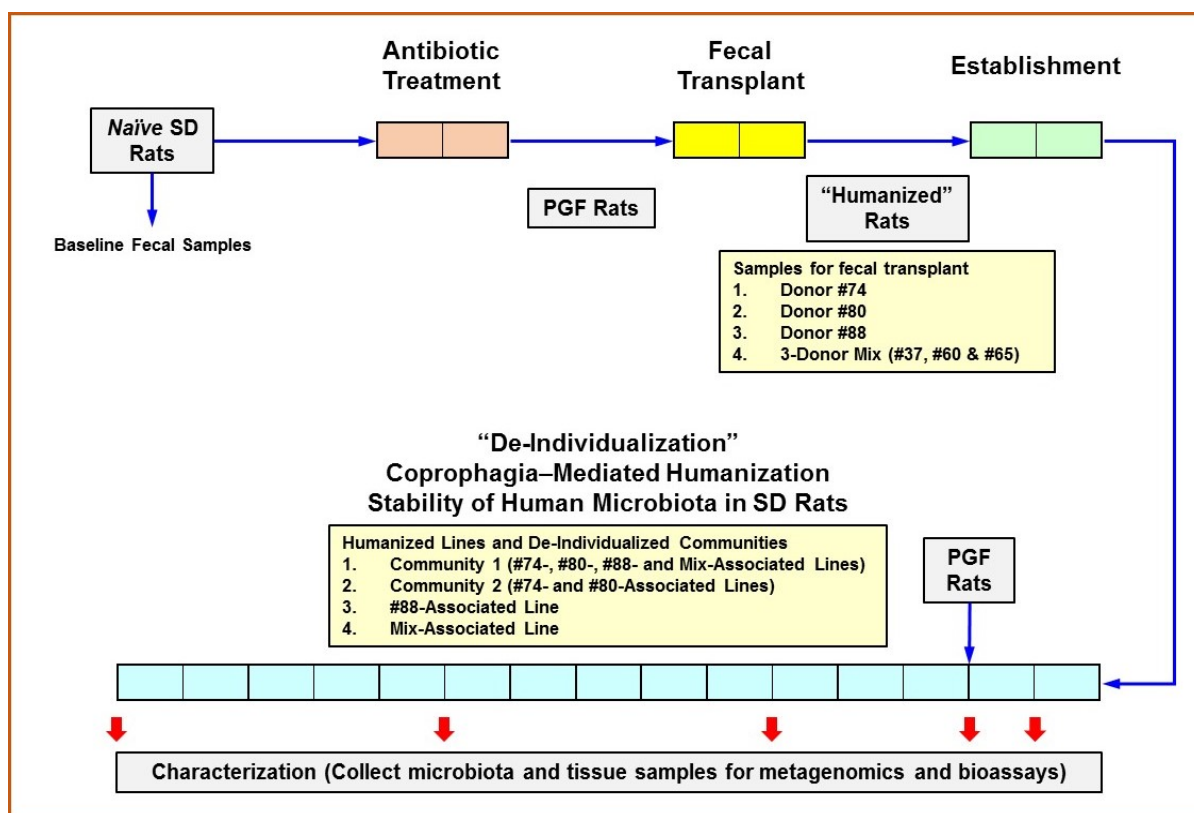


Figure 6. Experimental Design of De-Individualized Humanized Rat (DHR) Model Development

Each colored box represents one week. PGF rats were generated by a 12-day antibiotic treatment. Fecal transplant was performed via five intragastric gavage inoculations, at days 15, 17, 19, 22, and 25, using the material derived from a single donor or a mixture of three donors. Cecal content was sampled from randomly selected animals after 5 and 10 days of antibiotic treatment to monitor the depletion of the native rat gut microbiota, and at days 26 and 40 to assess fecal transplant and colonization of human microbiota in the rat hosts. Following fecal transplant and establishment of the human microbiota, the humanized animals were either randomly assigned to a de-individualized community, or maintained as independent humanized lines. Newly generated PGF rats, without prior inoculation of the human fecal materials, were also assigned to specific de-individualized communities. The bedding, together with fecal pellets, from each community was mixed and redistributed within that community, to result in coprophagia-mediated de-individualization and the formation of a homogenous profile of the gut microbiota within that community. Animals from De-Individualization Communities and Independent Lines were selected and euthanized at indicated time points, and samples collected for microbiota and host function analyses.

4.5 Metagenomic Analysis of De-Individualized Humanized Rat (DHR) Model

As described above, Sprague Dawley rats were first converted to PGF rats, which contained no culturable microbial cells in the cecal content after 3 days of antibiotic treatment. The PCR products of bacterial 16s rRNA variable regions, however, remained detectable in the fecal content from the treated animals even at the end of the 12-day

antibiotic treatment. This result was not completely unexpected, since PCR is much more sensitive than the culture method. Furthermore, microbial cells that cannot proliferate would still be detectable by the PCR method, as long as the microbial genomic DNA, especially the 16s rRNA gene, remains intact. Since the yield of the PCR products generated from the cecal contents of PGF rats were about a thousand fold less than that of the *naïve* rats at baseline, or the human donor samples, it was concluded that PGF rats were successfully generated. The PCR products generated from the samples of PGF rats were also included in the sequencing experiments to serve as a reference.

Figure 7 shows the result of metagenomic analysis of samples obtained after antibiotic treatment, as well as after fecal transplant using the samples from the humanized line derived from human donor #88. Antibiotic treatment of Sprague Dawley rats resulted in a shift in the alpha diversity with changes in almost all detectable bacterial phyla, compared with the baseline samples from the *naïve* rats. The microbiota community of the baseline rat fecal materials was very different from that of the human donors. However, the fecal microbiota in the humanized rats (transplanted with the fecal material from Donor #88) had a population structure resembling that of the donor sample (note the similarity between the #88-associated rats and Donor #88 in Figure 7). These results thus confirmed that the humanization procedure was highly effective, and successful colonization of human microbiota in the PGF rat hosts was achieved.

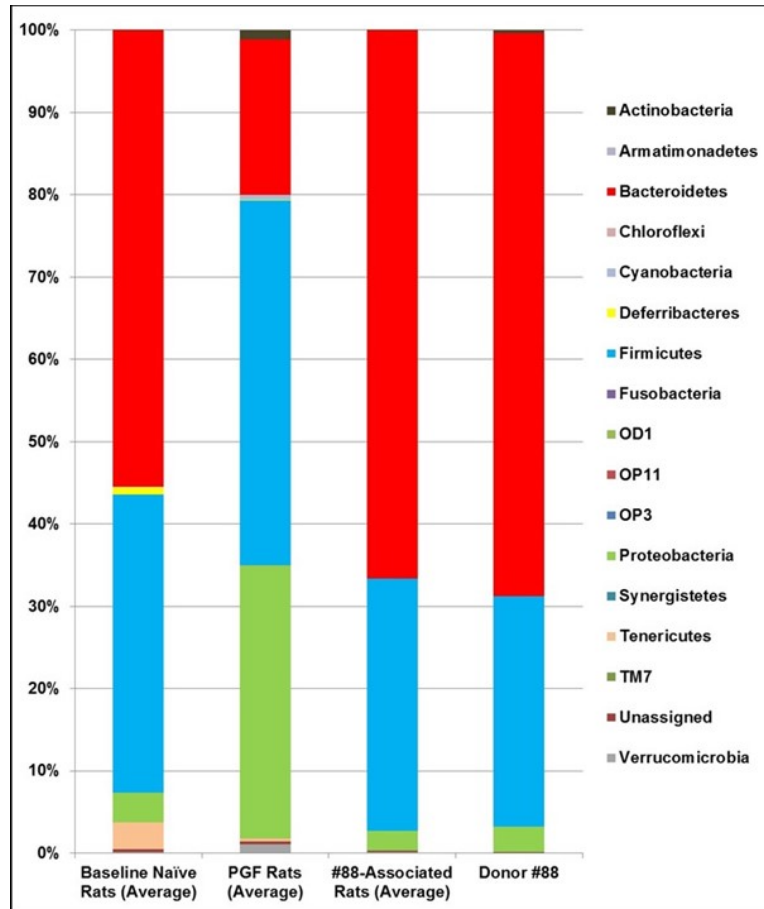


Figure 7. Alpha Diversity of the Fecal Microbiota from *Naïve* Sprague Dawley rats, Pseudo Germ-Free (PGF) Rats, Donor #88-Associated Rats, and Human Donor #88
The relative abundance of detectable bacterial phyla in the fecal microbiota were analyzed using metagenomics as described in Section 3.8. The average relative abundance from naïve rats and from PGF rats are compared with the abundance of the sample from Donor # 88 and the average of the samples from the humanized line generated using the fecal material from this donor. Samples were collected from #88-associated rats at day 40 (i.e. 15 days after the final fecal transplant and 2 days before the start of the de-individualization process).

Fecal microbiota from human donors, *naïve* Sprague Dawley rats, PGF rats, and humanized rats from de-individualized communities and independent humanized lines were also analyzed by metagenomics. Specifically, fecal samples collected from all animal groups at indicated time points throughout the entire experimental period were used, and the PCoA plots of the UniFrac distance metric between samples from different animal groups are shown in Figures 8 – 11.

Figure 8 shows the PCoA plot for data collected during the establishment period (before De-Individualization). All four humanized lines (i.e., #74-, #80-, #88-, and 3-donor-mix-associated lines) formed somewhat overlapping clusters that are distinct from that of the *naïve* Sprague Dawley rats at baseline and the control rats. Of the four humanized

lines, #74-associated rats formed a tight cluster while the clusters of the other three groups were less compact. Most of the human-associated rats (except a couple from the #80-humanized line and one from the mix-humanized line) are close to the human donors and far away from the samples from *naïve* baseline and control rats. This result indicates that the gut microbiota that was established in the PGF rats had greater similarity with the human microbiota than with the native rat microbiota, confirming successful colonization of the rat intestinal tract by the human microbiota.

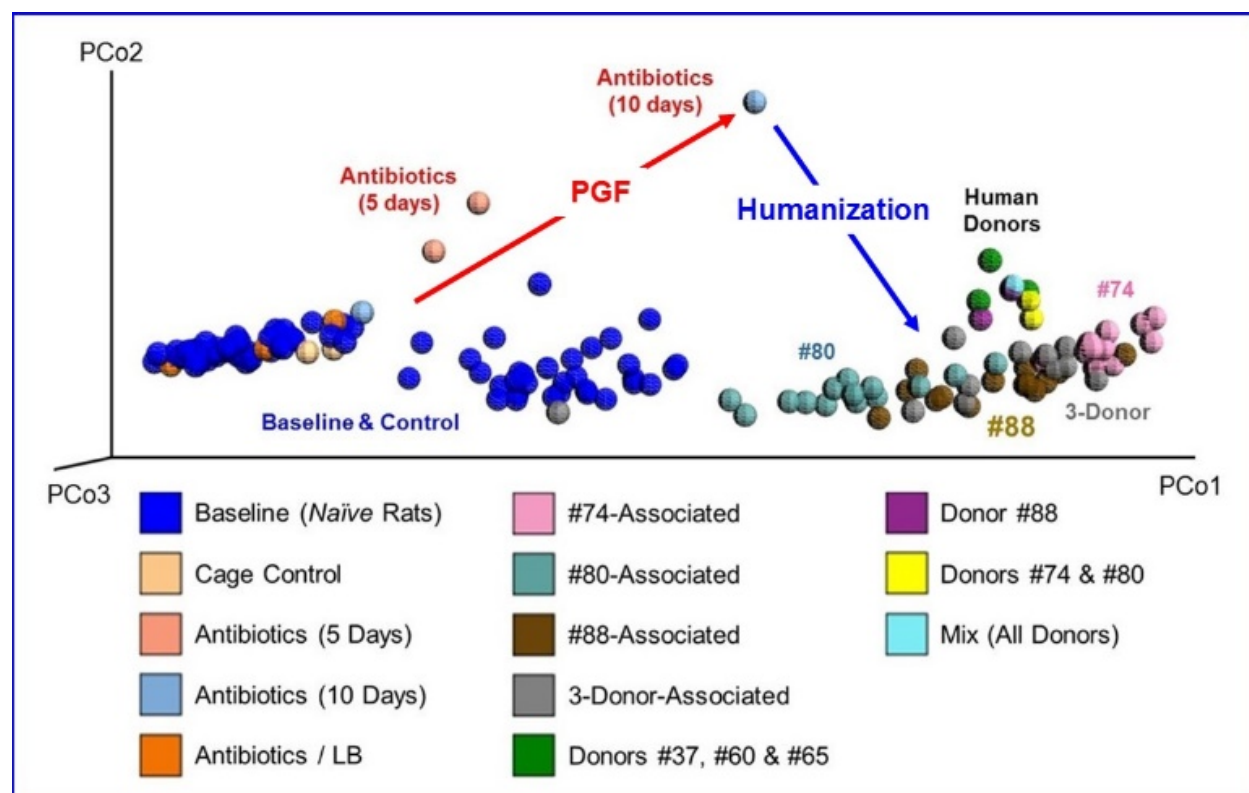


Figure 8. Principal Coordinate Analysis (PCoA) of Microbiota in Fecal Samples from Human Donors, *Naïve* Sprague Dawley Rats, and Humanized Rats, at Various Timepoints throughout the Experiment

Samples were from human-associated rats collected at day 12 of Establishment (2 days before the start of De-Individualization). Cage control rats were not treated with antibiotics nor fecal transplant. Antibiotic / LB refers to rats that were treated with antibiotics but received oral gavages of lysogeny broth with no human fecal material. “3-Donor-Associated” and “3-Donor-Line” indicate the Mix-Associated rats. “Donors” indicates individual fecal material or the mixture prepared from the indicated donors that were used for fecal transplant of PGF rats.

The rats in De-Individualized Community 1 appear to have maintained human microbiota throughout the entire 15-week period (Figure 9), and a similar profile of gut microbiota of human origin emerged in this community at the 10- and 15-week time points of the De-Individualization period. The samples collected at these time points form a tight cluster, indicating relatively similar gut microbiota population in the de-individualized rats

(see the red oval in Figure 9). The gut microbiota in the members of this community however showed a gradual change during the De-Individualization period that appeared to drift toward the clusters of the baseline and control samples. This observation suggests that the gut microbiota of Community 1 might not be completely stable, and its population structure gradually became similar to that of the *naïve* rat over time. Figure 9 also shows that humanization of PGF rats through coprophagia alone was not successful, since all of the samples from that group clustered near and between the baseline and control clusters.

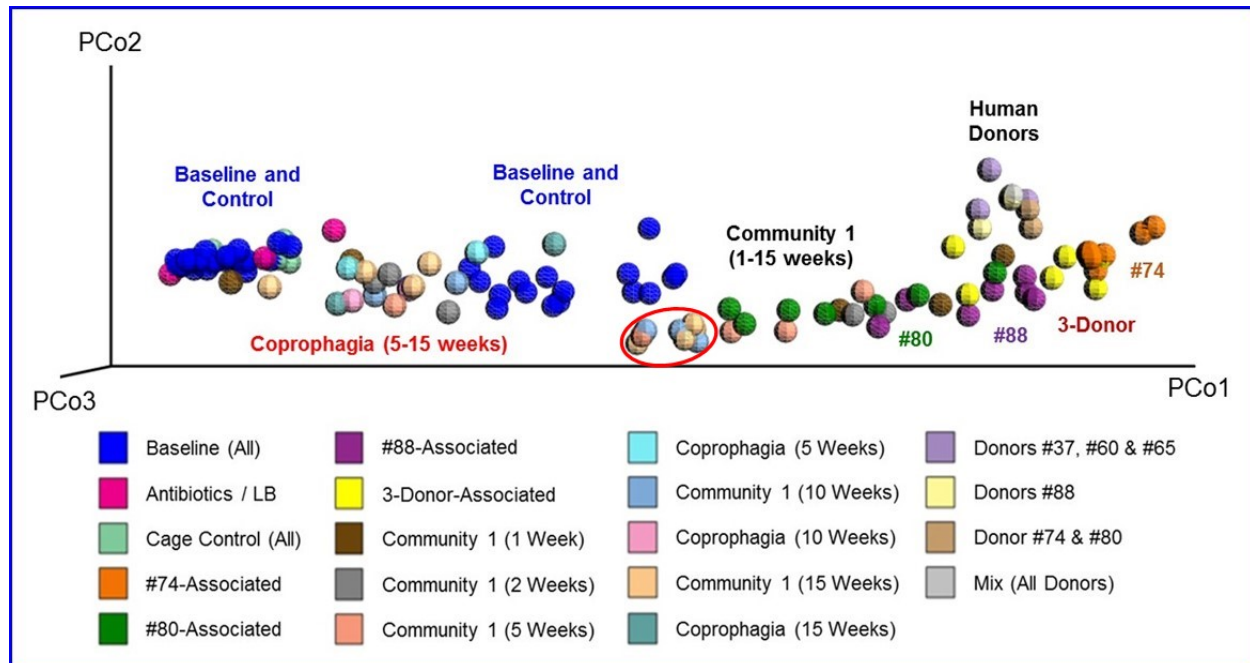


Figure 9. Principal Coordinate Analysis (PCoA) of Microbiota in Fecal Samples from Human Donors, *Naïve* Sprague Dawley Rats, and De-Individualized Community 1, at Different Time-points throughout the Experiment

Samples were from De-Individualized Community 1 collected at weeks 1, 2, 5, 10 and 15 of De-Individualization (red oval encircles samples collected at the last two time points). Cage control rats were not treated with antibiotics nor fecal transplant. Antibiotic / LB refers to rats that were treated with antibiotics but received oral gavages of lysogeny broth with no human fecal material. “3-Donor-Associated” and “3-Donor-Line” indicate the Mix-Associated rats. “Donors” indicates individual fecal material or the mixture prepared from the indicated donors that were used for fecal transplant of PFG rats.

De-Individualized Community 2 showed a similar pattern as Community 1. The emergence of a homogenous profile of gut microbiota in that community however occurred after 5 weeks of De-Individualization and maintained until the end of the experiment (see the red oval in Figure 10). It is not surprising that de-individualization occurred earlier for community 2 because it was developed from two humanized lines, while community 1 utilized all four humanized lines (with total six donors – 3 individual donors and the 3-donor mix). Thus, Community 2 required the merging of fewer and less

diverse microbiota communities. The gut microbiota in this community however also drifted towards the baseline and control cluster, but it remained well separated from that cluster even after 15 weeks. This is probably because the baseline samples from the rats in community 2 were part of the cluster farthest away from the human donors (see Figure 5 and the discussion above). Taking this into consideration, the gut microbiota in Communities #1 and #2 might in fact have a similar degree of continuous, gradual changes throughout the de-individualization period. Nevertheless, these results confirm that human microbiota can be established in PGF rats and maintained for at least 15 weeks. Moreover, the profile of human microbiota generated using different donors can be made into a homogenous community through the sharing of bedding materials that contain fecal pellets.

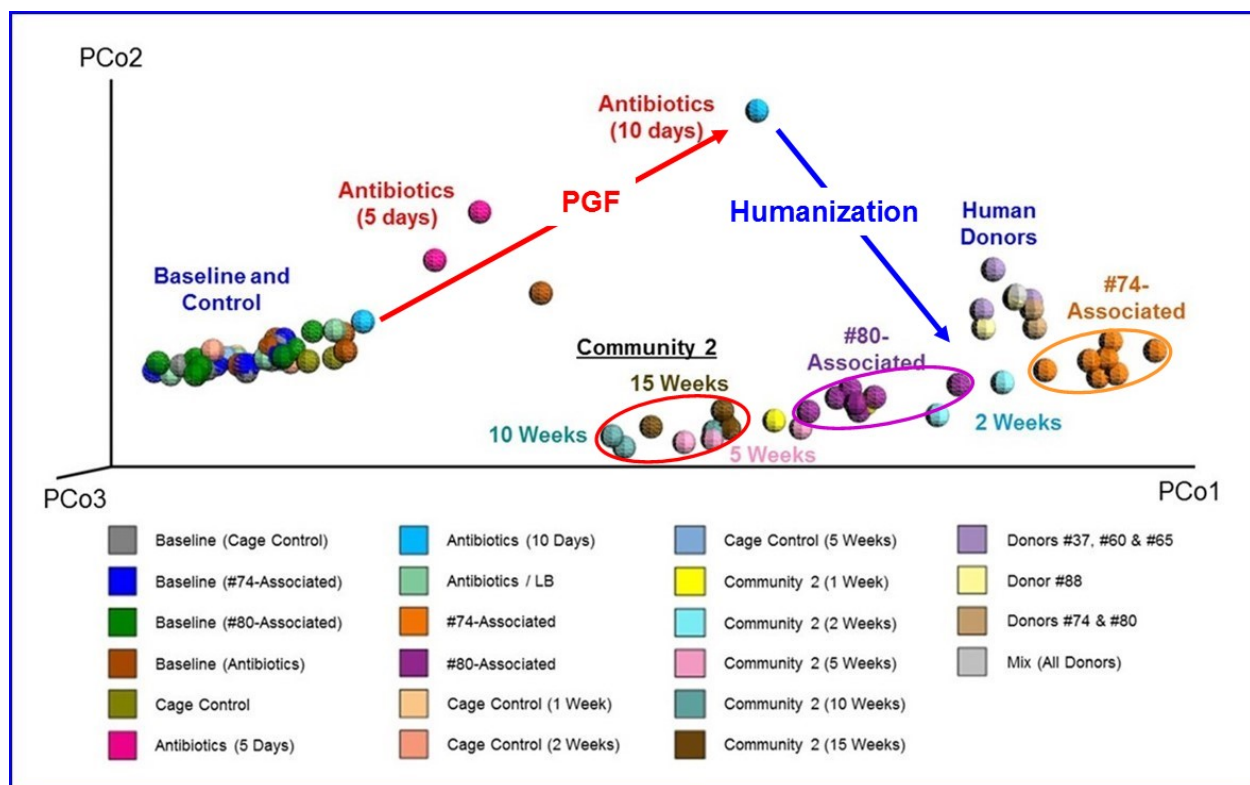


Figure 10. Principal Coordinate Analysis (PCoA) of Microbiota in Fecal Samples from Human Donors, *Naïve* Sprague Dawley Rats, and De-Individualized Community 2, at Different Time-points throughout the Experiment

Samples were from De-Individualized Community 2 collected at weeks 1, 2, 5, 10 and 15 of De-Individualization (red oval encircles samples collected at the last three time points). Cage control rats were not treated with antibiotics nor fecal transplant. Antibiotic / LB refers to rats that were treated with antibiotics but received oral gavages of lysogeny broth with no human fecal material. "Donors" indicates individual fecal material or the mixture prepared from the indicated donors that were used for fecal transplant of PFG rats.

The human microbiota from the rats that were maintained as individual transplanted lines were less stable than that of the two de-individualized communities. As shown in Figure 11, only the samples from #88-transplanted line collected at the 5-week time-point remained separated from the cluster(s) of the baseline and control samples from *naïve* rats. The samples from this transplanted line collected at the 10- and 15-week time points, as well as the samples from the 3-donor mix-associated line, were localized close to the microbiota samples from *naïve* / control rats.

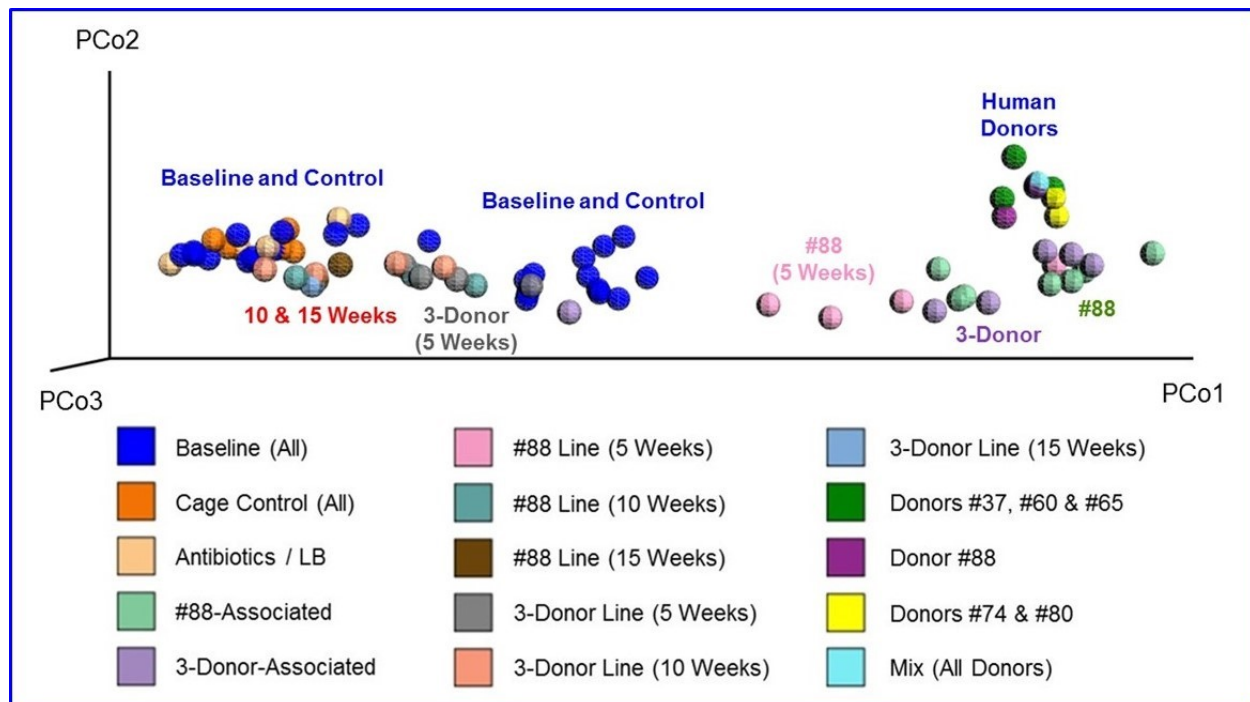


Figure 11. Principal Coordinate Analysis (PCoA) of Microbiota in Fecal Samples from Human Donors, *Naïve* Sprague Dawley Rats, and Individual Humanized Lines, at Different Time-points throughout the Experiment

Samples were from #88-associated and Mix-Associated lines collected at weeks 5, 10 and 15 of De-Individualization. Cage control rats were not treated with antibiotics nor fecal transplant. Antibiotic / LB refers to rats that were treated with antibiotics but received oral gavages of lysogeny broth with no human fecal material. “3-Donor-Associated” and “3-Donor-Line” indicate the Mix-Associated rats. “Donors” indicates individual fecal material or the mixture prepared from the indicated donors that were used for fecal transplant of PFG rats.

4.6 Analysis of Host Functions of De-Individualized Humanized Rat (DHR) Model

Several markers of the host functions and host-microbiota interaction in the DHR rats (including intestinal MPO activity, serum level of LBP, pro-inflammatory cytokines and hormones of the HPA axis) were examined. Overall, the results showed that there were no biologically significant changes that would indicate a negative reaction to the humanization process or a significant change in host functions or interaction with the gut microbiota. MPO activity in the intestinal tissues showed no significant change except for

community #1 at 15 weeks, which had an increase in activity in the small intestine, compared with other groups. This might indicate a reaction to the human microbiota (Table 2).

Table 2. Myeloperoxidase (MPO) Activity in Small Intestine Tissue Homogenate from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR)

Experimental Group	MPO Activity (μ U/mg Tissue)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	0.019 $\pm$ 0.002	0.028 $\pm$ 0.003	0.018 $\pm$ 0.003	0.010 $\pm$ 0.001	0.007 $\pm$ 0.000
De-Individualized Community #1	0.024 $\pm$ 0.002	0.030 $\pm$ 0.005	0.019 $\pm$ 0.002	0.011 $\pm$ 0.001	0.015 $\pm$ 0.001**
De-Individualized Community #2	0.030 $\pm$ 0.005	0.033 $\pm$ 0.003	0.028 $\pm$ 0.004	0.017 $\pm$ 0.005	0.009 $\pm$ 0.000
#88-Associated Line	N/A	0.026 $\pm$ 0.005	0.022 $\pm$ 0.002	0.008 $\pm$ 0.001	0.006 $\pm$ 0.000
3-Donor-Associated Line	N/A	0.027 $\pm$ 0.000	0.019 $\pm$ 0.003	0.010 $\pm$ 0.003	0.007 $\pm$ 0.001

*Value=Mean $\pm$ SE; 1 U of MPO activity = Amount of MPO needed to convert 1.0 μ mol of substrate to product per minute at 25°C; **difference from Cage Control is statistically significant ($p < 0.05$).

There was no change in the serum level of LBP, suggesting no increased production by the human microbiota or leakage of human microbiota or its endotoxins through the gastrointestinal tract of the rat hosts during the de-individualization period (Table 3). This was confirmed by the culturing data that did not find any bacterial cells in any of the extra-intestinal tissues including mesenteric layer, mesenteric lymph nodes, spleen and liver (data not shown).

Table 3. Serum Level of Lipopolysaccharide Binding Protein (LBP) in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR)

Experimental Group	LBP (μ g/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	10.87 $\pm$ 0.34	13.38 $\pm$ 2.37	6.93 $\pm$ 1.48	9.66 $\pm$ 0.53	4.21 $\pm$ 0.27
De-Individualized Community #1	4.41 $\pm$ 1.10	6.05 $\pm$ 1.58	4.37 $\pm$ 1.61	10.58 $\pm$ 2.11	2.99 $\pm$ 0.35
De-Individualized Community #2	10.98 $\pm$ 6.46	7.89 $\pm$ 0.70	10.43 $\pm$ 4.79	8.45 $\pm$ 2.91	7.59 $\pm$ 1.28
#88-Associated Line	N/A	6.97 $\pm$ 1.53	9.46 $\pm$ 3.51	9.67 $\pm$ 1.71	4.87 $\pm$ 1.91
3-Donor-Associated Line	N/A	9.19 $\pm$ 3.51	6.64 $\pm$ 0.34	5.70 $\pm$ 2.55	2.56 $\pm$ 1.21

* Value=Mean $\pm$ SE

For the hormones of the HPA axis, only sporadic/ inconsistent changes in the level of ACTH and corticosterone occurred, and were not likely a specific stress response to the experimental procedure or the colonization of the human microbiota (Tables 4a – 4c). For examples, in the #88-Associated Line, ACTH was significantly higher at 2 weeks and lower at 5 weeks than the Cage Control, and corticosterone levels were significantly higher than Control at 10 weeks. De-Individualized Community 1 had a significantly higher level of ACTH than the Cage Control at 15 weeks. The 3-donor mix-associated line had

significantly higher corticosterone than the Control at 15 weeks. Since these changes do not follow a specific pattern and do not correlate precisely with other markers, the biological implication of these changes is not completely understood at this point. There were small changes in some of the cytokines tested, but none were statistically significant after the correction for multiple testing (see Tables 5a – 5i).

Table 4. Serum Levels of Hypothalamic–Pituitary–Adrenal (HPA) Axis Hormones in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR)

4a. Peripheral Corticotropin-Releasing Factor

Experimental Group	Peripheral CRF Concentration (pg/μl)*				
	Week 1	Week 2	Week 5	Week 11	Week 17
Cage Control	0.107±0.004	0.111±0.004	0.099±0.003	0.118±0.007	0.106±0.000
De-Individualized Community #1	0.102±0.004	0.109±0.003	0.111±0.003	0.111±0.005	0.108±0.003
De-Individualized Community #2	0.113±0.012	0.103±0.005	0.102±0.001	0.105±0.001	0.114±0.003
#88-Associated Line	N/A	0.114±0.007	0.101±0.002	0.112±0.004	0.132±0.016
3-Donor-Associated Line	N/A	0.101±0.007	0.099±0.001	0.148±0.030	0.108±0.003

*Value=Mean±SE

4b. Adrenocorticotrophic Hormone

Experimental Group	ACTH (pg/μl)*				
	Week 1	Week 2	Week 5	Week 11	Week 17
Cage Control	7.04±1.52	3.36±0.71	9.03±1.01	8.97±2.72	7.20±1.36
De-Individualized Community #1	6.27±0.94	4.53±0.36	6.52±1.22	9.34±2.07	14.66±2.22**
De-Individualized Community #2	5.65±0.73	2.17±1.00	10.09±4.38	7.27±0.66	7.40±1.76
#88-Associated Line	N/A	12.70±3.22**	3.94±0.81**	12.49±4.30	13.39±2.12
3-Donor-Associated Line	N/A	2.36±0.63	8.69±4.94	8.07±1.59	10.49±0.00

*Value=Mean±SE; **difference from Cage Control is statistically significant ($p<0.05$).

4c. Corticosterone

Experimental Group	Corticosterone Concentration (pg/μl)*				
	Week 1	Week 2	Week 5	Week 11	Week 17
Cage Control	169.8±101.0	421.0±58.1	247.4±32.9	95.8±15.2	138.7±3.6
De-Individualized Community #1	229.8±19.4	620.6±122.1	235.1±44.9	99.5±21.0	166.6±12.8
De-Individualized Community #2	345.0±134.1	284.1±21.3	133.3±18.6	110.2±23.9	111.2±44.8
#88-Associated Line	N/A	229.7±23.7	395.3±71.1	213.5±74.9**	195.5±28.1
3-Donor-Associated Line	N/A	287.2±19.2	291.1±29.2	91.8±8.4	230.7±37.3**

*Value=Mean±SE; **difference from Cage Control is statistically significant ($p<0.05$).

Table 5. Serum Levels of Pro- and Anti-Inflammatory Cytokines in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR)

5a. IFN γ

Experimental Group	IFN γ (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	32.16 $\pm$ 1.99	26.33 $\pm$ 1.45	27.11 $\pm$ 1.34	23.20 $\pm$ 3.66	32.48 $\pm$ 0.44
De-Individualized Community #1	33.14 $\pm$ 0.78	33.09 $\pm$ 1.42	30.12 $\pm$ 2.80	35.21 $\pm$ 1.18	29.07 $\pm$ 1.92
De-Individualized Community #2	32.75 $\pm$ 0.06	35.53 $\pm$ 1.06	34.08 $\pm$ 5.19	26.84 $\pm$ 1.53	27.88 $\pm$ 0.34
#88-Associated Line	N/A	28.53 $\pm$ 0.08	28.74 $\pm$ 4.52	30.61 $\pm$ 3.68	40.29 $\pm$ 8.73
3-Donor-Associated Line	N/A	26.34 $\pm$ 1.10	31.86 $\pm$ 2.25	25.15 $\pm$ 2.37	28.81 $\pm$ 0.39

*Value=Mean $\pm$ SE

5b. IL-1 β

Experimental Group	IL-1 β (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	143.54 $\pm$ 0.64	131.92 $\pm$ 1.37	142.68 $\pm$ 5.65	143.44 $\pm$ 6.23	145.83 $\pm$ 2.27
De-Individualized Community #1	147.06 $\pm$ 1.83	143.45 $\pm$ 2.16	141.09 $\pm$ 3.03	149.09 $\pm$ 3.74	143.65 $\pm$ 2.14
De-Individualized Community #2	143.38 $\pm$ 9.38	153.52 $\pm$ 3.15	156.63 $\pm$ 15.74	140.97 $\pm$ 1.67	136.12 $\pm$ 9.66
#88-Associated Line	N/A	133.65 $\pm$ 0.58	138.97 $\pm$ 7.37	148.03 $\pm$ 9.80	157.31 $\pm$ 10.14
3-Donor-Associated Line	N/A	131.55 $\pm$ 1.05	142.22 $\pm$ 3.68	140.66 $\pm$ 3.13	139.95 $\pm$ 3.44

*Value=Mean $\pm$ SE

5c. IL-4

Experimental Group	IL-4 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	22.17 $\pm$ 4.25	21.04 $\pm$ 1.22	20.78 $\pm$ 0.26	24.91 $\pm$ 2.81	22.82 $\pm$ 7.30
De-Individualized Community #1	21.21 $\pm$ 2.26	21.96 $\pm$ 1.68	19.56 $\pm$ 2.06	20.66 $\pm$ 2.51	19.56 $\pm$ 1.22
De-Individualized Community #2	25.69 $\pm$ 0.52	26.47 $\pm$ 1.43	22.74 $\pm$ 0.38	17.30 $\pm$ 6.40	19.22 $\pm$ 2.26
#88-Associated Line	N/A	21.654.56 $\pm$	22.56 $\pm$ 2.37	24.00 $\pm$ 1.65	22.63 $\pm$ 1.37
3-Donor-Associated Line	N/A	12.52 $\pm$ 5.16	22.22 $\pm$ 1.21	28.30 $\pm$ 0.93	21.19 $\pm$ 6.32

*Value = Mean $\pm$ SE

5d. IL-5

Experimental Group	IL-5 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	22.92±4.37	15.64±1.30	14.45±0.53	17.19±4.34	26.59±7.89
De-Individualized Community #1	24.40±1.23	21.42±4.26	24.44±3.75	27.88±2.83	26.49±2.38
De-Individualized Community #2	20.45±3.70	35.15±11.79	21.36±3.39	23.22±2.52	24.01±6.79
#88-Associated Line	N/A	25.87±5.20	22.18±4.75	19.38±4.02	26.87±5.33
3-Donor-Associated Line	N/A	15.46±0.68	19.87±4.67	26.26±6.84	31.07±8.44

*Value = Mean±SE

5e. IL-6

Experimental Group	IL-6 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	899.9±88.3	748.2±35.6	764.1±56.3	746.3±104.3	930.7±54.1
De-Individualized Community #1	926.0±33.0	953.4±58.6	924.3±75.4	960.9±48.3	880.5±27.1
De-Individualized Community #2	878.4±75.4	979.3±152.5	910.0±149.6	897.7±73.1	801.6±41.0
#88-Associated Line	N/A	648.0±87.6	981.4±17.9	773.7±64.1	904.3±16.9
3-Donor-Associated Line	N/A	823.2±72.9	768.9±88.7	910.0±124.9	1096.5±203.4

*Value = Mean±SE

5f. CXCL1

Experimental Group	CXCL1 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	422.7±26.7	380.5±24.7	434.2±77.6	458.8±143.6	450.8±169.1
De-Individualized Community #1	472.8±69.6	478.1±72.5	497.8±46.9	461.3±43.0	451.7±65.6
De-Individualized Community #2	263.1±1.2	911.3±62.0	639.1±76.4	510.0±88.6	291.2±50.1
#88-Associated Line	N/A	292.6±22.8	533.4±43.9	475.5±118.0	302.1±4.0
3-Donor-Associated Line	N/A	247.0±123.4	372.0±93.7	364.8±113.9	742.7±228.0

*Value = Mean±SE

5g. IL-10

Experimental Group	IL-10 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	132.06±20.86	108.97±9.70	185.62±29.39	138.99±35.05	106.20±13.85
De-Individualized Community #1	122.82±24.52	153.02±13.65	157.85±13.37	112.95±11.33	130.94±16.37
De-Individualized Community #2	99.27±4.16	153.99±51.95	135.75±52.02	117.74±20.86	117.28±9.83
#88-Associated Line	N/A	121.44±18.01	146.37±30.86	181.93±20.55	158.84±18.01
3-Donor-Associated Line	N/A	85.42±14.69	120.98±7.26	136.21±29.24	113.82±3.46

*Value = Mean±SE

5h. IL-13

Experimental Group	IL-13 (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	25.69±0.19	24.86±0.24	25.22±0.05	24.56±0.48	26.39±0.53
De-Individualized Community #1	25.99±0.20	25.92±0.58	25.87±0.40	26.23±0.27	26.41±0.38
De-Individualized Community #2	25.57±0.24	26.50±0.43	25.63±0.41	26.23±0.26	25.74±0.54
#88-Associated Line	N/A	24.68±0.31	26.05±0.43	25.26±0.64	25.95±0.16
3-Donor-Associated Line	N/A	25.30±0.63	25.54±0.52	26.22±0.77	26.38±0.11

*Value = Mean±SE

5i. TNFα

Experimental Group	TNFα (pg/ml)*				
	Week 1	Week 2	Week 5	Week 10	Week 15
Cage Control	15.81±1.29	13.00±0.36	13.61±1.34	14.06±3.66	15.84±0.44
De-Individualized Community #1	15.92±0.35	15.47±0.86	15.50±2.80	16.00±1.18	17.15±1.92
De-Individualized Community #2	16.19±0.12	15.44±0.32±	15.26±5.19	16.36±1.53	16.51±0.34
#88-Associated Line	N/A	14.21±0.05	16.19±2.25	16.36±2.37	14.90±0.39
3-Donor-Associated Line	N/A	14.32±1.00	14.96±4.52	17.20±3.68	16.08±8.73

*Value = Mean±SE

Taken together, these results strongly suggest that the human microbiota likely interacts with the rat intestinal tract in a similar manner as the native rat microbiota, and is unlikely to cause any significant change in host functions.

4.7 Effect of Acute Sleep Deprivation on Gut Microbiota and Host Functions

4.7.1. Generation and Acute Sleep Deprivation Treatment of De-Individualized Humanized Rats (DHR)

Since the human gut microbiota can be stably maintained in the rat hosts for up to 15 weeks, it was decided to generate and use new sets of DHR for the sleep deprivation experiments. The overall design of the acute sleep deprivation experiment is shown in Figure 12. Initially, PGF and DHR were generated using the procedure as described in the Sections 3.5 and 3.7. Once the humanization and the de-individualization processes were confirmed, the DHR were subjected to 6 days of acute sleep deprivation (24 hours/day), using the Sleep Fragmentation Chamber (from Lafayette Instruments, Lafayette, IN, see Figure 13). Although this system is not as effective as the “flower pot” model in interfering with the rapid eye movement sleep of the animals, it is the only system that can be safely used in the germ-free isolators. In addition to DHR, control and sleep deprived groups of native Sprague Dawley rats were also included in this experiment. Thus, there were four animals groups in this experiment (Table 6). This design is based

on the expectation that the native rat groups will provide references for the sleep deprivation effect, which will facilitate the interpretation of the results of DHR groups.

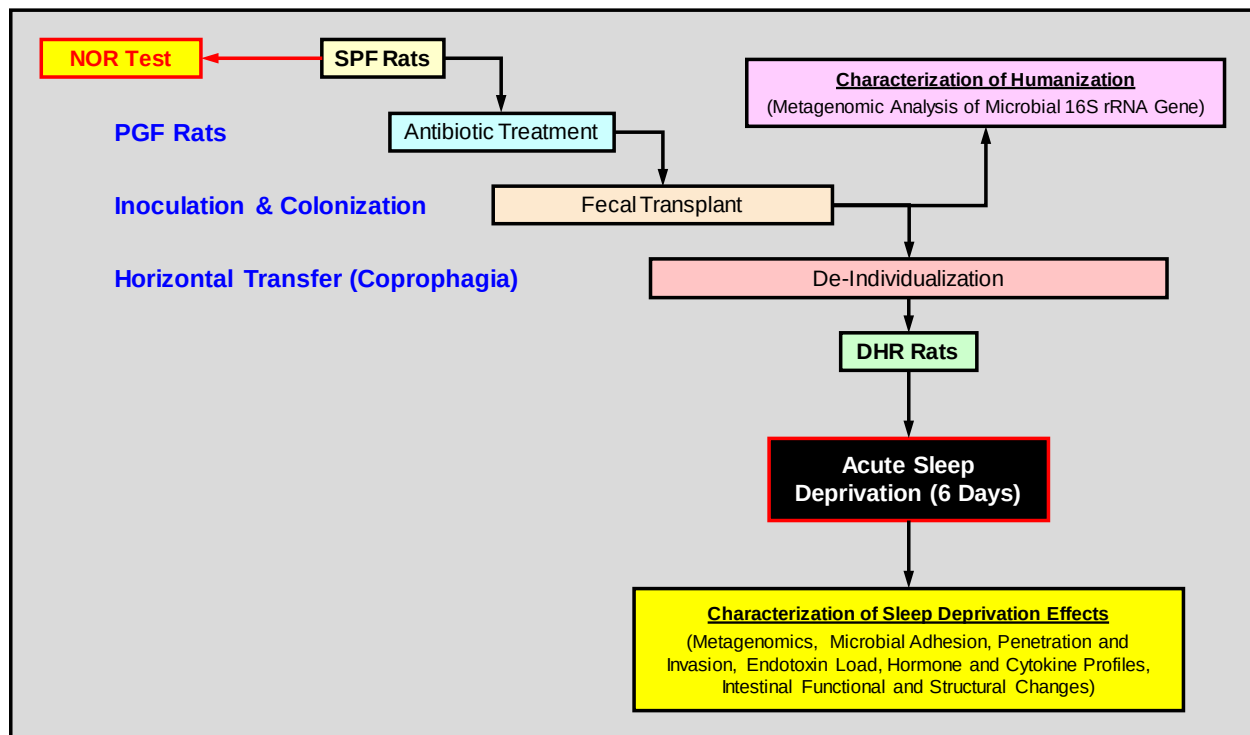


Figure 12. Overall Design of Acute Sleep Deprivation Experiment

The generation of PGF and DHR were as described in Sections 3.5 and 3.7. After the completion of the humanization and the de-individualization processes, the DHR (and native Sprague Dawley rats) were subjected to 6 days of acute sleep deprivation. Upon the completion of the sleep deprivation treatment, all animals were euthanized, and samples collected for microbiota and host function analyses.

Table 6. Experimental Groups of Acute Sleep Deprivation Experiment

Group	Humanization	Sleep Deprivation	Description
1	No	No	Control (for sleep deprivation and humanization)
2	No	Yes	Test (sleep deprivation); Control (for humanization)
3	Yes	No	Test (humanization); Control (for sleep deprivation)
4	Yes	Yes	Test (humanization); Test (sleep deprivation)



Figure 13. Sleep Fragmentation Chamber from Lafayette Instruments (Lafayette, Indiana)

The equipment contains an automated swipe bar. The movement of the bar will awake the rat subject, thereby resulting in sleep deprivation.

4.7.2. Effect of Acute Sleep Deprivation on Gut Microbiota of Native Sprague Dawley and De-Individualized Humanized Rat (DHR) Models

Upon the completion of the 6-day sleep deprivation treatment, all animal groups (including control native Sprague Dawley rats, sleep deprived native rats, control DHR, and sleep deprived DHR) were euthanized. Intestinal contents and tissue samples were collected, preserved and analyzed to investigate the effects of acute sleep deprivation on the gut microbiota and host functions (including metagenomic analysis of the intestinal contents, microbial adhesion, penetration and invasion, mucosal Inflammation, bacterial endotoxin load, cytokine profiles and HPA axis functions).

Metagenomic analysis revealed that significant changes in the alpha diversity of the gut microbiota can be detected in both native Sprague Dawley and DHR after six days of sleep deprivation. In the small intestine, the complexity of the microbiota population were reduced. Comparing these two animal models, the change in the native Sprague Dawley rats is much more dramatic than that in the DHR (see Figure 14). Surprisingly, an opposite change direction, an increase in the alpha diversity, was observed in the cecal content from both animal models (Figure 15). The large intestinal microbiota showed a pattern of changes very similar to that of small intestinal content (Figure 16). This result demonstrates that there are likely regional specific patterns of changes in the microbiota population along the intestinal tract after acute sleep deprivation, and strong suggests that analyzing the intestinal content from multiple regions systemically is needed in order to have a better understanding of microbiota perturbation following acute sleep deprivation.

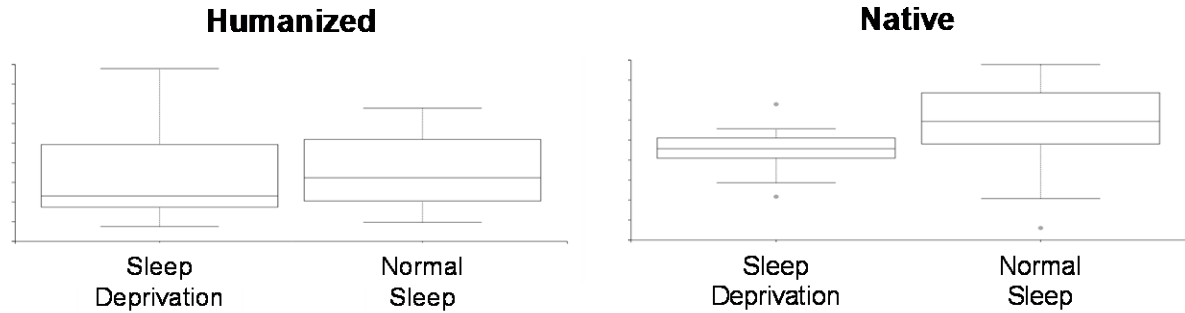


Figure 14. Alpha Diversity Boxplot of Small Intestinal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

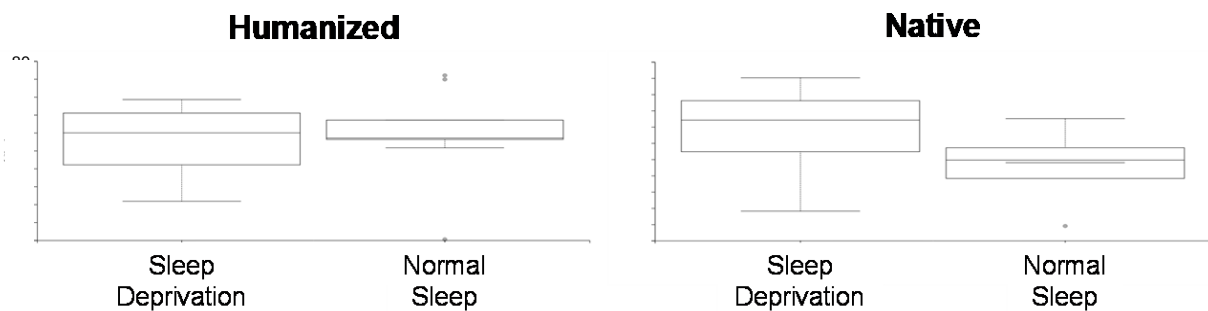


Figure 15. Alpha Diversity Boxplot of Cecal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

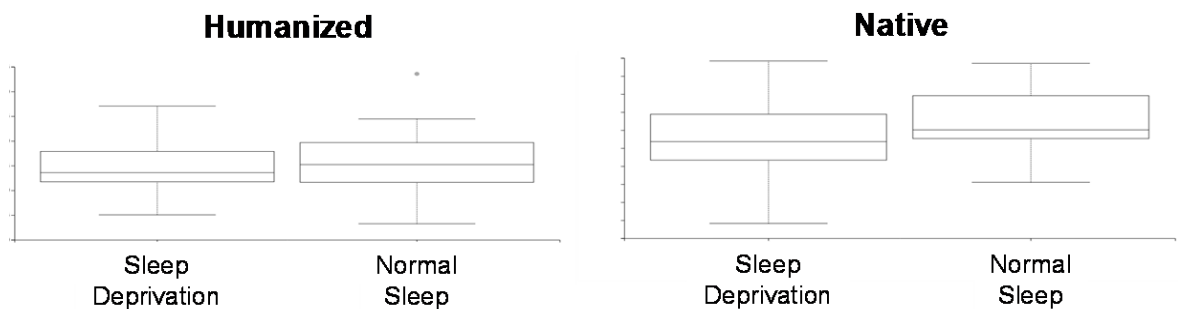


Figure 16. Alpha Diversity Boxplot of Large Intestinal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

Principal Coordinate Analysis of the UniFrac distance metric of the microbiota from native Sprague Dawley and DHR with or without acute sleep deprivation was also performed, and the results are shown in Figures 17 – 19. Overall, no clear separation of the clusters derived from normal sleep and sleep-deprived animals can be observed in

either animal model. Rotating the axes in different directions did not significantly improve the separation of the clusters. In the small intestine, the microbiota from animals with normal sleep forms a tighter cluster, while that from sleep-deprived animals scatter in a large area (Figure 17). However, such difference between normal sleep and sleep-deprived animals was not observed in the microbiota from the cecum or the large intestine, and they both form loose clusters with significant overlapping, (Figures 18 and 19). This result further confirms the existence of regional specific patterns of changes in the microbiota population along the intestinal tract. It also showed that acute sleep deprivation only caused subtle changes in the microbiota regardless of the animal model or the spatial locations in the intestinal tract.

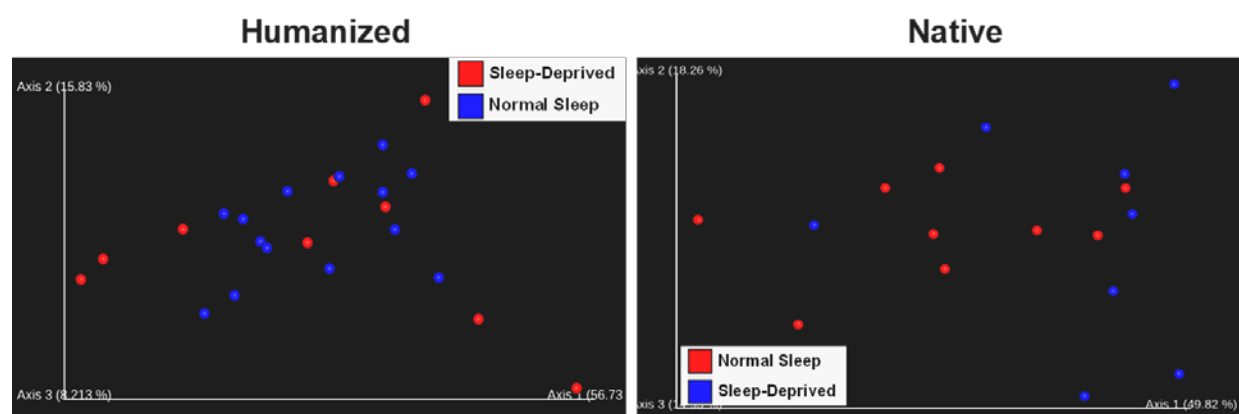


Figure 17. Principal Coordinate Analysis (PCoA) of Small Intestinal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

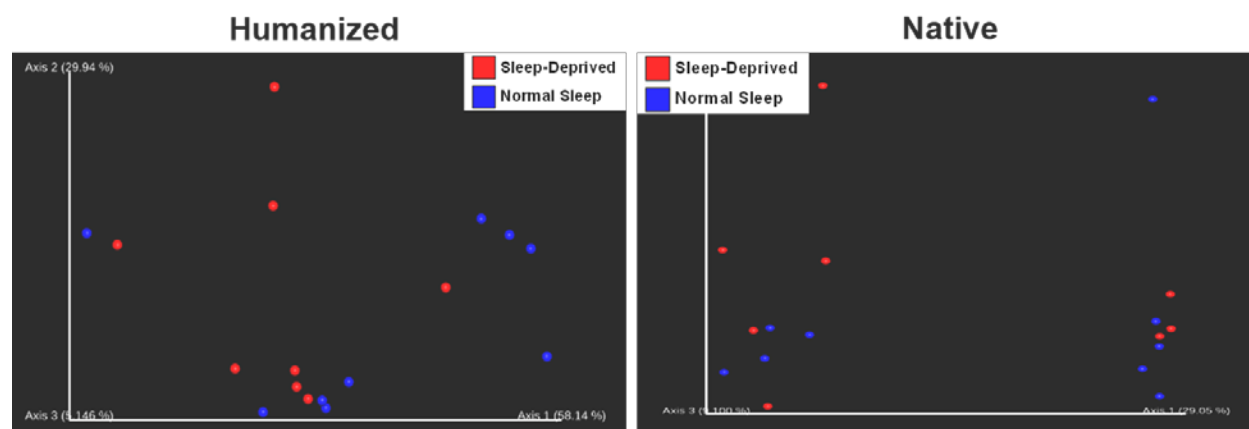


Figure 18. Principal Coordinate Analysis (PCoA) of Cecal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

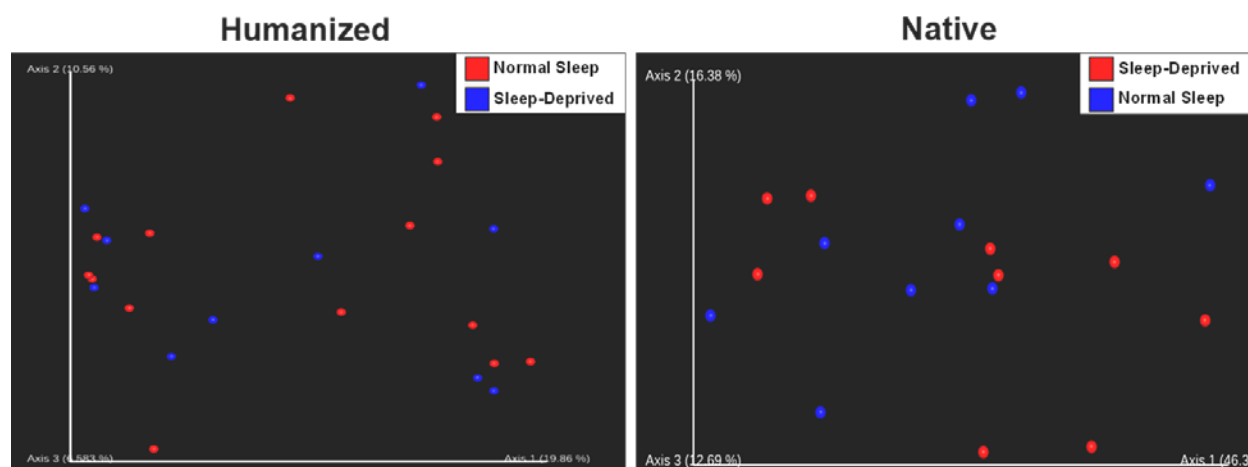


Figure 19. Principal Coordinate Analysis (PCoA) of Large Intestinal Microbiota of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats with Normal Sleep or Acute Sleep Deprivation

Even though no separation between the normal sleep and the sleep-deprived groups in the PCoA plots, further analysis revealed that concordant changes were detectable in the small intestinal microbiota after acute sleep deprivation in both the DHR and the native Sprague Dawley rats at all taxonomic levels. Representative results of bacterial families and genera with concordant changes are shown in Figures 20 and 21. However, there are also bacterial taxa showed changes in opposite directions (e.g. an unassigned phylum of Bacteroidaceae - the light yellow bar) between the native and DHR models.

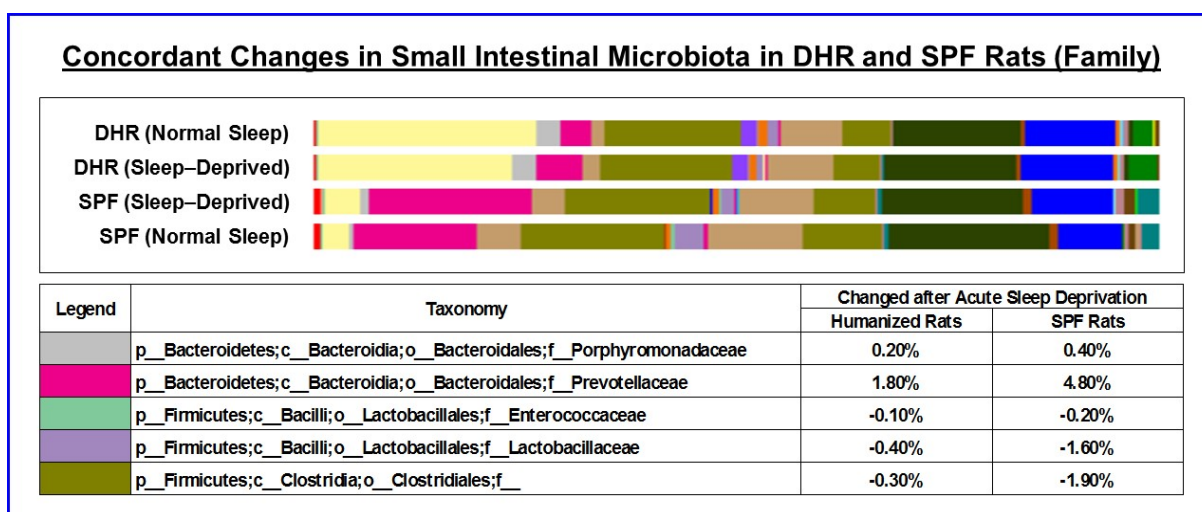


Figure 20. Concordant changes in Small Intestinal Bacterial Families of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats after Acute Sleep Deprivation

Five bacterial families showed concordant changes in DHR and native Sprague Dawley rats after acute sleep Deprivation.

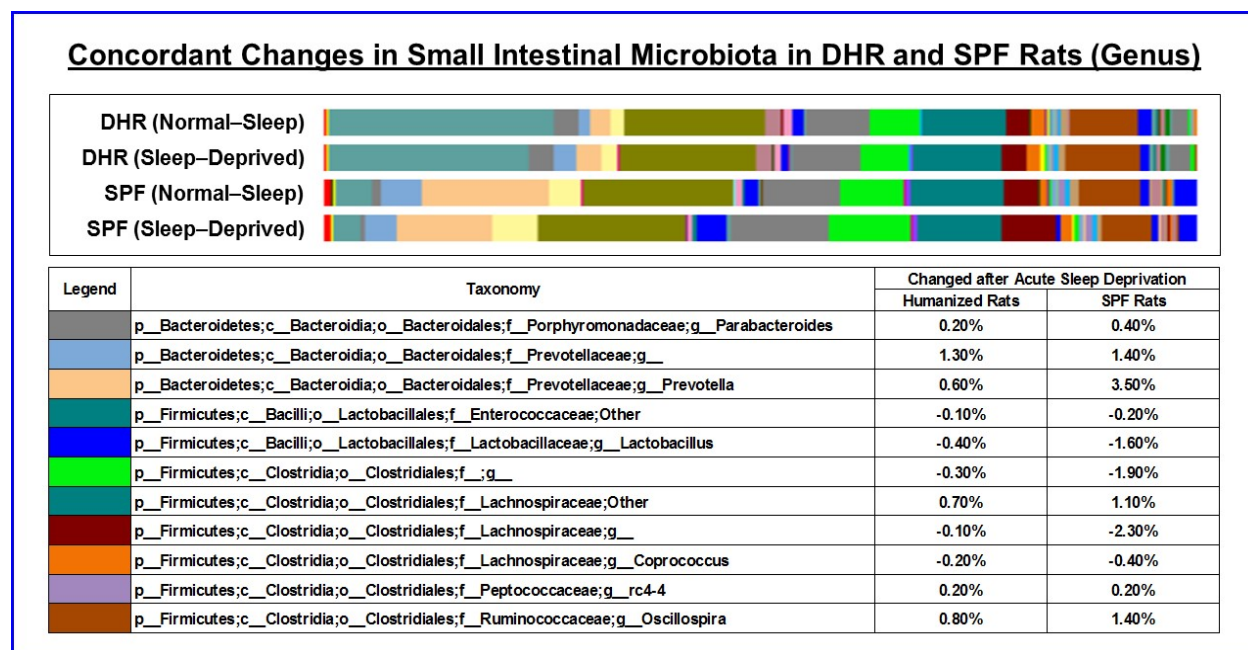


Figure 21. Concordant Changes in Small Intestinal Bacterial Genera of De-Individualized Humanized Rats (DHR) and Native Sprague Dawley Rats after Acute Sleep Deprivation

Eleven bacterial genera showed concordant changes in DHR and native Sprague Dawley rats after acute sleep Deprivation.

We subsequently analyzed the data set using the Gneiss analysis tool to identify the top 10 significantly altered microbial taxa. In the small intestine of DHR, acute sleep deprivation mainly resulted in a decrease in *Lactobacillus* and several undefined bacterial taxa (Figure 22). A completely different result was detected in the native Sprague Dawley rats, an increase in the *Turicibacter* were frequently observed, although several undefined bacterial taxa were also increased. Interestingly, both *Lactobacillus* and *Turicibacter* belong to the phylum Firmicutes, suggesting that this phylum is probably more susceptible to the influence of acute sleep deprivation.

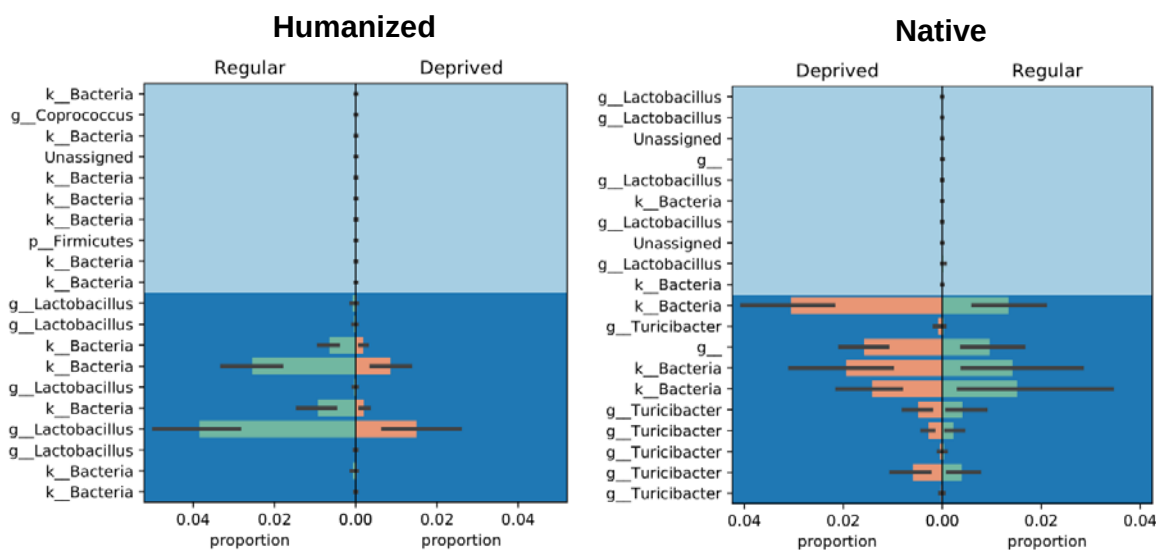


Figure 22. Top Ten Most Increased and Decreased Microbial Taxa in Small Intestinal Microbiota of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

Compared with the small intestinal microbiota, more dramatic changes in the cecal microbiota were observed in the DHR, in which *Succinivibrio* and *Bacteroides* were significantly decreased, while increases in *Bacteroidales* and *Firmicutes* were observed. As above, changes in undefined taxa were frequently observed. In contrast to the DHR, the cecal microbiota from native Sprague Dawley rats showed a relatively simple pattern in which *Clostridia* was significantly decreased (Figure 23).

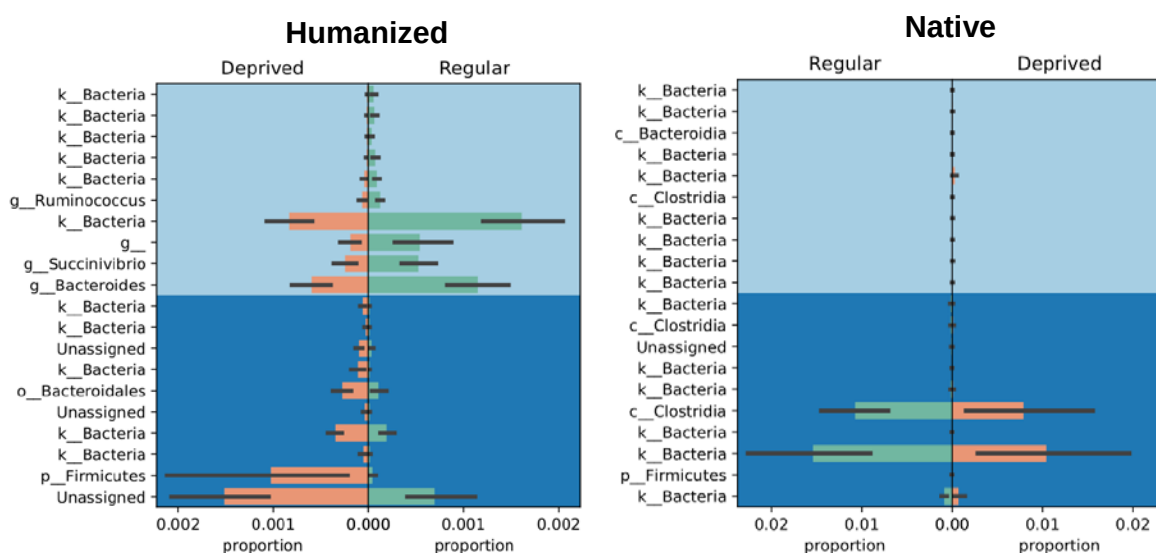


Figure 23. Top Ten Most Increased and Decreased Microbial Taxa in Cecal Microbiota of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

Interestingly, the large intestinal content from the DHR and native Sprague Dawley rats showed a very similar pattern with one taxon showing a dramatic decrease after acute sleep deprivation (Figure 24). Besides the large decrease in Oscillospira, acute sleep deprivation also resulted in a small decrease in Enterobacteriaceae and a small increase in Lachnospiraceae in DHR. Except an undefined bacterial taxon, only a decrease in Lactobacillus was detected in the large intestinal content from the native rats.

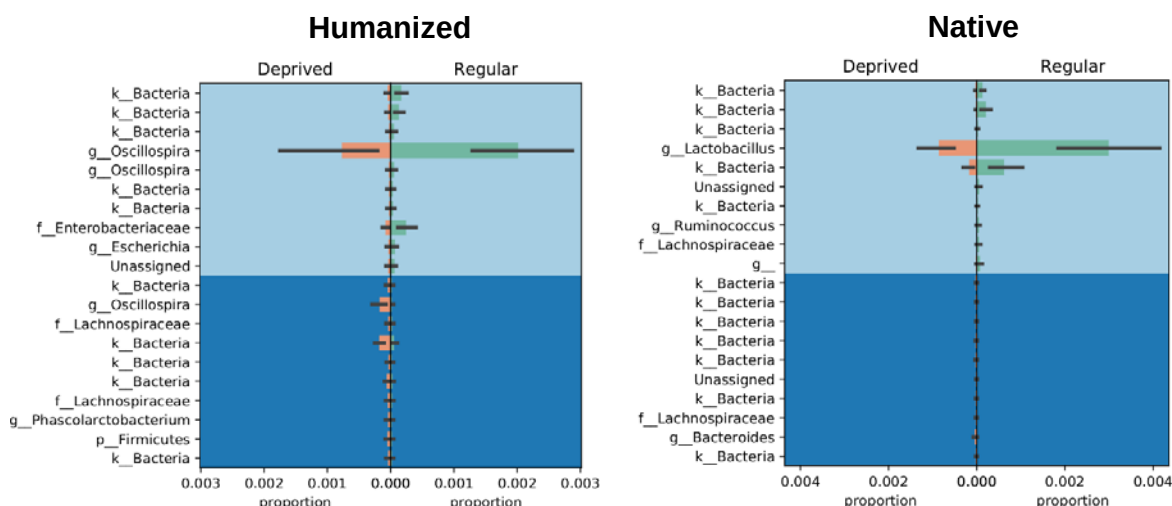


Figure 24. Top Ten Most Increased and Decreased Microbial Taxa in Large Intestinal Microbiota from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

To further explore the effect of acute sleep deprivation on gut microbiota population, the abundancy of all detectable OTUs in the large intestinal contents from sleep-deprived groups was compared with the animal groups with normal sleep, using the non-parametric Kruskal-Wallis H Test, with multiple testing correction. It was found that 348 and 279 OTUs in the large intestine were significantly altered after acute sleep deprivation in the native Sprague Dawley and DHR, respectively. However, further analysis revealed that the taxonomic profiles of the altered microbial taxa in these two animal models were very similar, due to the fact that multiple OTUs may map to microbial taxa of identical species, genus, family, and so on. For instance, eight taxa, two from Bacteroidetes (Rikenellaceae and S24-7), five from Firmicutes (Lachnospiraceae, Peptococcaceae, and three Ruminococcaceae families), and one from TM7 (F16) accounted for more than 80% of the microbiota populations (and OTUs) from the native SPF and DHR. In fact, the family S24-7 of Bacteroidetes represented more than 40% of the altered microbial OTUs induced by acute sleep deprivation in both native SPF and DHR. In contrast, there were only five unique taxa that were altered after acute sleep deprivation in these two animal models, with four in native SPF and one in DHR, as shown below.

a. Unique in Native Sprague Dawley Rats

- i. Actinobacteria; c_OPB41; o_; f_; g_; s_
- ii. Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_uniformis
- iii. Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Coprococcus
- iv. Firmicutes; c_Erysipelotrichi; o_Erysipelotrichales; f_Erysipelotrichaceae; g_Coprobacillus

b. Unique in De-Individualized Humanized Rats (DHR)

- i. Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_ovatus

In-depth analyses also showed that about 50% of the OTUs in the significantly changed microbiota population underwent opposite directions between the native SPF and DHR (see Groups 7 and 8 in Table 7). The representative families of specific phyla in each of these groups are shown in Table 8.

Table 7. Number of Significantly Changed Microbial Taxa (Operational Taxonomic Units, OTUs) in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

Group	Native Rats	DHR Rats	# OTUs
1	Increased	Increased	53
2	Decreased	Decreased	56
3	Increased	Absent	43
4	Decreased	Absent	26
5	Absent	Increased	2
6	Decreased	No Change	2
7	Decreased	Increased	30
8	Increased	Decreased	138
Total		350	

Table 8. Altered Bacterial Families (or Orders) in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

Group 1	Bacteroidetes (f_Bacteroidaceae, f_Rikenellaceae and f_S24-7)
	Firmicutes (f_Christensenellaceae, f_Mogibacteriaceae, f_Lachnospiraceae, f_Peptococcaceae and f_Ruminococcaceae)
	Proteobacteria (o_RF32)
	TM7 (f_F16)
Group 2	Bacteroidetes (f_Prevotellaceae and f_S24-7)
	Firmicutes (f_Erysipelotrichaceae, f_Lachnospiraceae, f_Mogibacteriaceae, f_Peptococcaceae and f_Ruminococcaceae)
	Tenericutes (o_RF39)
Group 3	Actinobacteria (f_Coriobacteriaceae)
	Bacteroidetes (f_Bacteroidaceae and f_S24-7)
	Firmicutes (f_Christensenellaceae, f_Dehalobacteriaceae, f_Erysipelotrichaceae, f_Lachnospiraceae, f_Lachnospiraceae, f_Mogibacteriaceae, f_Peptococcaceae and f_Ruminococcaceae)
	Tenericutes (o_RF39)
	TM7 (f_F16)
Group 4	Actinobacteria (c_OPB41)
	Bacteroidetes (f_Prevotellaceae and f_S24-7)
	Firmicutes (f_Dehalobacteriaceae, f_Lachnospiraceae, f_Erysipelotrichaceae, f_Ruminococcaceae and f_Streptococcaceae)
Group 5	Bacteroidetes (f_Bacteroidaceae)
Group 6	Bacteroidetes (f_S24-7)
	Firmicutes (f_Lachnospiraceae)
Group 7	Bacteroidetes (f_Bacteroidaceae and f_S24-7)
	Firmicutes (f_Clostridiaceae, f_Lachnospiraceae, f_Lactobacillaceae, f_Peptococcaceae and f_Ruminococcaceae)
	TM7 (f_F16)
Group 8	Actinobacteria (f_Coriobacteriaceae)
	Bacteroidetes (f_Bacteroidaceae, f_Rikenellaceae and f_S24)
	Deferribacteres (f_Deferribacteraceae)
	Firmicutes (f_Christensenellaceae, f_Dehalobacteriaceae, f_Erysipelotrichaceae, f_Lachnospiraceae, f_Mogibacteriaceae, f_Peptococcaceae, f_Ruminococcaceae and f_Streptococcaceae)
	Proteobacteria (o_RF32)
	Tenericutes (o_RF39)
	TM7 (f_F16)

Although these analyses are useful in identifying the changes in the gut microbiota community after acute sleep deprivation, they reveal little insight into the impacts of acute sleep deprivation on the functions of the gut microbiota. To address this issue, we conducted additional analyses to investigate the perturbation of specific pathways in gut microbiota following acute sleep deprivation in both native Sprague Dawley and DHR models. As shown in Table 9, 18 and 53 pathways were differentially regulated following acute sleep deprivation in the DHR and native rats, respectively. The majority of the altered pathways in the DHR rats occurred in the cecal microbiota. In contrast, the small intestinal microbiota of native Sprague Dawley rats contributed more than 60% of the dysregulated pathways. All the perturbed pathways contain multiple up- and down-regulated genes, except that one pathway contains more than ten up-regulated genes, but no down-regulated gene.

Table 9. Number of Altered Pathways in Small intestinal, Cecal and Large Intestinal Microbiota in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Acute Sleep Deprivation

Acute Sleep Deprivation	# Significantly Altered Pathways	
	DHR Rats	Native Rats
Small Intestine	6	34
Cecum	9	3
Large Intestine	3	16
Total	18	53

Comparison of the pathways between different regions in the same animal model, or that from the same spatial location between the two animal models, revealed that there is little commonality among these pathway lists, except several pathways that are related to microbial infections. The pathways of interest in the DHR include RIG-1-like receptor signaling pathway (small intestine), penicillin and cephalosporin biosynthesis (cecum), pertussis (cecum) and African trypanosomiasis (large intestine). As the Retinoic acid-inducible gene-1-like (RIG-I-like) receptors are involved in the recognition of viruses by the innate immune system, identification of differential regulation of these pathways thus provides some mechanistic insights into how sleep deprivation may increase the chance of pathogenic infections. Similarly, pathways related to infections, including African trypanosomiasis, pertussis, Shigellosis and Tuberculosis, were also differentially regulated in the small intestine of sleep-deprived native Sprague Dawley rats. Several metabolic pathways, including energy metabolism, phenylalanine metabolism, purine metabolism and tryptophan metabolism, as well as drugs and chemicals (including chemical toxicants) metabolism showed perturbation only in the native, but not in the DHR. It is also interesting that some pathways are altered in both native and DHR, but in different spatial locations. For example, the African trypanosomiasis pathway is differentially regulated in the small intestinal microbiota of native Sprague Dawley rats but in the large intestinal microbiota of DHR, while differential regulation of the pertussis pathway occurred in the small intestinal microbiota of native rats but in the cecal microbiota of DHR. These results again highlights the importance of analyzing the contents from multiple regions of the intestinal tract in order to gain more biologically meaningful results.

4.7.3. Changes in Intestinal Structures and Functions, Inflammatory Response, and Activity of Hypothalamic–Pituitary–Adrenal (HPA) Axis in Native Sprague Dawley and De-Individualized Humanized Rat (DHR) Models after Acute Sleep Deprivation

As described above, tissue samples were also collected for the investigation of the effects of acute sleep deprivation on perturbations of host functions, including structural and functional changes in three regions of the intestinal tract, microbial adhesion,

penetration and invasion, mucosal Inflammation, bacterial endotoxin load, cytokine profiles and HPA axis functions. As shown in Table 10, acute sleep deprivation did not result in significant changes in the MPO activity, an indicator of mucosal inflammation, in the small intestine. Negative results were also obtained from the cecum and large intestine (data not shown). These results suggest that acute sleep deprivation did not cause mucosal inflammation either animal model.

Table 10. Myeloperoxidase (MPO) Activity in Small Intestinal Tissue Homogenates from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Acute Sleep Deprivation

Group	MPO Activity* (μ Ua/mg Tissue)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	0.014 $\pm$ 0.001	N/A	N/A	N/A
SPF (Sleep–Deprived)	0.014 $\pm$ 0.001	8.60E-01	N/A	N/A
DHR (Normal Sleep)	0.014 $\pm$ 0.001	5.60E-01	N/A	N/A
DHR (Sleep–Deprived)	0.015 $\pm$ 0.001	N/A	8.00E-01	6.80E-01

*1 Unit MPO activity = Amount of MPO that oxidizes the APF substrate to generate 1.0 μ mol of fluorescein per minute at 25°C; NS = Normal Sleep; SD = Sleep–Deprived.

However, there is an increase in the bacteria-derived endotoxin load in the sleep-deprived DHR group, compared with its normal sleep counterpart (Table 11). Close examination of the data revealed that the DHR with normal sleep had a lower level of LBP than the native rats. This phenomenon was also observed in some humanized rats during the Model Development and Characterization Experiment, as shown in Table 3.

Table 11. Serum Levels of Lipopolysaccharide Binding Protein (LBP) in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Acute Sleep Deprivation

Group	Concentration (ng/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	2885.0 $\pm$ 159.0	N/A	N/A	N/A
SPF (Sleep–Deprived)	2782.0 $\pm$ 161.8	6.60E-01	N/A	N/A
DHR (Normal Sleep)	2594.4 $\pm$ 113.9	1.60E-01	N/A	N/A
DHR (Sleep–Deprived)	2976.9 $\pm$ 114.4	N/A	2.80E-02	3.40E-01

An increase in endotoxin load may be the result of structural changes in the intestinal tract. Therefore, specific structural parameters of the intestinal tract were determined. As shown in Table 12, there is no significant changes in the villus height in any experimental group, compared with native Sprague Dawley rats with normal sleep. Interestingly, the crypt depth was significantly decreased in the small intestine of sleep-

deprived native rats. As the intestinal crypts contain replicating stem cells and Paneth cells of the innate immune system. This structural change may have a negative impact on these important biological processes. However, the crypt depth in the large intestine of DHR were significantly decreased in both normal sleep and sleep-deprived groups, compared with the normal sleep native rats. This result thus suggest that these changes might be related to the humanization process, rather than the sleep deprivation treatment. No significant change in these measurements was observed in the cecum sample sets.

Table 12. Villus Height, Crypt Depth and Crypt Width in Small Intestine, Cecum and Large Intestine of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Acute Sleep Deprivation

Experiment	Subjects	Treatment	Villus Height (μm)	# Villi (per mm)	Crypt Depth (μm)	Crypt Width (μm)	# Crypts (per mm)
Small Intestine	Native Rats	Normal Sleep	411.76±21.83	8.52±0.66	74.17±1.92	71.26±2.26	9.02±0.63
		Sleep-Deprived	423.10±34.57	8.41±0.84	65.57±3.26 ^a	65.65±4.01	9.24±0.75
	Humanized Rats	Normal Sleep	424.78±18.43	8.87±0.70	68.63±4.60	64.22±4.47	8.99±0.74
		Sleep-Deprived	378.63±30.51	7.54±0.86	66.23±5.29	69.20±4.47	8.21±0.95
Cecum	Native Rats	Normal Sleep	139.94±9.54	NA	NA	66.18±2.46	NA
		Sleep-Deprived	153.66±8.94	NA	NA	66.10±2.46	NA
	Humanized Rats	Normal Sleep	158.61±10.96	NA	NA	62.45±2.42	NA
		Sleep-Deprived	145.73±3.78	NA	NA	64.58±2.74	NA
Large Intestine	Native Rats	Normal Sleep	290.42±14.80	21.65±1.49	90.25±7.37	49.76±2.71	16.85±0.82
		Sleep-Deprived	308.88±11.43	23.08±1.81	104.91±8.23	58.48±4.46	15.32±1.12
	Humanized Rats	Normal Sleep	307.41±13.56	19.84±0.80	55.03±7.53 ^b	53.53±3.66	15.82±0.98
		Sleep-Deprived	290.38±12.76	21.03±2.14	57.18±6.05 ^b	50.90±4.57	17.02±1.47

a – significantly different from normal sleep group (p<0.05); b - significantly different from its native rat counterpart (p<0.05); N/A – Data not available.

Despite an increase in the LBP in the sleep-deprived DHR, no significant changes was detected in any of the pro-inflammatory cytokines tested in this animal group (and other groups) (Tables 13a – 13i), indicating that there is no significant activation of the systemic inflammatory responses in these animals. The reason for this is not clear at this point.

Table 13. Serum level of Pro- and Anti-Inflammatory Cytokines in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Acute Sleep Deprivation

13a. IFN γ

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	20.67 $\pm$ 0.05	N/A	N/A	N/A
SPF (Sleep–Deprived)	20.52 $\pm$ 0.09	1.40E-01	N/A	N/A
DHR (Normal Sleep)	20.60 $\pm$ 0.06	3.40E-01	N/A	N/A
DHR (Sleep–Deprived)	20.60 $\pm$ 0.08	N/A	9.90E-01	4.90E-01

13b. IL-1 β

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	152.88 $\pm$ 0.35	N/A	N/A	N/A
SPF (Sleep–Deprived)	152.68 $\pm$ 0.21	4.30E-01	N/A	N/A
DHR (Normal Sleep)	153.02 $\pm$ 0.18	3.90E-01	N/A	N/A
DHR (Sleep–Deprived)	153.52 $\pm$ 0.23	N/A	9.30E-01	9.00E-01

13c. IL-4

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	11.11 $\pm$ 0.03	N/A	N/A	N/A
SPF (Sleep–Deprived)	11.11 $\pm$ 0.02	9.80E-01	N/A	N/A
DHR (Normal Sleep)	11.10 $\pm$ 0.03	7.40E-01	N/A	N/A
DHR (Sleep–Deprived)	11.08 $\pm$ 0.04	N/A	7.30E-01	5.50E-01

13d. IL-5

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	22.60 $\pm$ 0.10	N/A	N/A	N/A
SPF (Sleep–Deprived)	22.49 $\pm$ 0.08	3.80E-01	N/A	N/A
DHR (Normal Sleep)	22.61 $\pm$ 0.07	9.80E-01	N/A	N/A
DHR (Sleep–Deprived)	22.43 $\pm$ 0.12	N/A	2.20E-01	6.60E-01

13e. IL-6

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	198.27±6.82	N/A	N/A	N/A
SPF (Sleep-Deprived)	189.17±6.25	3.40E-01	N/A	N/A
DHR (Normal Sleep)	198.25±5.40	1.00E+00	N/A	N/A
DHR (Sleep-Deprived)	192.96±7.57	N/A	5.80E-01	7.00E-01

13f. Cxcl1

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	88.44±22.60	N/A	N/A	N/A
SPF (Sleep-Deprived)	114.88±31.25	5.80E-01	N/A	N/A
DHR (Normal Sleep)	71.05±7.62	5.60E-01	N/A	N/A
DHR (Sleep-Deprived)	68.92±6.86	N/A	8.60E-01	2.70E-01

13g. IL-10

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	24.01±0.43	N/A	N/A	N/A
SPF (Sleep-Deprived)	24.44±0.41	4.40E-01	N/A	N/A
DHR (Normal Sleep)	24.70±0.26	1.80E-01	N/A	N/A
DHR (Sleep-Deprived)	25.02±0.53	N/A	5.90E-01	4.20E-01

13h. IL-13

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	29.31±0.46	N/A	N/A	N/A
SPF (Sleep-Deprived)	29.27±0.73	4.20E-01	N/A	N/A
DHR (Normal Sleep)	29.30±0.34	8.50E-01	N/A	N/A
DHR (Sleep-Deprived)	29.26±0.82	N/A	2.40E-01	7.40E-01

13i. TNF α

Group	Concentration (pg/mL)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	19.14 $\pm$ 0.09	N/A	N/A	N/A
SPF (Sleep-Deprived)	19.17 $\pm$ 0.09	8.20E-01	N/A	N/A
DHR (Normal Sleep)	19.08 $\pm$ 0.05	5.70E-01	N/A	N/A
DHR (Sleep-Deprived)	19.10 $\pm$ 0.08	N/A	9.00E-01	5.50E-01

The levels of corticotropin-releasing factor (CRF), adrenocorticotrophic hormone (ACTH) and corticosterone were investigated to assess the effect of acute sleep deprivation on the activity of the HPA axis. CRF is secreted by the paraventricular nucleus of the hypothalamus in response to stress. It is also synthesized in peripheral tissues and T lymphocytes. Therefore, the serum levels of these hormones were determined. Interestingly, there was an increase in the CRF level in the DHR Model animals following acute sleep deprivation, compared with their non-sleep-deprived counterpart (Tables 14a – 14c). Similar to the result of LBP described above, this is due to a decrease in the CRF in the DHR with normal sleep, compared with their native Sprague Dawley counterpart. Sleep deprivation however induced an increase in ACTH in the native rats. Consistent with this result, the serum level of corticosterone was significantly increased in this animal group. In contrast to the native rats, no increase in ACTH was detected in sleep-deprived DHR, compared with DHR with normal sleep counterpart. Interestingly, DHR, regardless of the sleep conditions, had significantly increased ACTH levels, compared with that of the native Sprague Dawley rats with normal sleep. This observation thus suggest that the changes are likely due to the humanization process. As expected, the serum level of corticosterone was significantly increased in the DHR, further confirming that the HPA axis can be activated by both sleep deprivation and the humanization process.

Table 14. Serum Levels of Hypothalamic-Pituitary-Adrenal (HPA) Axis Hormones in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Acute Sleep Deprivation

14a. Peripheral CRF

Group	Concentration (pg/ μ L)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	0.147 $\pm$ 0.007	N/A	N/A	N/A
SPF (Sleep-Deprived)	0.136 $\pm$ 0.008	3.50E-01	N/A	N/A
DHR (Normal Sleep)	0.125 $\pm$ 0.002	1.50E-02	N/A	N/A
DHR (Sleep-Deprived)	0.147 $\pm$ 0.009	N/A	4.20E-02	4.00E-01

14b. ACTH

Group	Concentration (pg/ μ L)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	3.44 $\pm$ 0.75	N/A	N/A	N/A
SPF (Sleep–Deprived)	11.28 $\pm$ 3.29	3.50E-02	N/A	N/A
DHR (Normal Sleep)	15.74 $\pm$ 3.10	1.90E-03	N/A	N/A
DHR (Sleep–Deprived)	16.12 $\pm$ 4.83	N/A	9.50E-01	4.60E-01

14c. Corticosterone

Group	Concentration (pg/ μ L)	T-Test P-Value (v. SPF NS)	T-Test P-Value (v. DHR NS)	T-Test P-Value (v. SPF SD)
SPF (Normal Sleep)	172.79 $\pm$ 9.76	N/A	N/A	N/A
SPF (Sleep–Deprived)	215.18 $\pm$ 16.5	4.80E-02	N/A	N/A
DHR (Normal Sleep)	248.13 $\pm$ 13.87	3.20E-04	N/A	N/A
DHR (Sleep–Deprived)	228.78 $\pm$ 24.11	N/A	5.00E-01	6.50E-01

4.7.4. Effect of Acute Sleep Deprivation on Learning and Memory in *Naïve* Sprague Dawley Rats

It appears that acute sleep deprivation does not affect learning and memory in the *naïve* Sprague Dawley Rats, as assessed using the NOR test. Sleep-deprived SPF Sprague Dawley rats can recognize the novel object with a larger size at 24 hours or that of a different shape at 96 hours (Table 15). This result thus suggest that the acute sleep deprivation treatment employed in this study did not adversely affect the encoding and/or the retrieval of the episodic memory of the sample objects, as well as the preference for a novel object in these animals.

Table 15. Novel Object Recognition Test of Native Sprague Dawley Rats before and after Acute Sleep Deprivation

Condition*	Object 1	Object 2	Time Point	Object 1 Duration (%)	Object 2 Duration (%)	T-Test P-Value
Pre-SD Training	Rectangle	Rectangle	Day -14	45.1±6.7%	54.9±6.7%	3.10E-01
Pre-SD Testing (24h)	Rectangle	Rectangle (2x Size)	Day -13	42.1±4.9%	57.9±4.9%	3.60E-02
Pre-SD Testing (96h)	Rectangle	Disc	Day -11	27.4±3.1%	72.6±3.1%	6.30E-08
6-Day Sleep Deprivation (Days 1-6)						
Post-SD Training	Rectangle	Rectangle	Day 7	48.5±3.8%	51.5±3.8%	5.90E-01
Post-SD Testing (24h)	Rectangle	Rectangle (2x Size)	Day 8	37.2±5.9%	62.8±5.9%	8.80E-03
Post-SD Testing (96h)	Rectangle	Disc (Shape)	Day 11	25.9±3.2%	74.1±3.2%	4.10E-08

*Baseline and post-sleep-deprivation memory performance for was assessed after the retention periods of 24 and 96 hours.

4.8 Effect of Chronic Sleep Deprivation on Gut Microbiota Population and Host Functions

4.8.1. Generation and Chronic Sleep Deprivation Treatment of De-Individualized Humanized Rats (DHR)

The DHR animal set for the chronic sleep deprivation experiment was generated using the procedure as described above. Briefly, PGF rats were first generated by the treatment of Sprague Dawley rats with an antibiotic cocktail for 2 weeks. After the confirmation of the GF status of these animals, the fecal microbiota from five human donors were used to inoculate the PGF rats by oral gavage. The inoculated human microbiota was allowed to colonize the intestinal tracts of their respective hosts for two weeks, followed by the de-individualization procedure. After the confirmation of the emergence of a homogenous profile of gut microbiota in the DHR; the DHR were subjected to 6 weeks of chronic sleep deprivation treatment, using the sleep fragmentation chamber from Lafayette Instrument, as described in Section 3.9. After 6 weeks of sleep deprivation treatment, the animals were euthanized, and tissue samples collected for the analyses of microbial adhesion, penetration and invasion, mucosal Inflammation, bacterial endotoxin load, cytokine profile and HPA axis functions. As before, intestinal contents were also collected from three regions (small intestine, cecum and large intestine) of the intestinal tract and used for metagenomic analyses.

The overall design of this experiment is shown in Figure 25. As above, control and sleep-deprived groups of native SPF Sprague Dawley rats were also included, resulting in total animal groups in this experiment (including native Sprague Dawley rats with normal sleep or chronic sleep deprivation, and DHR with normal sleep or chronic sleep-

deprivation, see Table 16). As discussed above, the inclusion of native rats in this experiment will facilitate the interpretation of the results of this experiment.

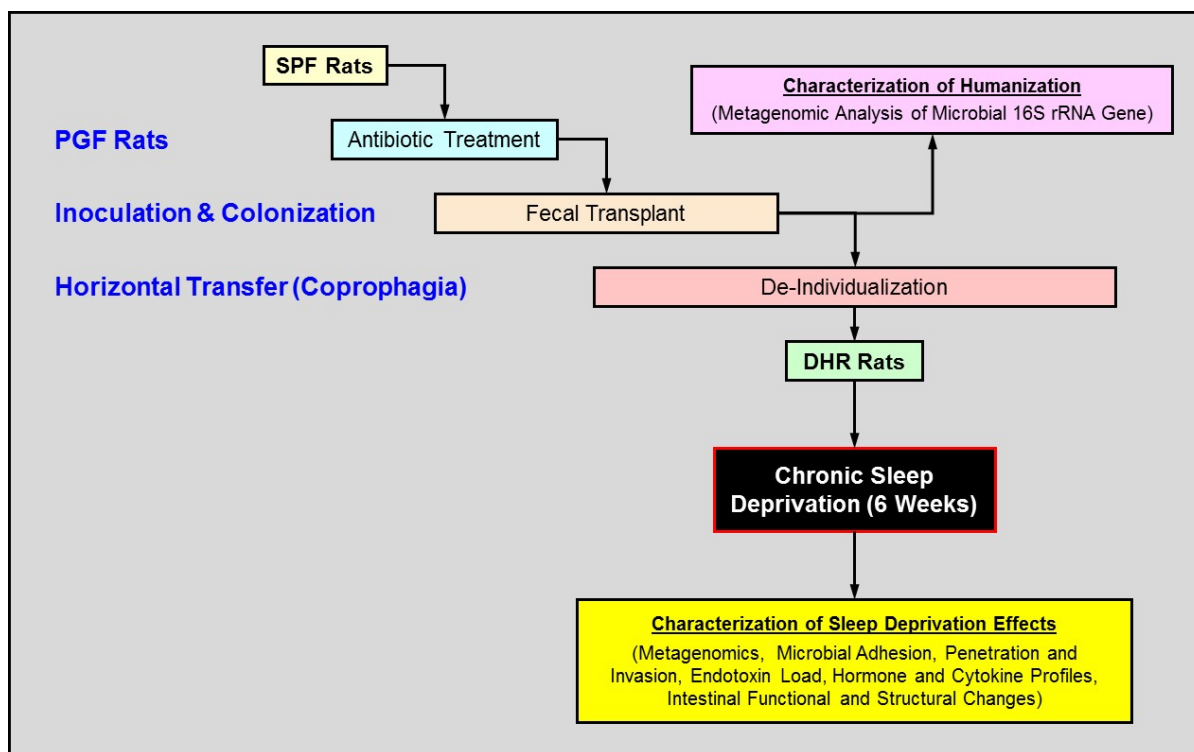


Figure 25. Study Design of Chronic Sleep Deprivation Experiment

Table 16. Experimental Groups of Chronic Sleep Deprivation Experiment

Group	Humanization	Sleep Deprivation	Description
1	No	No	Control (for sleep deprivation and humanization)
2	No	Yes	Test (sleep deprivation); Control (for humanization)
3	Yes	No	Control (for sleep deprivation); Test (humanization)
4	Yes	Yes	Test (sleep deprivation); Test (humanization)

4.8.2. Effect of Chronic Sleep Deprivation on Gut Microbiota of Native Sprague Dawley and De-Individualized Humanized Rat (DHR) Models

The titers of the intestinal contents collected from the animals groups were determined as described above, using the plate culture method. After six weeks of sleep deprivation, the titer of the large intestinal content was decreased in both native Sprague Dawley and DHR, while that from the cecum were not affected. In the small intestine, the DHR showed a significant decrease in the microbial titer, while the native rats showed an increase in microbial titer, although not statistically significant (Table 17).

Table 17. Microbial Titers of Gut Microbiota from Different Regions of Intestinal Tract of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Microbial Titer (CFU/g)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Baseline (Fecal)	6.70E+08	2.20E+08	3.80E+08	5.20E+08
Distal Ileal Content	2.30E+05	5.90E+05	3.70E+05	1.20E+05*
Cecal Content	1.20E+08	1.30E+08	1.80E+08	2.20E+08
Proximal Colonic Content	1.00E+08	5.90E+07*	1.80E+08	8.40E+07*
Fecal Pellet	3.10E+08	3.00E+08	1.20E+08	1.60E+08

* p<0.05

Microbial cells corresponding to the fractions of microbial adhesion, penetration and invasion were recovered using the procedure we developed. Their titers were determined using the plate culture method. As shown in Table 18, microbial adhesion was decreased in the large intestine of DHR after chronic sleep deprivation, which is associated with increased microbial invasion in the same region in this animal group (Table 20), suggesting the adhered cells may have a protective role in these animals. Increased microbial invasion in the large intestine of native Sprague Dawley rats undergoing chronic sleep deprivation was also observed. However, it is associated with decreased penetrated cells (Table 19). This may suggest that the penetrated cells may have a higher propensity to invade into deeper layers of the intestine in the native rats after chronic sleep deprivation.

Table 18. Microbial Adhesion in Different Regions of Intestinal Tract of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Microbial Titer (CFU/g tissue)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Distal Ileum	1.20E+05	1.00E+04	8.10E+04	8.90E+04
Cecum	2.50E+05	1.50E+04	2.70E+05	2.20E+05
Proximal Colon	1.50E+05	1.80E+04	7.00E+05	2.10E+05*

* p<0.05

Table 19. Microbial Penetration in Different Regions of Intestinal Tract of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Microbial Titer (CFU/g tissue)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Distal Ileum	4.30E+05	4.60E+05	3.60E+06	3.60E+06
Cecum	3.40E+05	9.30E+04	1.90E+06	1.50E+06
Proximal Colon	8.30E+05	3.10E+05*	4.20E+06	2.70E+06

* p<0.05

Table 20. Microbial Invasion in Different Regions of Intestinal Tract of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Microbial Titer (CFU/g tissue)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Distal Ileum	8.50E+00	0.00E+00	1.40E+01	5.70E+00
Cecum	1.50E+02	2.50E+02	3.20E+02	6.00E+02
Proximal Colon	2.30E+02	4.80E+02 *	1.30E+03	9.10E+03 *

* p<0.05

Extra-intestinal invasion of microbial cells into the mesenteric layer, mesenteric lymph nodes and other organs including the spleen and liver were also investigated. As shown in Table 21, chronic sleep deprivation resulted in an increase in the microbial titers in the spleen and the liver of native Sprague Dawley and DHR rats, respectively. Since these two animal models have different microbiota population, this result may suggest that the human and rat microbiota contain different microbial taxa that can invade and become overgrown in distal organs in a target-site-specific manner after chronic sleep deprivation.

Table 21. Extra-intestinal Microbial Invasion of Different Organs of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Microbial Titer (CFU/g tissue)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Mesentery	6.30E+03	5.80E+03	3.10E+03	4.50E+03
Mesenteric Lymph Nodes	4.30E+03	6.70E+03	6.60E+03	5.70E+03
Spleen	3.30E+02	2.00E+03*	5.40E+02	5.20E+02
Liver	1.40E+02	3.70E+02	8.40E+02	2.10E+03*

* p<0.05

After six weeks of sleep deprivation, significant decreases in the alpha diversity were detected in the intestinal contents from all three regions of the DHR, with the cecal content showing the most dramatic effect (Figure 26). Unlikely the result of the acute sleep deprivation experiment, chronic sleep deprivation consistently reduced the complexity of the microbiota community in all three spatial locations of the intestinal tracts of the DHR. In contrast, only the large intestinal microbiota from sleep-deprived native rats showed a significant decrease in the community complexity, while the small intestinal and cecal microbiota appear to have recovered at some point of this prolonged period of chronic sleep deprivation (Figure 27).

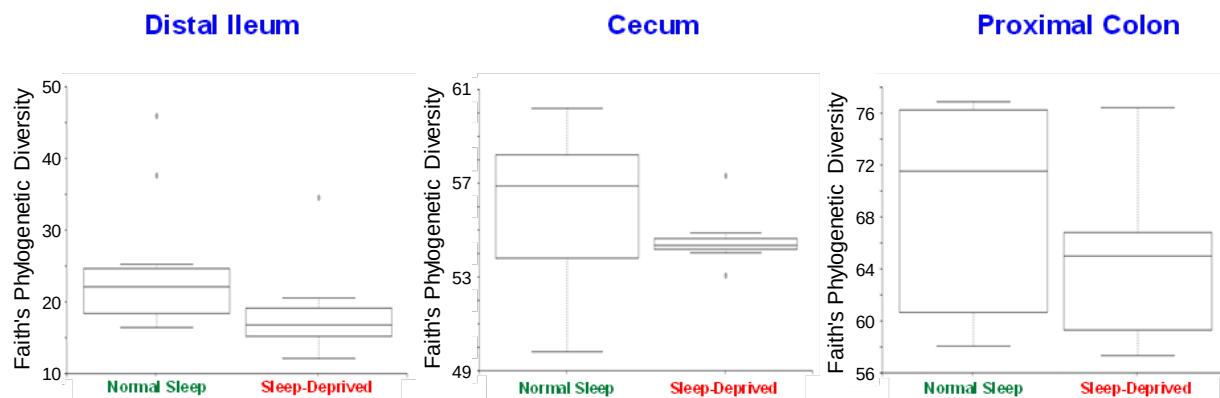


Figure 26. Alpha Diversity Boxplots of Gut Microbiota from Small Intestine, Cecum and Large Intestine of De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

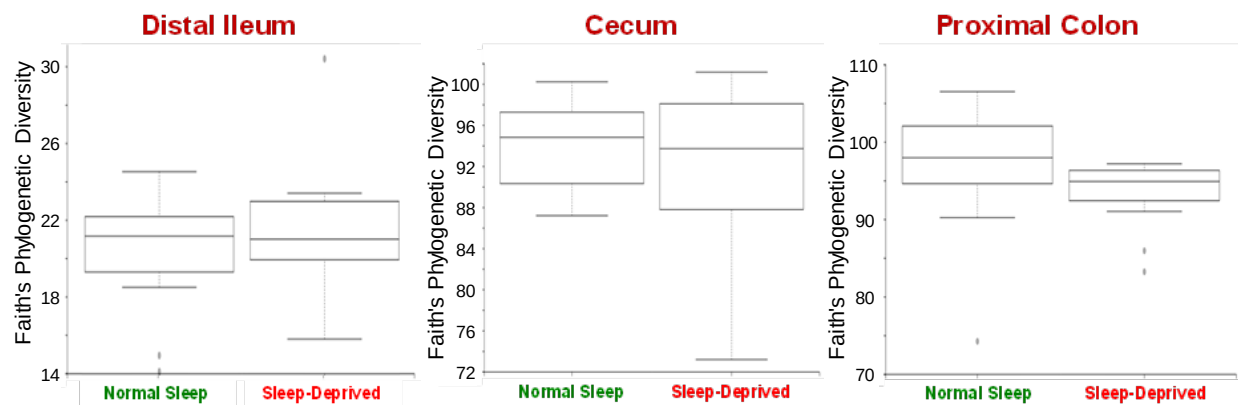


Figure 27. Alpha Diversity Boxplots of Gut Microbiota from Small Intestine, Cecum and Large Intestine of Native Sprague Dawley Rats with Normal Sleep or Chronic Sleep Deprivation

As above, PCoA of the UniFrac distance metric of the microbiota from normal sleep and sleep-deprived native Sprague Dawley and DHR rats were performed, and the results are shown in Figures 28 – 30. The microbiota in all three regions from the DHR with normal sleep and chronic sleep deprivation form two relatively well-separated clusters in this analysis, indicating that the changes in the microbiota population structure can be readily detected in the DHR after chronic sleep deprivation. In contrast, incomplete separation of the clusters was observed in the native Sprague Dawley rats, with the cecal content showing the best separation. These results suggest that the DHR is probably a more sensitive model for detecting microbiota perturbation induced by chronic sleep deprivation (or perhaps the exposure to other stressors as well).

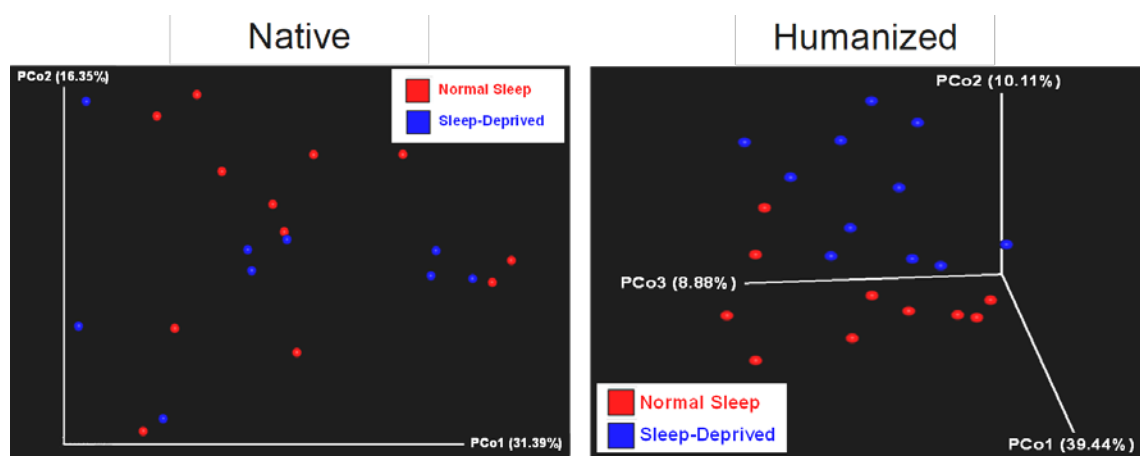


Figure 28. Principal Coordinate Analysis (PCoA) of Small Intestinal Microbiota from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

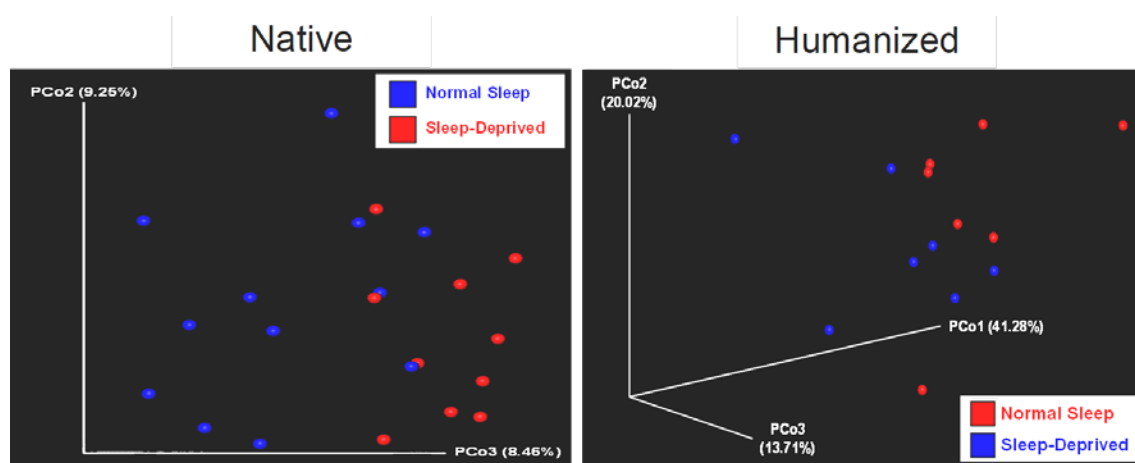


Figure 29. Principal Coordinate Analysis (PCoA) of Cecal Microbiota from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

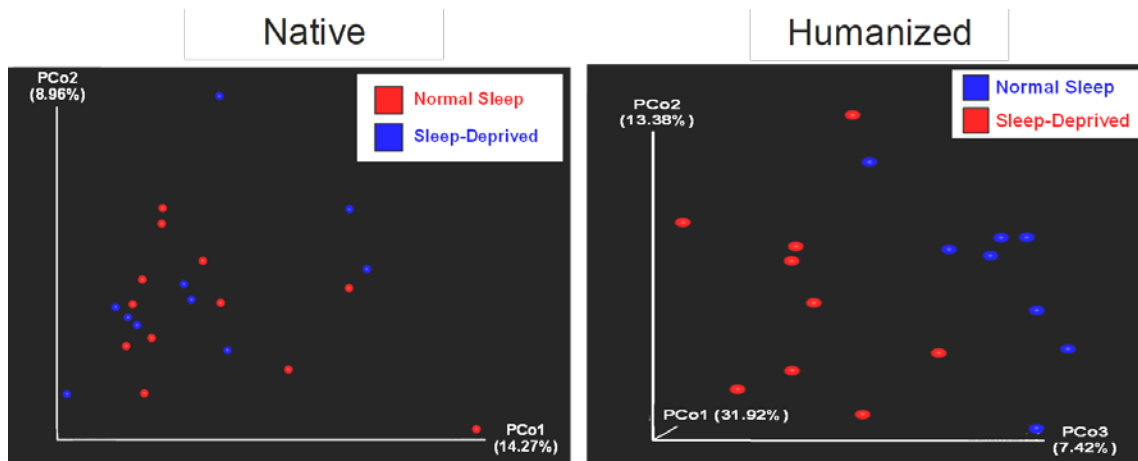


Figure 30. Principal Coordinate Analysis (PCoA) of Large Intestinal Microbiota from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

The top 10 most increased and decreased microbial taxa in the sleep-deprived subjects were also determined, using the Gneiss analysis tool. As shown in Figure 31, Erysipelotrichaceae (especially the genus *Turicibacter*) and Clostridiaceae (genus 02d06) were the most increased microbial cells in the small intestine of sleep-deprived native rats, while *Proteus* and *Lactobacillus* were dramatically decreased (Figure 32). It should be noted that decreased population of *Turicibacter* in the small intestinal of native rats was also observed after acute sleep deprivation. *Peptostreptococcaceae* and *Staphylococcaceae* (genus *Staphylococcus*) and *Enterobacteriaceae* were most up-regulated in DHR rats after chronic sleep deprivation (Figure 31). On the other hand, *Streptococcaceae* (genus *Streptococcus*), *Lachnospiraceae*, and *Bacteroidaceae* (genus *Bacteroides*) were decreased in DHR after chronic sleep deprivation (Figure 32).

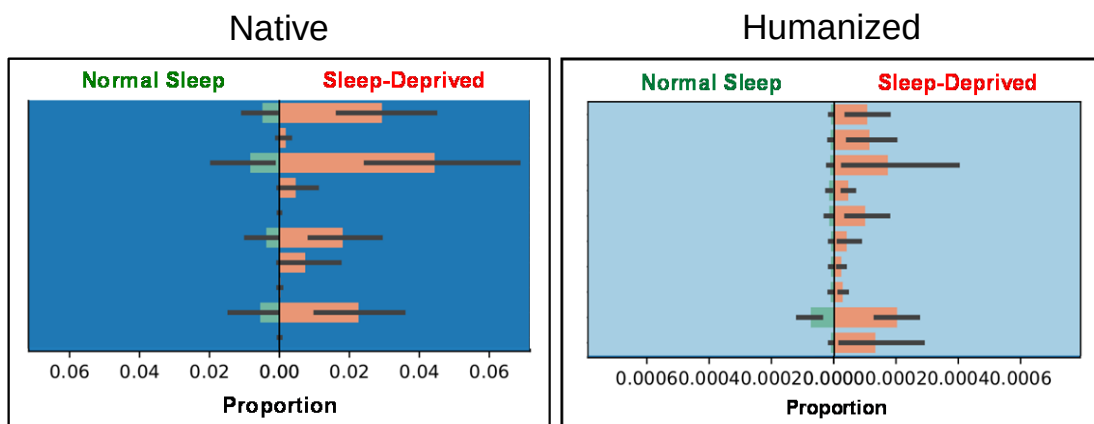


Figure 31. Top Ten Most Increased Microbial Taxa in Small Intestinal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

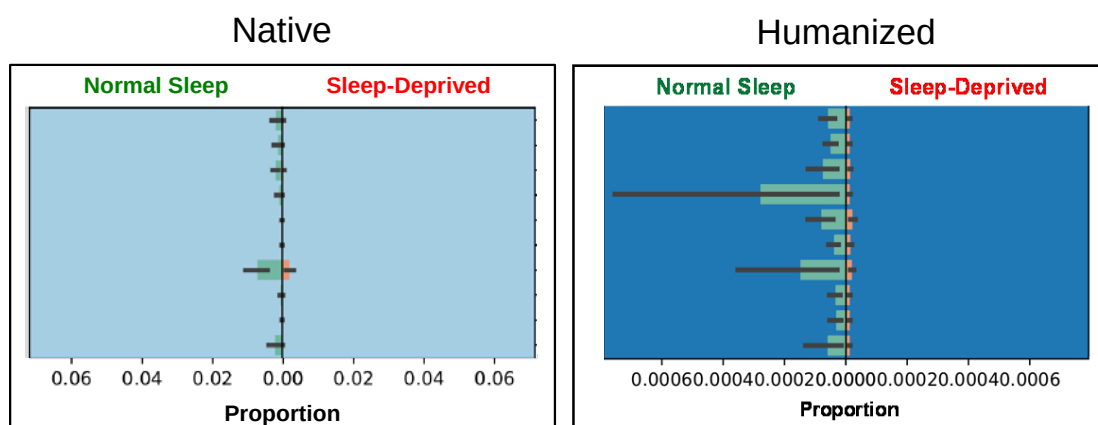


Figure 32. Top Ten Most Decreased Microbial Taxa in Small Intestinal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

In the cecal content of sleep-deprived native Sprague Dawley rats, Ruminococcaceae (especially genus *Oscillospira*) and Streptococcaceae (genus *Streptococcus*) were the most increased microbial cells. Similarly, Ruminococcaceae (genus *Oscillospira*) was also increased in the cecal content of sleep-deprived DHR, together with an increase in Lachnospiraceae. (Figure 33). Interestingly, Ruminococcaceae (genus *Oscillospira*) was also the most decreased taxon in this sample set (Figure 34), due to the fact that many OTUs map to microbial taxa with the same taxonomic classification – this was also observed in the acute sleep deprivation data set. In addition, Lachnospiraceae, Bacteroidaceae and Enterobacteriaceae were also decreased in the cecal content of sleep-deprived DHR (Figure 34).

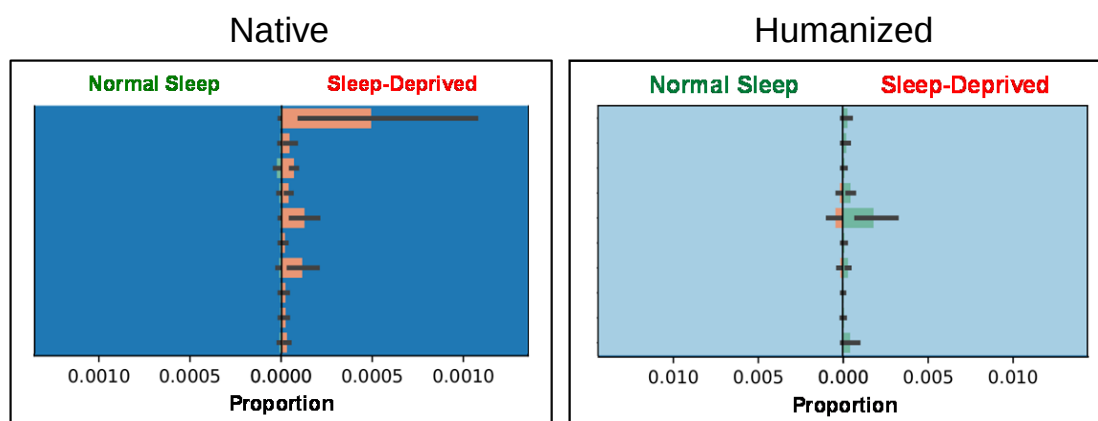


Figure 33. Top Ten Most Increased Microbial Taxa in Cecal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

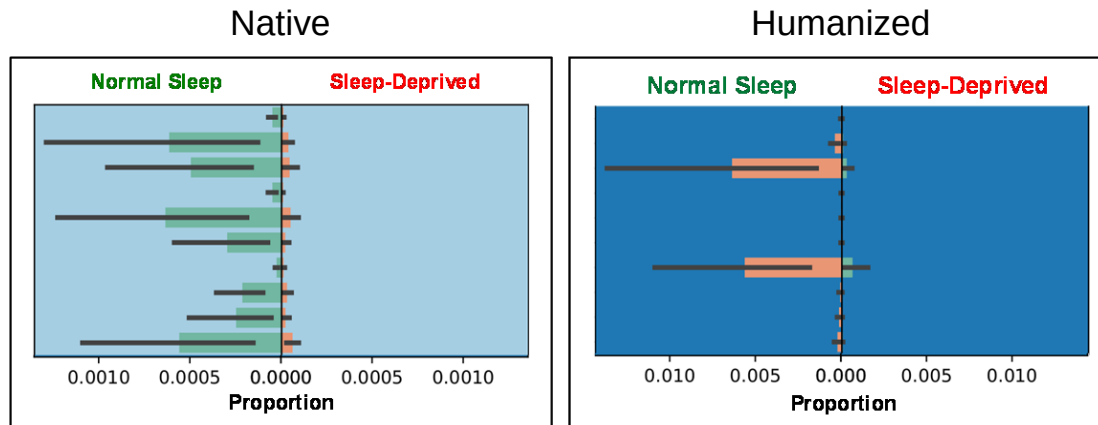


Figure 34. Top Ten Most Decreased Microbial Taxa in Cecal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

As shown in Figures 35 and 36, Sutterellaceae (genus *Sutterella*) and Erysipelotrichaceae (genus *Turicibacter*) were increased, while Ruminococcaceae, Lactobacillaceae (genus *Lactobacillus*) and Porphyromonadaceae (genus *Parabacteroides*) were decreased in the large intestinal content of sleep-deprived native Sprague Dawley rats. Increased Erysipelotrichaceae (genus *Turicibacter*) also occurred in small intestinal content, but not in the cecal content from this group of animals. Ruminococcaceae (genus *Oscillospira*) and Lachnospiraceae were increased in the large intestinal content of sleep-deprived DHR. In contrast, a more widespread decrease of Bacteroidaceae (genus *Bacteroides*), Desulfovibrionaceae (genus *Bilophila*), Lachnospiraceae (genus *Anaerostipes*), Barnesiellaceae and Rikenellaceae were detected in the DHR after chronic sleep deprivation.

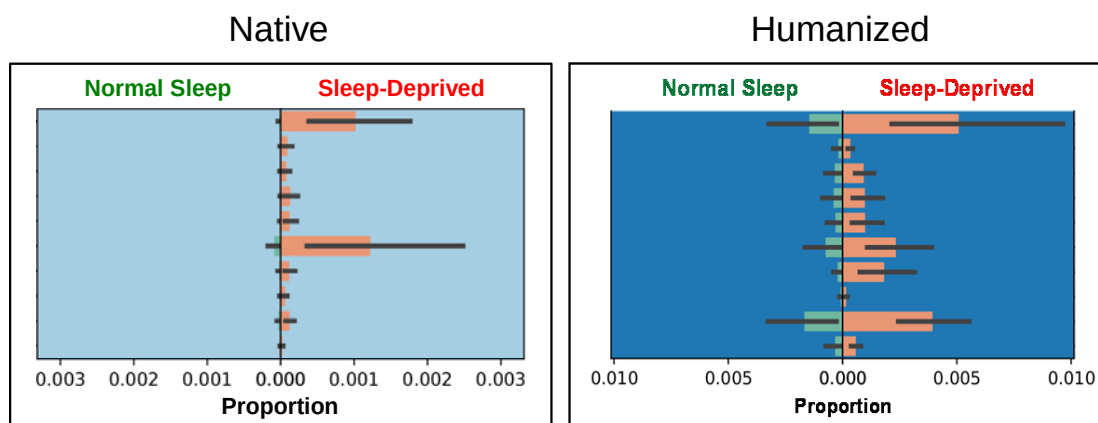


Figure 35. Top Ten Most Increased Microbial Taxa in Large Intestinal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

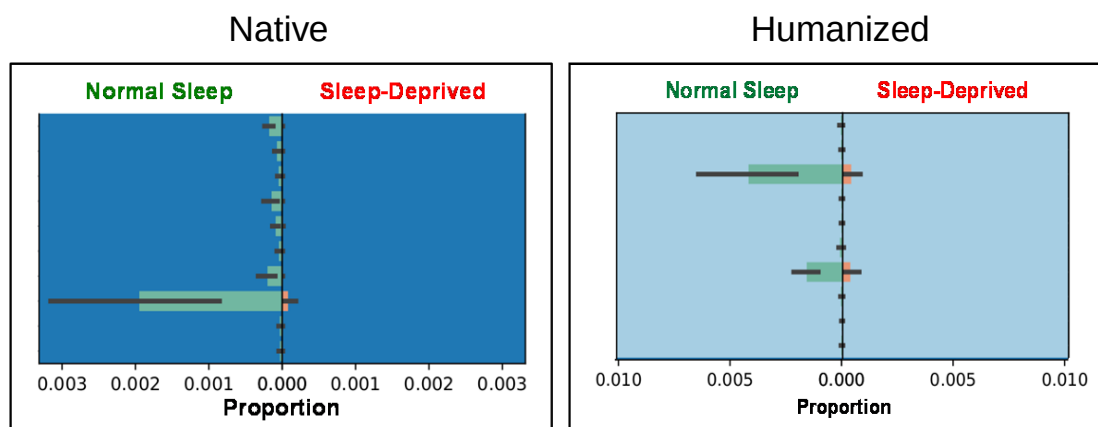


Figure 36. Top Ten Most Decreased Microbial Taxa in Large Intestinal Content from Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

The result of the Gneiss analysis is summarized in Tables 22 – 24. Additionally, we also performed the Kruskal-Wallis H Test (with multiple test correction) and identified 251 and 448 OTUs were significantly altered in the native Sprague Dawley and DHR, respectively. Interestingly, chronic sleep deprivation caused only slightly more OTUs undergoing significant changes than acute sleep deprivation. The result of the Kruskal-Wallis H Test is summarized in Table 25.

Table 22. Top Ranking Increased and Decreased Microbial Taxa in Small Intestine of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

Taxon	Change Direction	# OTUs	Host
Firmicutes;c_Bacilli;o_bacillales;f_Staphylococcaceae;g_Staphylococcus	Increased	2	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_02d06	Increased	1	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae;g_	Increased	1	Humanized Rats
Firmicutes;c_Erysipelotrichia;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Turicibacter	Increased	5	Native SPF Rats
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_	Increased	1	Humanized Rats
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides	Decreased	1	Humanized Rats
Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	Decreased	3	Native SPF Rats
Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	Decreased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_;;g_	Decreased	2	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	Decreased	2	Humanized Rats
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Morganellaceae;g_Proteus	Decreased	2	Native SPF Rats

Table 23. Top Ranking Increased and Decreased Microbial Taxa in Cecum of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

Taxon	Change Direction	# OTUs	Host
Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	Increased	1	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_;g_	Increased	4	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_;g_	Increased	3	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	Increased	2	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus	Increased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_	Increased	1	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_	Increased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	Increased	2	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	Increased	1	Humanized Rats
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides	Decreased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_;g_	Decreased	6	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	Decreased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_	Decreased	1	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	Decreased	1	Native SPF Rats
Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_	Decreased	1	Humanized Rats

Table 24. Top Ranking Increased and Decreased Microbial Taxa in Large Intestine of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

Taxon	Change Direction	# OTUs	Host
Firmicutes;c_Clostridia;o_Clostridiales;f_g_	Increased	4	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	Increased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	Increased	1	Humanized Rats
Firmicutes;c_Erysipelotrichia;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Turicibacter	Increased	1	Native SPF Rats
Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;c_Betaproteobacteria;f_Sutterellaceae;g_Sutterella	Increased	1	Native SPF Rats
Bacteroidetes;c_Bacteroidetes;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides	Decreased	1	Native Rats
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Barnesiellaceae];g_	Decreased	1	Humanized Rats
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides	Decreased	1	Humanized Rats
Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_	Decreased	1	Humanized Rats
Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	Decreased	1	Native SPF Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	Decreased	2	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerostipes	Decreased	1	Humanized Rats
Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_	Decreased	1	Native SPF Rats
Proteobacteria;c_Deltaproteobacteria;o_Desulfobivionales;f_Desulfobivionaceae;g_Bilophila	Decreased	1	Humanized Rats

Table 25. Significantly Increased and Decreased Operational Taxonomic Units (OTUs) in Cecal Content of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

	Native Rats	DHR Rats
Total	251	448
Increased	152	366
Detectable only after sleep deprivation	68	214
Increased after sleep deprivation	84	152
Decreased	99	82
Undetectable after sleep deprivation	67	49
Decreased after sleep deprivation	32	33

Taxa that were significantly altered in both animal models, as well as those that exhibited changes in a model-specific manner are shown in Tables 26 – 31. Interestingly,

the majority of the up-regulated taxa showed increases in both acute and chronic sleep deprivation, while the majority of significantly decreased taxa were not affected by acute sleep deprivation.

Table 26. Significantly Increased Taxa in Both Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

All taxa are also altered after acute sleep deprivation.

p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_acidifaciens
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Porphyromonadaceae; g_Parabacteroides
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Porphyromonadaceae; g_Parabacteroides; s_distasonis
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_ ; s_
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_Alistipes; s_massiliensis
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_S24-7; g_ ; s_
p_Firmicutes; c_Bacilli; o_Lactobacillales
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_ ; s_
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Coprococcus; s_
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Oscillospira; s_
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Ruminococcus; s_
p_Proteobacteria; c_Gammaproteobacteria; o_Enterobacteriales; f_Enterobacteriaceae

Table 27. Significantly Increased Taxa in De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_[Barnesiellaceae]; g_ ; s_ ★
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_caccae ★
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_fragilis ★
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides; s_uniformis ★
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Porphyromonadaceae; g_Parabacteroides; s_gordonii ★
p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Rikenellaceae; g_Alistipes; s_indistinctus ★
p_Firmicutes; c_Bacilli; o_Lactobacillales; f_Lactobacillaceae; g_Lactobacillus ★
p_Firmicutes; c_Bacilli; o_Lactobacillales; f_Lactobacillaceae; g_Lactobacillus; s_reuteri ★
p_Firmicutes; c_Bacilli; o_Turicibacteriales; f_Turicibacteraceae; g_Turicibacter; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Christensenellaceae; g_Christensenella; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Clostridiaceae; g_02d06; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Clostridiaceae; g_Clostridium; s_perfringens ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Dehalobacteriaceae; g_Dehalobacterium; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_[Ruminococcus]; s_gnavus ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Anaerostipes; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Dorea; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Peptostreptococcaceae; g_ ; s_ ★
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Anaerofilum; s_
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Anaerotruncus; s_
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Oscillospira; s_guilliermondii
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Ruminococcus; s_flavifaciens
p_Firmicutes; c_Clostridia; o_Clostridiales; f_Veillonellaceae; g_Veillonella; s_dispar
p_Firmicutes; c_Erysipelotrichi; o_Erysipelotrichales; f_Erysipelotrichaceae; g_Clostridium; s_cocleatum
p_Firmicutes; c_Erysipelotrichi; o_Erysipelotrichales; f_Erysipelotrichaceae; g_Clostridium; s_saccharogumia
p_Proteobacteria; c_Betaproteobacteria; o_Burkholderiales; f_Oxalobacteraceae ★
p_Proteobacteria; c_Gammaproteobacteria; o_Enterobacteriales; f_Enterobacteriaceae; g_Proteus; s_ ★

★ - Significantly changed after acute sleep deprivation.

Table 28. Significantly Increased Taxa in Native Sprague Dawley Rats after Chronic Sleep Deprivation

p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__ ; s__	★
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__	★
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Peptococcaceae; g__rc4-4; s__	★
p__Proteobacteria; c__Alphaproteobacteria; o__RF32; f__ ; g__ ; s__	★
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Klebsiella; s__	★
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Morganella; s__morganii	
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Sodalis; s__	
p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__Pasteurellaceae; g__Haemophilus; s__parainfluenzae	★
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	★
p__Tenericutes; c__Mollicutes; o__RF39; f__ ; g__ ; s__	★

★ - Significantly changed after acute sleep deprivation.

Table 29. Significantly Decreased Taxa in Both Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Adlercreutzia; s__	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Rikenellaceae; g__Alistipes; s__massiliensis	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__S24-7; g__ ; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Christensenellaceae; g__ ; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__ ; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__[Ruminococcus]; s__gnavus	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Anaerotruncus; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Oscillospira; s__	

Table 30. Significantly Decreased Taxa in De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__[Barnesiellaceae]; g__ ; s__	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__caccae	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__eggerthii	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__ovatus	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Parabacteroides; s__	
p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__	
p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__ruminis	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Clostridium; s__hathewayi	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Dorea; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Lachnospira; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__	
p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Phascolarctobacterium; s__	
p__Proteobacteria; c__Alphaproteobacteria; o__RF32; f__ ; g__ ; s__	
p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Oxalobacter; s__formigenes	
p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Succinivibrionaceae	
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae	
p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Trabulsiella; s__	
p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas	

Table 31. Significantly Decreased Taxa in Native Sprague Dawley Rats after Chronic Sleep Deprivation

p	Bacteroidetes; c	Bacteroidia; o	Bacteroidales; f	Bacteroidaceae; g	Bacteroides; s	acidifaciens
p	Firmicutes; c	Bacilli; o	Lactobacillales; f	Lactobacillaceae; g	Lactobacillus; s	reuteri
p	Firmicutes; c	Clostridia; o	Clostridiales; f	Dehalobacteriaceae; g	Dehalobacterium; s	
p	Firmicutes; c	Clostridia; o	Clostridiales; f	Lachnospiraceae; g	Coprococcus; s	
p	Firmicutes; c	Clostridia; o	Clostridiales; f	Lachnospiraceae; g	Roseburia; s	
p	Tenericutes; c	Mollicutes; o	RF39; f			

As mentioned above, while these analyses are useful in identifying the changes in the gut microbiota community, they reveal little insight into the functional impact of chronic sleep deprivation on gut microbiota. As above, we also conducted the analyses of microbiota pathway perturbation of the small intestinal, cecal and large intestinal contents in both native Sprague Dawley and DHR after chronic sleep deprivation. As shown in Table 32, the number of dysregulated pathways in native Sprague Dawley rats is more than twice of that in the DHR. In DHR, more than 60% of the dysregulated pathways occurred in small intestine. In contrast, almost 45% of the altered pathways of the native rat occurred in the cecum.

Table 32. Number of Differentially Regulated Pathways in Small Intestinal, Cecal and Large Intestinal Contents of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) after Chronic Sleep Deprivation

Chronic Sleep Deprivation	# Differentially Regulated Pathways	
	Native SPF Rats	DHR Rats
Small Intestine	31	30
Cecum	45	9
Large Intestine	26	9
Total	102	48

A number of pathways are altered in both native Sprague Dawley and DHR, but in different spatial locations. For example, epithelial cell signaling pathway in *Helicobacter pylori* infection was differentially regulated in the cecal content of native rats but in the large intestinal content of DHR. The *Vibrio cholerae* infection pathway was altered in the small intestinal content of native rats but in the large intestinal content of DHR. As in the case of acute sleep deprivation, many infection-related pathways were dysregulated following chronic sleep deprivation in the DHR. These include penicillin and cephalosporin biosynthesis, RIG-like receptor signaling pathway, *Vibrio cholerae* pathogenic cycle, bacterial chemotaxis, epithelial cell signaling in *Helicobacter pylori* infection and *Vibrio cholerae* infection. Alterations in infection-related pathways in the

native Sprague Dawley rats include bacterial invasion of epithelial cells, bacterial toxins, lipopolysaccharide biosynthesis, *Vibrio cholerae* infection, and epithelial cell signaling in *Helicobacter pylori* infection. Similar to the results of acute sleep deprivation, more than 16 metabolic pathways were dysregulated in the native rats, of which only four (4) showed differential regulation in the DHR.

4.8.3. Changes in Intestinal Structures and Functions, Inflammatory Response and Activity of Hypothalamic–Pituitary–Adrenal (HPA) Axis in Native Sprague Dawley and De-Individualized Humanized Rat (DHR) Models after Chronic Sleep Deprivation

Since changes in microbial adhesion, penetration and invasion after chronic sleep deprivation were observed, it would be of interest to determine if structural changes occurred in the intestinal tract of these animals. In DHR, chronic sleep deprivation resulted in decreased villus height in the large intestine, as well as decreased crypt width in the small intestine. However, crypt depth in the small intestine and the numbers of villi and crypts (per mm), as well as crypt width in the large intestine were significantly altered in native Sprague Dawley rats after chronic sleep deprivation (Table 33). The humanization process appears to affect these structures in a regional-specific manner, with the most dramatic effect observed in the villus height in the cecum of DHR.

Table 33. Villus Height, Crypt Depth and Crypt Width in Small Intestine, Cecum and Large Intestine of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Experiment	Subjects	Treatment	Villus Height (μm)	# Villi (per mm)	Crypt Depth (μm)	Crypt Width (μm)	# Crypts (per mm)
Small Intestine	Native Rats	Normal Sleep	406.65±16.79	9.04±1.02	64.98±3.44	63.58±3.57	9.22±1.06
		Sleep-Deprived	435.13±20.06	7.64±0.58	78.82±4.12 ^a	72.82±3.90	8.39±0.53
	Humanized Rats	Normal Sleep	419.96±19.80	7.93±0.79	77.91±2.53 ^b	73.91±3.36	8.68±0.74
		Sleep-Deprived	431.14±23.10	8.01±0.46	71.44±3.33	66.16±2.66 ^a	9.11±0.47
Cecum	Native Rats	Normal Sleep	243.25±6.59	NA	NA	66.61±1.87	NA
		Sleep-Deprived	216.30±15.34	NA	NA	64.11±2.32	NA
	Humanized Rats	Normal Sleep	175.92±7.11 ^b	NA	NA	63.05±2.23	NA
		Sleep-Deprived	170.85±8.91 ^b	NA	NA	64.14±3.13	NA
Large Intestine	Native Rats	Normal Sleep	293.31±16.33	15.76±0.48	29.52±1.55	54.58±1.45	14.36±0.46
		Sleep-Deprived	336.73±21.10	17.63±0.72 ^a	32.88±1.59	50.21±2.05 ^a	16.79±0.81 ^a
	Humanized Rats	Normal Sleep	318.90±11.20	16.71±0.74	33.96±1.19 ^b	52.08±1.75	15.52±0.45
		Sleep-Deprived	275.07±15.01 ^{a,b}	17.86±0.66	31.22±1.68	51.73±2.60	16.04±0.68

a – significantly different from normal sleep group (p<0.05); b - significantly different from its native rat counterpart (p<0.05); N/A – Data not available.

Since structural changes and altered microbial penetration and invasion in both native Sprague Dawley and DHR were observed after chronic sleep deprivation, we investigated if these changes were associated with mucosal inflammation, activation of immune response and HPA axis dysregulation. As shown in Table 34, no up-regulation of MPO activity in any of the intestinal regions tested, suggesting that no detectable mucosal inflammation in these animals after chronic sleep deprivation. Similarly, no change in the bacterial endotoxin load was detected in either animal model after chronic sleep deprivation (Table 35).

Table 34. Myeloperoxidase (MPO) Activity in Small Intestine, Cecum and Large Intestine of Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

MPO Activity (pmol/min/ml)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Distal Ileum	0.1742±0.0141	0.1775±0.0162	0.1839±0.0185	0.2018±0.0649
Cecum	0.0841±0.0106	0.0923±0.0279	0.0927±0.0323	0.1009±0.0326
Proximal Colon	0.1288±0.0273	0.1213±0.0253	0.194±0.0631	0.1971±0.0506

Table 35. Serum Level of Lipopolysaccharide binding protein (LBP) in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Endotoxin Load	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Serum LBP (mM)	127.61±32.9	113.17±29.06	109.43±41.93	130.85±41.27

To further evaluate the status of inflammatory and immune responses in the native Sprague Dawley and DHR after chronic sleep deprivation, a panel of nine cytokines and chemokines were studied. While Chemokine (C-X-C motif) ligand 1 (i.e., CXCL1) showed significant changes in the native Sprague Dawley and DHR rats after chronic sleep deprivation, other cytokines (and chemokines) were unchanged (Table 36). CXCL1, which is expressed by macrophages, neutrophils and epithelial cells, has neutrophil chemoattractant activity. It is involved in inflammatory response (resulted from bacterial infection) and wound healing. Up-regulation of this cytokine thus suggests inflammatory response at the systemic level indeed occurred in the chronically sleep-deprived subjects.

Table 36. Serum Level of Pro- and Anti-Inflammatory Cytokines in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep or Chronic Sleep Deprivation

Cytokines / Chemokines (pg/ml)	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
IFN γ	117.76 $\pm$ 11.44	104.22 $\pm$ 27.08	116.99 $\pm$ 7.87	115.89 $\pm$ 10.72
IL-1 β	479.7 $\pm$ 14.76	479.43 $\pm$ 23.13	472.74 $\pm$ 1.05	473.85 $\pm$ 2.3
IL-4	79.5 $\pm$ 5.02	75.66 $\pm$ 16.68	80.26 $\pm$ 4.63	79.69 $\pm$ 6.76
IL-5	16.23 $\pm$ 3.66	15.69 $\pm$ 3.12	15.31 $\pm$ 3.71	16.43 $\pm$ 2.67
IL-6	438.21 $\pm$ 26.91	429.29 $\pm$ 43.87	389.66 $\pm$ 13.2	390.14 $\pm$ 23.3
IL-10	167.26 $\pm$ 26.27	173.72 $\pm$ 28.93	167.01 $\pm$ 22.26	141.39 $\pm$ 18.02
IL-13	112.93 $\pm$ 9.48	109.12 $\pm$ 2.63	109.08 $\pm$ 1.05	110.07 $\pm$ 3.52
CXCL1	41.27 $\pm$ 11.7	131.28 $\pm$ 15.56*	51.01 $\pm$ 9.87	153.6 $\pm$ 20.21*
TNF α	54.04 $\pm$ 3.92	53.8 $\pm$ 3.74	53.13 $\pm$ 1.54	53.47 $\pm$ 3.23

*p<0.05

As discussed above, the level of the hormones of the HPA axis are useful markers of stress response. As shown in Table 36, the level of peripheral CRF was not significantly altered in either animal model after chronic sleep deprivation. Consistently, the levels of ACTH was not significantly affected by chronic sleep deprivation in these animals. However, corticosterone was increased after chronic sleep deprivation in native Sprague Dawley rats, consistent with our previous observations. Close examination revealed that DHR showed a significant decrease in the ACTH level regardless of the sleep conditions, compared with the native rats. However, a higher level of corticosterone was detected in the DHR with normal sleep, which was further increased after chronic sleep deprivation. However, these differences are not statistically significant, due to the large standard deviations in these values.

Table 37. Serum Levels of Hypothalamic–Pituitary–Adrenal (HPA) Axis Hormones in Native Sprague Dawley Rats and De-Individualized Humanized Rats (DHR) with Normal Sleep and Chronic Sleep Deprivation

HPA Axis	Native Rats		Humanized Rats	
	Normal Sleep	Sleep Deprived	Normal Sleep	Sleep Deprived
Peripheral CRF (pg/ml)	171.69 $\pm$ 12.62	174.51 $\pm$ 13.46	169.47 $\pm$ 4.23	171.44 $\pm$ 4.03
ACTH (pg/ml)	986.95 $\pm$ 124.06	817.04 $\pm$ 185.13	482.55 $\pm$ 75.06	472.01 $\pm$ 129.49
Corticosterone (ng/ml)	112.61 $\pm$ 27.26	161.49 $\pm$ 66.97	204.62 $\pm$ 85.95	251.33 $\pm$ 96.81

5.0. DISCUSSION

5.1 Development of Pseudo Germ-Free (PGF) Rats and De-Individualized Humanized Rat (DHR) Model

Recent publications strongly suggest gut microbiota have an important roles in the functions and well-being of the host. However, studies of human microbiota are mostly limited to the analysis of the fecal microbiota, due to the difficulties in accessing the intestinal tract for sample collection using non-invasive methods. As a result, there is a significant knowledge gap concerning the responses of gut microbiota (especially human gut microbiota that reside in the small intestine) to a variety of physiological and psychological stressors. Although this gap may be partially addressed by animal studies using laboratory rodents, there are dramatic differences between the human and rodent microbiota. Thus, their responses to the same stimuli (or stressors) will likely be different. For this reason, the availability of an adequate animal model(s) harboring the human microbiota will be an extremely useful tool for microbiome research. Since gut microbiota is highly individualized (personalized), an animal model generated by mono-association with the sample from a single human donor will likely have limited coverage and representation of the entire spectrum of the human microbiota. To address these issues, a novel humanized model, the De-Individualized Humanized Rat Model, was therefore developed for the investigation of the effect of sleep deprivation on microbiota population and functions in three spatial locations of the intestinal tract, namely the small intestine, cecum and large intestine.

Naïve Sprague Dawley rats were first converted to PGF rats by the treatment with an antibiotic cocktail developed specific for this purpose that is capable of depleting all culturable microbial cells in the cecal content after 3 days. This represented at least an 8-order of magnitude reduction in the microbial titer. Others have reported the use of 1 to 4 antibiotics in the drinking water or by oral gavage for 3 to 21 days in mice, and they achieved reductions of 1 to 5 orders of magnitude (Manichanh et al. 2010; Reikvam et al. 2011; Wos-Oxley et al. 2012). Since mice that experienced 2- to 5-orders of magnitude reduction in bacterial load after antibiotic treatment resembled GF mice anatomically and physiologically (Reikvam et al. 2011), these authors thus assumed that these antibiotic-treated animals represented a valid germ-free (germ-free-like) model for microbiome research. Nevertheless, we decided to employ a 12-day full course of antibiotic treatment in our protocol, to ensure that a near-complete PGF status could be achieved.

Our result also showed that the humanized rats produced using the procedure we developed had a gut microbiota that closely resembled that of the human donors. Similar results have been reported by others in mice, when a highly effective antibiotic treatment was used (Wos-Oxley et al. 2012) or when using GF animals (Hintze et al. 2014). Smaller reductions in microbial load prior to the transplant of human fecal material, however, had a negative impact on the humanization process: the diversity of the established microbiota was reduced and it maintained greater similarity to the native mouse gut microbiota of the *naïve* animals than that from the donors (Manichanh et al. 2010; Wos-Oxley et al. 2012). In this study, the antibiotic treatment was able to deplete all culturable microbial cells in the cecal content after 3 days, but viable cells might have remained because the native

rat microbiota partially recovered in animals that had been treated with antibiotics for 12 days but did not receive fecal transplant (the Antibiotic / LB group in Figures 5). Alternatively, this could be due to recolonization of the intestinal tract by the microbiota on other locations of the body (e.g. skin microbiota) that can readily survive antibiotic treatment. If this were the case, it would suggest that the environmental factors can influence the microbiota population structure in the gut, since the microbiota recovered from antibiotic treatment clustered near the baseline and control samples in the PCoA plots. The unsuccessful humanization by coprophagic acquisition somewhat confirmed this notion. PGF rats, which were introduced into the de-individualized community without direct fecal transplant, did not acquire sufficient amount of the human microbiota through coprophagia to overcome the recovery and/or recolonization of the rat native microbiota.

To date, the longest reported stability in a humanized rodent model for gut microbiota was 12 weeks (Hintze et al. 2014), but the mice in that study were generated using individual donors and had distinct microbiota and metabolomic profiles. In this study, humanized rats generated from multiple donors with diverse microbiota profiles were de-individualized in order to eliminate donor-specific attributes to produce a high degree of similarity in their gut microbiota. This homogenous profile of human microbiota were maintained in the humanized rats for up to 15 weeks. Interestingly, members of the de-individualized communities could maintain the human microbiota better than the independent transplanted lines, regardless if the independent lines were generated from a single donor or a mix of three donors. This difference probably resulted from a difference between the ability of the transplanted human microbiota to compete with the recovering native rat microbiota of the recipient. In the case of the 3-donor mix, the optimal population structure of this mixture has not yet been established prior to the transplantation into rat hosts, which will undoubtedly decrease its ability to compete with the native rat microbiota.

Taken together, these results confirmed the general utilities of our methods for generating PGF and DHR for microbiome research. They not only provide a means to create PGF stocks from commercially purchased animals, but also allow the creation of animal colonies that stably maintain human microbiota in the intestinal tract for at least 15 weeks after fecal transplant. Unlike those humanized models that showed donor-specific characteristics as described previously in the literature, our method was designed to generate a homogenous profile of human microbiota from human samples with diverse microbiota profiles. This method may also be used to develop a homogeneous humanized model directly from GF rodents. Since the number of strains of rats and mice (including genetically modified models) available for research is vast, and since GF animals of most of these models are not commercially available, a procedure such as the one described herein, which can reproducibly generate PGF animals suitable for subsequent fecal transplant, is highly valuable for microbiome research. More importantly, this DHR Model can be considered as a standard humanized model, and the use of such model in microbiome research will thus allow the comparison and validation of results across multiple laboratories and institutions.

5.2 Effects of Sleep Deprivation on Gut Microbiota Community Structure and Functions

Sleep is an essential biological process that has a critical role in metabolic regulation. Inadequate sleep can activate a classical stress response with increases in HPA-axis activity and cortisol release. Sleep deprivation has enormous impacts on metabolic homeostasis, leading to increased risk of a range of metabolic disorders, including cardiovascular and gastrointestinal morbidity, peptic ulcers, obesity, insulin resistance, and type 2 diabetes. A relationship between sleep deprivation and prevalence of weight gain and obesity has been shown (Bollinger et al, 2009). Interestingly, perturbation of gut microbiota presents a pattern of metabolic abnormalities mirroring those associated with sleep deprivation. Despite the similar patterns of metabolic disorders induced by the perturbation of gut microbiota and sleep deprivation, it is not clear how these two conditions interact with and influence each other. This study was designed to address these issues. Specifically, the hypothesis to be tested is that sleep deprivation initially degrades the functional and structural integrity of the gastrointestinal tract, resulting in an altered metabolic homeostasis and intestinal permeability. While the former induces a perturbation of the gut microbiota with the overgrowth of pathogenic microbes, the latter results in compromised tight junction structure of the intestinal tract resulting in the leakage of pathogenic microbial cells and factors such as endotoxin LPS. The combined effects of these events can trigger mucosal and systemic inflammation, increased levels of cortisol and the dysregulation of the HPA axis.

Stress is known to increase intestinal permeability, allowing gut microbiota an opportunity to translocate across the intestinal mucosa and directly interact with immune cells (Gareau et al, 2008; Teitelbaum et al, 2008). Degradation in the structural integrity of the intestinal tract can result in increased intestinal permeability and subsequently the leakage of gut microbial cells and factors both externally and interstitially, causing exposure to degrading factors, including LPS. This degraded epithelial state in turn, triggers mucosal and systemic inflammation. In addition, changes in intestinal functions can cause changes in the gut microbiota composition and metabolism. It has been reported that sleep deprivation results in gut barrier dysfunction, facilitating bacterial translocation from the gut lumen into systemic circulation (Everson and Toth, 2000). Sleep deprivation also increases oxidative damage and cell death in the intestine in rodents, and infection of body tissues by the pathogenic bacteria found in the intestine after sleep deprivation (Everson et al., 2014). Intestinal overgrowth of total aerobes, and total facultative anaerobes, including several pro-inflammatory and pathogenic species has been shown to occur following 10 days of near total sleep deprivation (Everson and Toth, 2000). Sleep loss also induces a systemic low-grade inflammation characterized by the release of several molecules, such as cytokines and acute-phase proteins, including TNF- α , IL-1 β , IL-6, IL-17A and C-Reactive Protein (CRP) (Hurtado-Alvarado et al. 2016). These inflammatory molecules in turn degrade the functions of tight junction proteins and promote disruption in cellular components of the gut barrier and blood-brain barrier.

Besides the changes in the immune and endocrine systems, sleep-disrupted animals consumed more food than control animals (Everson and Toth, 2000; Poroyko et al., 2016). Increased food consumption may therefore comprise an indirect mechanism by which sleep deprivation impacts the gut microbiota (Benedict et al. 2016; Zhang et al. 2017). This may explain the observations of Poroyko et al. (2016) that taxa known to feed on undigested nutrients (Lachnospiraceae and Ruminococcaceae), were increased following sleep fragmentation. The importance of the gut microbiome in metabolic disorders such as obesity and diabetes has been demonstrated and certain mixes of gut microbiota may protect or predispose the host to obesity. A shift in gut microbiota populations towards the genus of Firmicutes from Bacteroidetes has been observed in obese individuals. In contrast, if an obese individual loses weight, a shift towards Bacteroidetes occurs (Cryan & Dinan, 2012). Obesity, in part, is characterized by systemic inflammation, in which the gut-brain axis is likely to be involved (Tahergorabi and Khazaei, 2013).

Consistent with the reports in the literature, significant changes in the gut microbiota can be detected in both native Sprague Dawley and DHR after six days of sleep deprivation, as well as after 6 weeks of chronic sleep deprivation, in the treatment-, host- and location-specific manners. Despite this, concordant changes in the gut microbiota can be detected in the native and DHR, strongly implicating the specific effect of sleep deprivation. For instance, Bacteroidaceae, Rikenellaceae and S24-7 families of Bacteroidetes, Christensenellaceae, Mogibacteriaceae, Lachnospiraceae, Peptococcaceae and Ruminococcaceae families of Firmicutes, RF32 order of Proteobacteria, and F16 family of TM7 were increased in the large intestine of both native and DHR rats. Similarly, decreases in Prevotellaceae and S24-7 families of Bacteroidetes, Erysipelotrichaceae, Lachnospiraceae, Mogibacteriaceae, Peptococcaceae and Ruminococcaceae families of Firmicutes, and RF39 order of Tenericutes were detected in the large intestine of these animals.

However, more dramatic changes in the gut microbiota were observed after chronic sleep deprivation, with much more OTUs significantly altered. In contrast, the number of microbial taxa that were increased or decreased in both native and DHR hosts are about the same as that of the acute sleep deprivation experiment. Specifically, Bacteroidaceae, Porphyromonadaceae, Rikenellaceae and S24-7 families of Bacteroidetes, Lachnospiraceae and Ruminococcaceae families (and Lactobacillales order) of Firmicutes, and Enterobacteriaceae family of Proteobacteria were significantly increased in both native Sprague Dawley and DHR after chronic sleep deprivation. Decreases in Coriobacteriaceae family of Actinobacteria, Rikenellaceae and S24-7 families of Bacteroidetes, and Christensenellaceae, Lachnospiraceae and Ruminococcaceae families of Firmicutes were detected in both native and DHR after chronic sleep deprivation. Interestingly, the majority of the up-regulated taxa showed increases in both acute and chronic sleep deprivation, while the majority of significantly decreased taxa were not affected by acute sleep deprivation. This observation suggests

that the increased taxa might have a more significant role in the metabolic disorders induced by sleep deprivation.

Although these results identify the changes in the gut microbiota population, they reveal little insight into the changes in the gut microbiota that function collectively as a community. This is important, since microbial taxa in the microbiota do not function in isolation. Instead, they must cooperate with other members in the microbiota community and together they provide the functional outputs as a single entity, such as providing the essential roles that no other organs of the host can fulfill. For this reason, the gut microbiota has been coined as an extra organ of the host. In fact, the elucidation of the functional changes, rather than solely the changes in the community members, is essential in microbiome research. Therefore, we conducted additional analyses to investigate pathway perturbation in the gut microbiota following sleep deprivation in both native Sprague Dawley and DHR models. The results showed that the number of perturbed pathways in the native rats is much more than that in its DHR counterpart (53 vs. 18) after acute sleep deprivation. While the small intestinal microbiota of the native rats contributed more than 60% of the differentially regulated pathways after acute sleep deprivation, about half of the altered pathways in the DHR occurred in the cecal microbiota. Interestingly, acute sleep deprivation resulted in dysregulation of a large number (total 23) of metabolic pathways in the native rats, including the metabolisms of sugars, amino acids, fatty acids, carbohydrates, proteins and lipids. In contrast, only five metabolic pathways, which are mostly involved lipid metabolism, were detected in the DHR.

In comparison, chronic sleep deprivation resulted in dysregulation of 102 and 48 pathways in native Sprague Dawley and DHR, respectively, which are significantly higher than that induced by acute sleep deprivation. Again, the number of altered pathways in the native rats are significantly more than that in its DHR counterpart. Unlike the acute sleep deprivation experiment, most of the altered pathways (almost 45%) in the native rats occurred in the cecum, while DHR showed the higher number of altered pathways in the small intestine, accounting for more than 60% of the total number in this host. A number of pathways are commonly altered in both native and DHR, but in different spatial locations. For instance, lipid metabolism and pentose phosphate pathways were altered in the large intestine of native Sprague Dawley rats, but they were differentially regulated in the small of DHR. Overall, perturbation of 25 metabolic pathways occurred in the native rats. This number is similar to that observed in the acute sleep deprivation experiment, despite that a much higher number of altered pathways was detected in the chronic sleep deprivation experiment. These results suggest that perturbation of metabolism might have reached plateau. However, close examination revealed that less than ten of these pathways are common in these two experiments.

In addition to the perturbation of metabolic pathways, acute sleep deprivation resulted in pathway perturbations that are related to microbial infections in both native and DHR. There are several common pathways observed in these two animal models, including African trypanosomiasis, penicillin and cephalosporin biosynthesis, Retinoic acid-Inducible gene-1-like (RIG-1-like) receptor signaling pathway, Shigellosis, pertussis and Tuberculosis. Similarly, chronic sleep deprivation also resulted in dysregulation of pathways related to microbial infections in both native and DHR, including bacterial chemotaxis, bacterial invasion of epithelial cells, bacterial toxins, epithelial cell signaling pathway in *Helicobacter pylori* infection, penicillin and cephalosporin biosynthesis, RIG-like receptor signaling pathway, *Vibrio cholerae* infection and *Vibrio cholerae* pathogenic cycle. Some of these pathways were altered in both acute and chronic conditions, suggesting they are particularly sensitive to sleep conditions. Taken together, these results provide the mechanistic insight into the roles of microbiota perturbation in increased susceptibility to microbial infections after acute and chronic sleep deprivation.

5.3 Effects of Sleep Deprivation on Host Functions

Although significant changes in the community structure and functions of gut microbiota have been observed in both native Sprague Dawley and DHR after acute sleep deprivation, changes in the host functions were relatively minor and insignificant. For instance, no significant change in the marker of mucosal inflammation, MPO activity could be detected, nor any change in the pro-inflammatory cytokines tested, in either animal model. Although acute sleep deprivation had no detectable effect on the CRF level in the native Sprague Dawley rats, it did increase the levels of ACTH and corticosterone significantly, highlighting the impacts of sleep deprivation on the activity of the HPA axis.

On the other hand, DHR appeared to be more sensitive to the acute sleep deprivation treatment. The crypt depth, a marker of mucosal epithelial cell proliferation and mucosal regeneration, was significantly decreased in the small intestine of sleep-deprived native rats. Although this structural change can potentially increase the gut permeability, resulting in enhanced microbial invasion and bacteria-derived endotoxin load, such abnormalities were not observed. Acute sleep deprivation also resulted in an increased bacteria-derived endotoxin load in the DHR, mainly due to a decrease in the DHR with normal sleep, suggesting that the human gut microbiota may have a higher activity than the native rat microbiota in protecting the mucosal layer of the intestine against microbial invasions. This enhanced protection may be related to the structural changes, such as the decreased crypt depth in the DHR, compared to the native rats. Consistent with these results, the CRF level was also increased in the DHR following acute sleep deprivation, again due to decreased CRF in the DHR with normal sleep (compared with their native SPF counterpart). Unexpectedly, DHR, regardless of the sleep conditions, had significantly increased ACTH and corticosterone levels, compared with the native Sprague Dawley rats with normal sleep. These results suggest that these two hormones might have reached the upper limit in the DHR, which could not be further increased by acute sleep deprivation.

However, the changes in the gut microbiota, intestinal structures and the HPA axis in these animals did not appear to have any significant impacts on the learning and memory of these animals. We found that the preference for a novel object was not significantly affected in the *naïve* Sprague Dawley Rats even after 6 days of acute sleep deprivation, as assessed using the NOR test. This result indicates that the encoding and/or retrieval of the episodic memory of the familiar object remained intact in these animals. Although this test was performed using a different set of animals than that used for the analyses of the gut microbiota and host functions, we expect that both sets of animals should have very similar performance in learning and memory functions.

In addition to the significant changes in the community structure and functions of the gut microbiota, chronic sleep deprivation also has profound effects on the host functions in both animal models. For instance, crypt depth in the small intestine, as well as the number of villus and crypts in the large intestine were significantly increased in the native rats, while decreased crypt width in the large intestine were observed. In contrast, crypt width in the small intestine and villus height in large intestine were decreased in the DHR. Changes in these structures associated with intestinal injuries and dysfunctions have been previously reported in the literature (Drucker et al. 1999; Kaya et al. 2007; Sever et al. 2009; Abimosleh et al. 2012; Adas et al. 2013; Lima-Júnior et al. 2014; Wu et al. 2014; Yang et al. 2014; Miousse et al. 2017). Therefore, these structural changes in the intestinal tract likely represent the mechanistic basis of increased microbial invasion in the large intestine, as well as in the spleen and liver of both native and DHR. However, the biological significance of this observation is not clear at this point, since no mucosal inflammation could be detected in all three intestinal regions tested, nor is there any change in the bacterial endotoxin load, in either animal model after chronic sleep deprivation. Nevertheless, these results suggest that chronic sleep deprivation, resulting in increased microbial invasion of intra-intestinal and extra-intestinal tissues is a common phenomenon. Consistent with this notion, chemokine CXCL1, which is involved in bacterial infection, inflammatory response and wound healing, was significantly up-regulated in both native and DHR after chronic sleep deprivation. This suggests that chronic sleep deprivation might trigger a relatively confined, rather than a wide-spread inflammatory response.

The levels of peripheral CRF and ACTH were not changed after chronic sleep deprivation. Although corticosterone was increased in both native and DHR after chronic sleep deprivation, these changes are not statistically significant, due to the large standard deviations in these values. These results thus suggest that there is no HPA axis activation after 6 weeks of sleep deprivation. However, the possibility that HPA axis activation occurred at earlier time points and then returned to the normal level cannot be ruled out, since activation of the HPA axis was detected after 6 day of sleep deprivation.

6.0. CONCLUSION

This study represents a comprehensive investigation of the effects of sleep deprivation on the gut microbiota community structure and functions, as well as on the host functions. The strength of this study is that both acute and chronic sleep deprivation were studied using two animal models, the native Sprague Dawley and DHR. Furthermore, changes in the gut microbiota and host functions were investigated in three different locations along the intestinal tract, and the functional significance of these changes identified using a pathway analysis approach. Comparison of the results obtained from different animal models not only allows the identification of effects of sleep deprivation in general, it also provides important insight into specific responses of native rat microbiota and human microbiota to sleep deprivation. Similarly, comparison of the results obtained from the acute and chronic sleep deprivation experiments reveal treatment-specific effects on gut microbiota and host functions. Comparison of the results obtained from different locations of the intestinal tract, especially using the DHR model, probably has the most far-reaching implications. Because the analysis of human gut microbiota has been limited to the use of fecal materials, which provides little information concerning how human microbiota interacts with the host within the intestinal tract and how this interaction may change under acute and chronic sleep deprivation. The DHR model developed in this study thus effectively addresses the limitations of most human microbiome studies in the literature. The novel procedures we have developed for the generation of pseudo germ-free animals and for the de-individualization of humanized animals will significantly facilitate future microbiome research.

7.0. REFERENCES

- Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS et al. (2012) Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig Dis Sci.* 57:887-96.
- Adas G, Adas M, Arikan S, Sarvan AK, Toklu AS. Et al. (2013) Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa. *World J Gastroenterol.* 19:2904-12.
- Afra K, Laupland K, Leal J et al. (2013) Incidence, risk factors, and outcomes of *Fusobacterium* species bacteremia. *BMC Infect Dis* 13: 264-2334-13-264.
- Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H et al. (2012) Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology.* 37:1885-95.
- Aldabal L and Bahammam AS. (2011) Metabolic, endocrine, and immune consequences of sleep deprivation. *Open Respir Med J.* 5:31-43.
- Aleisa AM, Helal G, Alhaider IA, et al. (2011) Acute nicotine treatment prevents REM sleep deprivation-induced learning and memory impairment in rat. *Hippocampus.* 21:899-909.
- Alhaider IA, Aleisa AM, Tran TT, Alkadhi KA. (2010) Caffeine prevents sleep loss-induced deficits in long-term potentiation and related signaling molecules in the dentate gyrus. *Eur J Neurosci.* 31:1368–76.
- Alzoubi KH, Khabour OF, Salah HA, Abu Rashid BE. (2013) The combined effect of sleep deprivation and Western diet on spatial learning and memory: role of BDNF and oxidative stress. *J Mol Neurosci.* 50:124-33.
- Anderson JR, Carroll I, Azcarate-Peril MA, Rochette AD, Heinberg LJ et al. (2017) A preliminary examination of gut microbiota, sleep, and cognitive flexibility in healthy older adults. *Sleep Med.* 38:104-7.
- Backhed F, Ding H, Wang T, et al. (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 101:15718-23.
- Becker N, Kunath J, Loh G, et al. (2011) Human intestinal microbiota: characterization of a simplified and stable gnotobiotic rat model. *Gut Microbes* 2:25-33.
- Benedict C, Vogel H, Jonas W, Woting A, Blaut M et al. (2016) Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. *Mol Metab.* 5:1175-86.
- Bollinger T, Bollinger A, Skrum L, et al. (2009) Sleep-dependent activity of T cells and regulatory T cells. *Clin Exp Immunol.* 155:231-8.
- Broadbent NJ, Gaskin S, Squire LR, Clark RE. (2009) Object recognition memory and the rodent hippocampus. *Learn Mem.* 17:5-11.
- Caporaso JG, Kuczynski J, Stombaugh J, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335-336.

- Cirelli C and Bushey D. (2008) Sleep and wakefulness in *Drosophila melanogaster*. *Ann N Y Acad Sci USA*. 1129:323-9.
- Cirelli C and Tononi G. (2008) Is sleep essential? *PLoS Biol*. 6:1605-11.
- Cirelli C, Gutierrez CM, Tononi G. (2004) Extensive and divergent effects of sleep and wakefulness on brain gene expression. *Neuron*. 41:35–43.
- Collins J, Auchtung JM, Schaefer L, et al. (2015) Humanized microbiota mice as a model of recurrent *Clostridium difficile* disease. *Microbiome* 3:35-015-0097-2.
- Cryan JF, and Dinan TG. (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci*. 13:701-12.
- DeSantis TZ, Hugenholtz P, Larsen N, et al. (2016) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72: 5069-5072.
- Dhaliwal J, Singh DP, Singh S, Pinnaka AK, Boparai RK et al. (2018) *Lactobacillus plantarum* MTCC 9510 supplementation protects from chronic unpredictable and sleep deprivation-induced behavior, biochemical and selected gut microbial aberrations in mice. *J Appl Microbiol*. 125:257-69.
- Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL. (1999) Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. *Am J Physiol*. 276:79-91.
- Eckburg PB, Bik EM, Bernstein CN, et al. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635-1638.
- Ennaceur A and Delacour J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav Brain Res*. 31:47-59.
- Ennaceur A. (2010) One-trial object recognition in rats and mice: methodological and theoretical issues. *Behav Brain Res*. 215:244-54.
- Everson CA, and Toth LA. (2000) Systemic bacterial invasion induced by sleep deprivation. *Am J Physiol Regul Integr Comp Physiol*. 278:R905-16.
- Gareau MG, Jury J, MacQueen G, et al. (2007) Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut*. 56:1522-8.
- Gareau MG, Silva MA, Perdue MH. (2008) Pathophysiological mechanisms of stress-induced intestinal damage. *Curr. Mol. Med*. 8:274-81.
- Gaskin S, Tardif M, Cole E et al. (2010) Object familiarization and novel-object preference in rats. *Behav Processes*. 83:61-71.
- Guan Z, Peng X, Fang J. (2004) Sleep deprivation impairs spatial memory and decreases extracellular signal-regulated kinase phosphorylation in the hippocampus. *Brain Res*. 1018:38–47.
- Hammond RS, Tull LE, Stackman RW. (2004) On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiol Learn Mem*. 82:26-34.

- Hintze KJ, Cox JE, Rompato G, et al. (2014) Broad scope method for creating humanized animal models for animal health and disease research through antibiotic treatment and human fecal transfer. *Gut Microbes* 5: 183-191.
- Human Microbiome Project Consortium. (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207-214.
- Hurtado-Alvarado G, Domínguez-Salazar E, Pavon L, Velázquez-Moctezuma J, Gómez-González B. (2016) Blood-Brain Barrier Disruption Induced by Chronic Sleep Loss: Low-Grade Inflammation May Be the Link. *J Immunol Res*. 2016:4576012.
- Jazwinska EC. (1986) Sleep deprivation alters the cell population kinetics in the jejunum of the male Syrian hamster (*Mesocricetus auratus*). *Cell Tissue Kinet*. 19:335-50.
- Ji SK, Yan H, Jiang T, et al. (2017) Preparing the Gut with Antibiotics Enhances Gut Microbiota Reprogramming Efficiency by Promoting Xenomicrobiota Colonization. *Front Microbiol* 8: 1208-22.
- Kaya E, Ceylan A, Kara N, Güven H, Yildiz L. (2007) The effect of L-glutamine on mucosal healing in experimental colitis is superior to short-chain fatty acids. *Turk J Gastroenterol*. 18:89-94.
- Lima-Júnior RC, Freitas HC, Wong DV, Wanderley CW, Nunes LG. et al. (2014) Targeted inhibition of IL-18 attenuates irinotecan-induced intestinal mucositis in mice. *Br J Pharmacol*. 171:2335-50.
- Lozupone C and Knight R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71:8228-35.
- Lozupone C, Lladser ME, Knights D, et al. (2011) UniFrac: an effective distance metric for microbial community comparison. *ISME J* 5:169-72.
- Mackiewicz M, Shockley KR, Romer MA, et al. (2007) Macromolecule biosynthesis: a key function of sleep. *Physiol Genomics*. 31:441–57.
- Manichanh C, Reeder J, Gibert P, et al. (2010) Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome Res* 20:1411-9.
- McDermott CM, LaHoste GJ, Chen C, et al. (2003) Sleep deprivation causes behavioral, synaptic, and membrane excitability alterations in hippocampal neurons. *J Neurosci*. 23:9687-95.
- Miousse IR, Pathak R, Garg S, Skinner CM, Melnyk S et al. (2017) Short-term dietary methionine supplementation affects one-carbon metabolism and DNA methylation in the mouse gut and leads to altered microbiome profiles, barrier function, gene expression and histomorphology. *Genes Nutr*. 12:22-33.
- Miousse IR, Pathak R, Garg S, Skinner CM, Melnyk S. et al. (2017) Short-term dietary methionine supplementation affects one-carbon metabolism and DNA methylation in the mouse gut and leads to altered microbiome profiles, barrier function, gene expression and histomorphology. *Genes Nutr*. 12:22-33.
- Mirmiran M. (1986) The importance of fetal/neonatal REM sleep. *Eur J Obstet Gynecol Reprod Biol* 21:283-91.

- Moggras MA, Guillem F, Brazzini-Poisson V, et al. (2009) The effects of total sleep deprivation on recognition memory processes: a study of event-related potential. *Neurobiol Learn Mem* 91:343–52.
- Mooser C, Gomez de Agüero M and Ganai-Vonarburg SC. (2018) Standardization in host-microbiota interaction studies: challenges, gnotobiology as a tool, and perspective. *Curr Opin Microbiol* 44:50-60.
- Morrissey MJ, Duntley SP, Anch AM, Nonneman R. (2004) Active sleep and its role in the prevention of apoptosis in the developing brain. *Med Hypotheses* 62:876-9.
- National Research Council of the National Academies, Guide for the Care and Use of Laboratory Animals, Washington, DC: The National Academies Press, 2010.
- Poroyko VA, Carreras A, Khalyfa A, Khalyfa AA, Leone V et al. (2016) Chronic Sleep Disruption Alters Gut Microbiota, Induces Systemic and Adipose Tissue Inflammation and Insulin Resistance in Mice. *Sci Rep*. 6:35405-16.
- Possemiers S, Bolca S, Eeckhaut E, et al. (2007) Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol Ecol* 61:372-83.
- Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229-41.
- Reikvam DH, Erofeev A, Sandvik A, et al. (2011) Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS One* 6:e17996.
- Sabino M, Cappelli K, Capomaccio S, Pascucci L, Biasato I. et al. (2018) Dietary supplementation with olive mill wastewaters induces modifications on chicken jejunum epithelial cell transcriptome and modulates jejunum morphology. *BMC Genomics*. 19:576-88.
- Sever M, Klicek R, Radic B, Brcic L, Zoricic I et al. (2009) Gastric pentadecapeptide BPC 157 and short bowel syndrome in rats. *Dig Dis Sci*. 54:2070-83.
- Staley C, Kaiser T, Beura LK, et al. (2017) Stable engraftment of human microbiota into mice with a single oral gavage following antibiotic conditioning. *Microbiome* 5:87-017-0306-2.
- Sudo N, Chida Y, Aiba Y, et al. (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol*. 558:263-75.
- Taglialatela G, Hogan D, Zhang WR, Dineley KT. (2009) Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. *Behav Brain Res*. 200:95-9.
- Tahergorabi Z, Khazaei M. (2013) The relationship between inflammatory markers, angiogenesis, and obesity. *ARYA Atheroscler*. 9:247-53.
- Teitelbaum AA, Gareau MG, Jury J, et al. (2008) Chronic peripheral administration of corticotropin-releasing factor causes colonic barrier dysfunction similar to psychological stress. *Am. J. Physiol. Gastrointest. Liver Physiol*. 295:G452-9

- Thompson CL, Wisor JP, Lee CK, et al. (2010) Molecular and anatomical signatures of sleep deprivation in the mouse brain. *Front Neurosci* . 4:165-84.
- Turnbaugh PJ, Ridaura VK, Faith JJ, et al. (2009) The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 1:6ra14.
- Turner TH, Drummond SP, Salamat JS, Brown GG. (2007) Effects of 42 hr of total sleep deprivation on component processes of verbal working memory. *Neuropsychology*. 21:787–95.
- Vazquez-Baeza Y, Pirrung M, Gonzalez A, et al. (2013) EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* 2:16-217X-2-16.
- Vogel-Ciernia A, Wood MA. (2014) Examining object location and object recognition memory in mice. *Curr Protoc Neurosci*. 69:8.31.1-8.31.17.
- Wos-Oxley M, Bleich A, Oxley AP, et al. (2012) Comparative evaluation of establishing a human gut microbial community within rodent models. *Gut Microbes* 3:234-49.
- Wu J, Chen J, Wu W, Shi J, Zhong Y et al. (2014) Enteral supplementation of bovine lactoferrin improves gut barrier function in rats after massive bowel resection. *Br J Nutr*. 112:486-92.
- Yang G, Wang H, Kang Y, Zhu MJ. (2014) Grape seed extract improves epithelial structure and suppresses inflammation in ileum of IL-10-deficient mice. *Food Funct*. 5:2558-63.
- Zhang SL, Bai L, Goel N, Bailey A, Jang CJ et al. (2017) Human and rat gut microbiome composition is maintained following sleep restriction. *Proc Natl Acad Sci USA*. 114:E1564-E1571.

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

ACTH	Adrenocorticotrophic hormone
CFU/g	Colony forming unit per gram of material
CNS	Central Nervous System
CRF	Corticotropin Releasing Factor
CRP	C - reactive protein
CXCL1	Chemokine(C-X-C motif) ligand 1
DHR	De-Individualized Humanized Rat
DNA	Deoxyribonucleic acid
GF	Germ-free
HPA	Hypothalamus-pituitary-adrenal gland
IL-1 β	Interleukin-1beta
IL-6	Interleukin-6
IL-7A	Interleukin-7A
LPS	Lipopolysaccharides
LBP	Lipid Binding Protein
mg/L	milligram/liter
Mm	millimeters
MPO	Myeloperoxidase
NOR	Novel Object Recognition
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
pM	picomoles
OTUs	Operational taxonomic units
PBS	Phosphate buffered saline
PGF	Pseudo germ-free
QIIME	Quantitative Insights into Microbial Ecology
rDNA	ribosomal Deoxyribonucleic Acid
RIG-1-Like	Retinoic acid-inducible gene-1-like

rRNA	ribosomal Ribonucleic Acid
SPF	Specific pathogen-free
TNF- α	Tumor Necrosis factor alpha