Temporal and region-specific effects of sleep fragmentation on gut microbiota and intestinal morphology in Sprague Dawley rats

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ABSTRACT
Sleep is a fundamental biological process, that when repeatedly disrupted, can result in severe health consequences. Recent studies suggest that both sleep fragmentation (SF) and dysbiosis of the gut microbiome can lead to metabolic disorders, though the underlying mechanisms are largely unclear. To better understand the consequences of SF, we investigated the effects of acute (6 days) and chronic (6 weeks) SF on rats by examining taxonomic profiles of microbiota in the distal ileum, cecum and proximal colon, as well as assessing structural and functional integrity of the gastrointestinal barrier. We further assayed the impact of SF on a host function by evaluating inflammation and immune response. Both acute and chronic SF induced microbial dysbiosis, more dramatically in the distal ileum (compared to other two regions studied), as noted by significant perturbations in alpha- and beta-diversity; though, specific microbial populations were significantly altered throughout each of the three regions. Furthermore, chronic SF resulted in increased crypt depth in the distal ileum and an increase in the number of villi lining both the cecum and proximal colon. Additional changes were noted with chronic SF, including: decreased microbial adhesion and penetration in the distal ileum and cecum, elevation in serum levels of the cytokine KC/GRO, and depressed levels of corticotropin. Importantly, our data show that perturbations to microbial ecology and intestinal morphology intensify in response to prolonged SF and these changes are habitat specific. Together, these results reveal consequences to gut microbiota homeostasis and host response following acute and chronic SF in rats.

Introduction
A disrupted night’s sleep is an increasingly common occurrence in today’s society that can result in more than just an unrefreshing night’s rest and excessive daytime fatigue. Recurrent sleep fragmentation (SF) can adversely impact health, cognition, and physical performance.¹⁻⁴ In fact, insufficient sleep has been linked to a wide range of conditions such as impaired attention and memory, diminished mood regulation, and hypersensitivity to pain.⁵,⁶ More alarmingly, SF can have deleterious consequences to our health, disrupting metabolic homeostasis and increasing our risk for obesity, diabetes, cardiovascular disease, stroke and ultimately, a shortened lifespan.⁷,⁸

Gut microbiota makes essential contributions to host health and longevity. To put this contribution into perspective, while the human genome contains about 20,500 genes, the hologenome (the host and its microbiome) contains over 250,000 unique genes.⁹ This large reserve of microbial genetic material affects numerous physiological pathways of the host, facilitating nutrient and drug metabolism, immune function and development, and production of neurotransmitters, vitamins, and other nutrients.¹⁰ Moreover, the overall health, well-being and development of the host are directly influenced by the symbiotic relationship with its microbiota communities.¹¹,¹² Environmental, psychological and physical stressors can promote dysbiosis, or an imbalance in microbial composition and function, which can significantly impact host health and lead to pre-disease and disease states.¹³ Microbial dysbiosis has been associated with immunosuppression, cognitive detriments, inflammation, and increased permeability of the epithelial barrier,
which can lead to translocation of gut bacteria and toxins into systemic circulation and surrounding tissues.\textsuperscript{14,15} Interestingly, dysbiosis can further give rise to a pattern of metabolic abnormalities, such as obesity and diabetes, mirroring that associated with sleep disturbances.\textsuperscript{16–18} This suggests that SF and gut microbiota perturbation likely have intertwined pathways that have yet to be elucidated.

A few studies have studied dysbiosis of the gut microbiome following the stress of SF; however, microbiota profiles are chiefly constructed from expelled fecal material.\textsuperscript{19–22} This sampling method, while simple and noninvasive, provides an incomplete profile of the intestinal microbiome as the composition and function of microbiota varies across different regions of the gastrointestinal tract.\textsuperscript{23} The overarching aim of this research was to assess the effects of both acute and chronic SF upon microbiota populations by examining 16S rRNA material found within three different locations of the gastrointestinal tract – the distal ileum, cecum and proximal colon. Furthermore, we evaluated systemic host response to SF, including mucosal inflammation, immune response, markers of hypothalamus-pituitary-adrenal (HPA) axis dysregulation, and the functional and structural maintenance of the gastrointestinal tract.

Materials and methods

Animals

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”.\textsuperscript{24}

Specific Pathogen Free (SPF) Sprague Dawley rats were purchased from Charles River (4-week old). Upon arrival to the vivarium, rats were quarantined and monitored for a two-week period. During this time, rats were socially housed in standard rat cages using conventional bedding (CellZorb, Cincinnati Lab Supply, #00009). Rats were provided for food (LabDiet Formulab Diet 5008, Cincinnati Lab Supply, #5008) and water \textit{ad libitum}. All animal rooms were climate controlled (20–26°C, 30–70% humidity) with a 12-h light/dark cycle (on at 0600).

Sleep fragmentation

After the quarantine period, animal feed was switched to irradiated chow (Picolab Rodent Irradiated Chow, Charles River, #5053), and autoclaved water (both provided \textit{ad libitum}) as there was a concern that the regular diet could potentially contain microbial cells that could alter the microbiome. Rats (aged 7 weeks) were singly housed in automated SF chambers (Lafayette Instrument Company; Lafayette, IN; model 80391) with sterile bedding (Alpha Dri Irradiated Bedding, Charles River, #ADT IRR), located in semi-rigid isolators (Charles River) for a two-week acclimation period to their new environment. These SF chambers had an automated sweeper bar that moved horizontally across the bottom of the cage that would wake the rat, as it was obliged to step over the sweeper bar at set intervals. Once the rats reached 9 weeks of age, the SF chambers were turned on. For the acute SF study, the sweeper bar moved across the bottom of the cage every 3 min, 24 h/day, for a period of 6 days. As the chronic SF study was more strenuous and demanding on the animals, a 3-h rest period (from 12 p.m. to 3 p.m.) was required for animal safety. Therefore, the sweeper bar moved across the bottom of the cage every 3 min for only 21 h/day for the 6-week chronic study. For the control (normal sleep) group, the animals were housed in the same SF chambers under the same conditions in different isolators; however, the sweeper bar remained stationary.

Sample collection and DNA extraction

Immediately following the 6 day or 6-week period of SF, rats were euthanized by CO\textsubscript{2} asphyxiation (random order, between 8:30 a.m. and 1:00 p.m.) and trunk blood was collected. Liver, spleen, mesentery, mesenteric lymph nodes, serum, plasma, leukocytes and erythrocytes were isolated and preserved separately at –80°C. Intestines were incised under sterile conditions and the content was removed from the
entirety of the cecum, and from a 2–3 inch section of the ileum and colon proximal to the cecum. The content was flash-frozen in liquid nitrogen and stored at −80°C. The corresponding intestinal tissues were cut open lengthwise and rinsed 3 times with PBS. Half of the tissue samples were wrapped in foil and placed on ice for bacterial adhesion and penetration assays (performed within 2–4 h); while the other half were flash frozen in liquid nitrogen and stored at −80°C until analysis. Total DNA was isolated from 250 mg of intestinal content using QIAamp DNA Stool Mini Kit (Qiagen, #51504) following manufacturer’s instructions. DNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Fisher Scientific).

**Multi-amplicon 16S rRNA amplification and DNA library preparation**

Hypervariable regions of 16S rDNA (along with negative and positive controls) were amplified using the Ion 16S Metagenomics Kit (Fisher Scientific, #A26216) per 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. Following PCR amplification, 5 μl of the product was run on a 2% agarose gel to confirm DNA amplification. DNA was purified using Agencourt AMPure XP magnetic beads (Fisher Scientific, #A63880). DNA was quantified with the 4200 TapeStation (Agilent) using D1000 screen tape and reagents (Agilent, #5067-5582/#5067-5583). Subsequently, 50 ng of the amplified and purified DNA of each sample were enzymatically sheared, end-repaired and adapter ligated using the Ion Xpress Plus Fragment Library Kit (Fisher Scientific, #4471269) according to manufacturer’s directions. Each sample was individually labeled to allow for multiplexing of the samples using Ion Xpress Barcode Adapters 1–96 Kit (Fisher Scientific, #4474517).

**Metagenomic sequencing and data processing**

Each DNA library was diluted to 60 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 (Life Technologies, #A30010). DNAs were sequenced using the Ion Torrent S5 Semiconductor Sequencer (Fisher Scientific, v.5.2) and Ion S5 Sequencing Kit (Fisher Scientific, #A33208). The metagenomic library was constructed with 400-bp inserts for each sample. Sequence reads were automatically processed by Ion Torrent Suite v.5.2, which removed: polyclonal sequences, low-quality sequences (Q < 10), low-quality 3’-ends, and bar-coded adapters.

**Taxonomic analysis**

Taxonomic analysis of the resultant raw FASTQ files were carried out using Quantitative Insights into Microbial Ecology (QIIME2) software (v2018.11). Raw sequences were trimmed to 250-bp, merged and quality-filtered at Phred Quality score of 20 (Q < 20). Feature tables were compiled to assign operational taxonomic units (OTUs) into different taxonomic levels based on a 99% sequence identity to the GreenGenes reference database (v13.8). Alpha-diversity evenness and richness of species were measured using Pielou’s metric and Menhinick’s index, respectively. Significance was determined by the Kruskal-Wallis test (p < .05). Beta-diversity was calculated using Bray-Curtis dissimilarity and weighted normalized Unifrac methodology, with significance established using permutational multivariate analysis of variance (PERMANOVA; p < .05, 999 permutations). PERMANOVA is a non-parametric multivariate statistical test used to test differences (similar to ANOVA, but used for more numerous variables, i.e. OTU abundances). Three-dimensional principal coordinate analysis (PCoA) plots of beta-diversity were visualized with Emperor. Perturbations of the microbiota population were evaluated using Gneiss balance correlation clustering (Ward’s hierarchical) and Analysis of Composition of Microbiomes (ANCOM).

**Bacterial adhesion, penetration and invasion**

Non-adhered microbial cells were removed from fresh tissue from each of the three intestinal tract sections by incubation with gentle shaking in 1x PBS containing 1 mM DDT for 15 min. The tissues were then washed 4 times with 1.5 ml PBS (60 sec with gentle shaking). The supernatant of the final wash
was preserved in 15% glycerol and stored at –80°C for monitoring the removal of the non-adhered microbial cells. To isolate adhered microbial cells, 1.5 ml of PBS was added to each piece of tissue and vortexed vigorously for 60 s. This step was repeated and the supernatants were stored at –80°C in 15% glycerol. The vortex-wash was repeated two more times and stored at –80°C for future culturing. The washed tissues were used for the isolation of penetrated and invaded microbial cells. A piece of tissue was cut from each section and placed in a freshly prepared 1 ml antibiotic solution (0.3 mg/ml of ampicillin and 0.3 mg/ml of gentamicin in PBS). After incubating in the antibiotic solution at room temperature for 1 h, tissues were washed 3 times in 1.5 ml PBS (60 sec with gentle shaking). The supernatant was preserved in 15% glycerol to assess the effect of antibiotic treatment. Tissues, with and without antibiotic pretreatment, were placed in separate tubes for homogenization to begin isolation of penetrated and invaded microbial cells. Samples were homogenized in 500 μl PBS with five 2-mm zirconium oxide homogenization beads in a Bullet Blender Gold (Next Advance) at speed 8 for 2 min. Cells were pelleted at 9,000xg (5 min, 4°C) and resuspended in 1 ml of 15% glycerol in lysogeny broth (LB) and stored at –80°C for future culturing.

**Determination of microbial titer**

Intestinal content resuspended in PBS, adhesion tissue PBS washes, or tissue homogenate with and without antibiotic treatment, were spread evenly onto 5% sheep blood agar plates with TSA (Fisher Scientific, #R02050) and ran in duplicate. Both aerobic and anaerobic colonies were grown, with anaerobic bacteria cultured using BD GasPak EZ Gas Generating System (Fisher Scientific, #B260684) following manufacturer’s instructions. Colonies were counted automatically using the Scan 300 Automatic Colony Counter (Interscience, #436300), and colony forming units (CFU) per gram of material was calculated.

**Evaluation of mucosal inflammation, immune response, bacteria-derived endotoxin load, and hypothalamic-pituitary-adrenal (HPA) axis**

Mucosal inflammation was evaluated using myeloperoxidase (MPO) activity as an indicator (MPO Activity Assay Kit, Abcam, #ab105136). Serum levels of proinflammatory cytokines (IFN-Gamma, IL-1 Beta, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-Alpha) were measured using V-PLEX Proinflammatory Panel 2 Rat Kit (Meso Scale Discovery, #K15059D-1) and serum lipopolysaccharide binding protein (LBP) levels were assayed with Rat Lipopolysaccharide Binding Protein Kit (MyBioSource, #MBS014875). The hypothalamic-pituitary-adrenal (HPA) axis was evaluated using: Corticosterone ELISA Kit (Arbor Assays, #K014-H5), ACTH (Adrenocorticotropic hormone, corticotropin) ELISA kit (MyBioSource, #MBS2502683), and Peripheral Corticotropin Releasing Factor (CRF) ELISA kit (Kamiya Biomedical Company, KT-383). All kits were used according to the manufacturer’s directions.

**Immunohistochemistry**

Intestinal tissues were blocked in optimal cutting temperature medium and sliced transversely into 12 mm slices and fixed to gel-coated microscope slides by incubation on a slide warmer at 40°C for 10 min or less. The slides were stored at –80°C until they underwent automated staining with hematoxylin and eosin (H&E). The stained tissue was imaged using a customized Leica microscope and measurements were obtained using Leica Application Suite X, (v.3.3.3.16958, Leica Microsystems CMS GmbH). Images were collected at 5-fold and 20-fold magnification.

Villus length was measured from the luminal surface of the epithelium to the start of the submucosal, muscle layer, and only those villi with both landmarks clearly discernable were measured. Crypt depth was measured from the cell layer closest to the submucosa to the area where the crypt walls narrowed or pinched inward to form a protective pocket. The number of villi or crypts per millimeter of tissue were obtained by dropping a line across the tissue, counting the villi and crypts that the line intercepted, and then dividing the count by the length of the line in millimeters. The number of measurements obtained per animal was limited only by the quality of the image, but only the mean value for each subject was used for statistical analysis.
Statistical analysis was accomplished using Prism 7 for Windows (GraphPad Software, v.7.04). Each endpoint was analyzed by two-way ANOVA of treatment (with or without SF) against length of treatment (acute or chronic). Tukey’s and Sidak’s multiple comparisons were used to establish statistical significance ($p < .05$).

**Results**

**Phylogenetic analysis of gut microbiota community richness**

Specific pathogen free (SPF) Sprague Dawley rats underwent SF for either 6 days (acute, SF n = 9, control n = 10) or 6 weeks (chronic, SF n = 10, control n = 12). We assessed gut microbiota composition by sampling content from the distal ileum, cecum, and proximal colon for high-throughput analysis of 16S rRNA amplicons. Across all 122 sequenced samples, 51,408,485 high-quality (250-bp single-end reads) 16S rRNA gene sequences were retained after Ion Torrent sequencing and quality filtering with an average of 241,381 reads and 214 OTUs per sample (Supplemental Tables 1 & 2). Rarefaction analysis with Faith’s phylogenetic diversity index was used to validate adequate sequencing capture of microbiota gene diversity (Supplemental Figure 1) in the (A) acute and (B) chronic SF studies for both SF and normal sleep subjects. Notably, in both studies the phylogenetic diversity and abundance in the distal ileum (represented by the lower line groupings in the two charts) were less than that of the cecum or proximal colon (upper lines) and this difference is clearly observed in the graphs. Based on the rarefaction results, a species profiling depth of 30,000 reads was selected for alpha- and beta-diversity studies in order to retain all samples in the analysis.

**The microbiota profile of the distal ileum differs from the cecum and proximal colon**

To evaluate the microbiota populations of the three intestinal regions, we measured the within-sample (alpha) diversity and the (beta) diversity between all samples, including normal sleep subjects (Figure 1). When comparing the alpha-diversity using Faith’s

![Figure 1](image_url)
phylogenetic diversity index, the species diversity and richness in the distal ileum was significantly distinct from species in the cecum and proximal colon (Kruskal-Wallis, pair-wise, \( p < .001 \)). This striking difference was observed in both the (A) acute and (B) chronic metagenomic studies, regardless of sleep variable. Significant alterations to alpha-diversity between the cecum and proximal colon species were also noted in the acute SF study (\( p = .0047 \)). Beta-diversity between microbiota communities was evaluated using the weighted normalized Unifrac metric, comparing pairwise distances between samples. Three-dimensional sample Unifrac distances, visualized with PCoA plots, are shown in Figure 1. The total-captured diversity of the top three principal coordinates (PC) was 81% in the (C) acute and 79% in the (D) chronic SF experiments. While significant dissimilarities (PERMANOVA, 999 permutations, \( P < .001 \)) were observed in the beta diversity of the gut microbiome between all three different intestinal regions (regardless of sleep variable), the microbiota population of the distal ileum was least similar to the populations of the other two intestinal regions. In addition, chronic SF gave rise to increased dispersion between samples of the same intestinal region. Specifically, longer periods of SF brought about a wider diversity of microbiota populations in the distal ileum (between study subjects); while, microbiota population profiles between the cecum and the proximal colon appear more similar (D). This trend of increasing sample dispersion with increased SF is further reinforced by the tight PCoA sample groupings of normal sleep subjects in both the (C) acute and (D) chronic studies for all three intestinal regions examined.

**Intestinal microbiota profiles are altered by acute and chronic sleep fragmentation**

The richness of alpha diversity of each intestinal region, with or without SF, was quantified using Menhinick’s index and is shown in Figure 2. For the acute SF study, the number of taxa within the (A) distal ileum exhibited a significant change with SF, while there were no significant changes in the richness of the (B) cecal or (C) proximal colon microbial communities. For the chronic SF study, the taxa richness of the (D) distal ileum was again altered, though not reaching the significance threshold (\( p = .056 \)). Additionally, the microbiota population located in the (E) cecum showed significant changes, while changes in the taxa of the (F) proximal colon were not evident with SF. Alpha diversity evenness of species was measured.

![Figure 2](image_url). Altered alpha-diversity richness of intestinal bacterial communities induced by sleep fragmentation. Alpha-diversity (Menhinick’s richness index) of samples located in the distal ileum (a and d), cecum (b and e), or proximal colon (c and f) from SPF rats that underwent either acute (a–c) or chronic (d–f) sleep fragmentation compared to normal sleep subjects. Acute SF: normal sleep, \( n = 10 \); fragmented sleep, \( n = 9 \) (two normal sleep samples were removed from the acute analysis due to inadequate sequencing depth – one from the distal ileum and one from the proximal colon). Chronic SF: normal sleep, \( n = 12 \); fragmented sleep, \( n = 10 \). Kruskal-Wallis test: n.s. non-significant, \(^* p < .05, ^{**} p = .056.\)
using Pielou’s evenness metric (Supplemental Figure 2). The only significant change of relative evenness occurred within the acute SF group in the microbial population of the distal ileum (p = .041). Although absolute abundance of microbiota were unaltered by SF, the data are presented in Supplemental Figure 3, with a visualization of the alpha-diversity of observed OTUs.

To examine the effect of SF upon the beta diversity of the gut microbiota between the three different regions, Bray-Curtis dissimilarity and weighted normalized Unifrac distance matrixes were used. Figure 3, which visualizes the Bray-Curtis dissimilarity results with PCoA plots, shows that while acute SF did not significantly perturb microbiota populations, chronic SF did result in altered beta-diversity in all three intestinal regions. In addition to dissimilarity, weighted normalized Unifrac distance matrices also take into account the phylogenetic relatedness and abundance of microbiota. Interestingly, significant differences (PERMANOVA, p < .05) were observed only in the microbial population in the distal ileum of the acute SF subjects (Supplemental Figure 4). Of interest, differences in the microbial populations of the proximal colon and distal ileum approached significance (p = .063 and p = .086, respectively) in chronic SF subjects.

**Individual OTU analysis**

Gneiss balance trees and Analysis of Composition of Microbiomes (ANCOM) were used to evaluate significant shifts in microbiota populations at different taxonomic levels. With acute SF, ANCOM analysis identified only two significantly altered microbiota populations: an increase in the Enterobacteriaceae family in the distal ileum and an increase of the Proteobacteria phylum in the cecum (Supplemental Table 3). Gneiss balance profiling, which compares balances between subsets of microbial communities, identified additional features altered with acute SF in the distal ileum, which are presented in Figure 4a, showing decreases

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**Figure 3.** Sleep fragmentation in SPF rats alters microbiota profiles. Beta diversity was measured using Bray-Curtis dissimilarity and visualized with PCoA plots. Significant shifts occurred only in the chronic sleep fragmentation studies (PERMANOVA, p ≤ 0.025). Acute SF: normal sleep, n = 10; fragmented sleep, n = 9 (one acute, normal sleep, proximal colon sample was removed from analysis due to inadequate sequencing depth). Chronic SF: normal sleep, n = 12; fragmented sleep, n = 10. The percent diversity captured by each axis (PC) is shown.
in the Lactobacillaceae, Lachnospiraceae, F16, and Alcaligenaceae families and increases in S24-7 and Porphyromonadaceae families. Figure 4b shows the results of the balance analysis of the distal ileum microbiota in the chronic study, where SF lead to decreases in Enterobacteriaceae and Lactobacillaceae families and increases in Turicibacteraceae and Clostridiaceae families. These results are confirmed in Table 1, which summarizes the ANCOM analysis of the same data, detecting 10 significantly altered features in the distal ileum. The most altered phylum was the Firmicutes, with decreases in the families Lactobacillaceae and Veillonellaceae and increases in the families Staphylococcaceae, Clostridiaceae, and Turicibacteraceae in the distal ileum. Gneiss balance analysis of cecum microbiota found that

Table 1. ANCOM analysis listing significant differential abundant features (taxa) in the distal ileum, cecum, and proximal colon that were altered by chronic sleep fragmentation in SPF rodents. The log fold change and W statistic are provided for the features at the various taxonomic levels.

| Intestinal region | Feature | Taxonomic level | Mean difference | W |
|-------------------|---------|-----------------|-----------------|---|
| Distal Ileum      | k_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales | 4 | 1.3 | 7 |
|                   | k_Bacteria;p_Firmicutes;c_Bacilli;o_Turicibacteraceae | 4 | 2.4 | 12 |
|                   | k_Bacteria;p_Firmicutes;c_Bacilli;o_Turicibacteraceae;g_Turicibacter | 6 | 2.9 | 33 |

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acute SF (Supplemental Figure 5 A) resulted in a decrease in the order Clostridiales and increases in the Ruminococcaceae family and in the genus Oscillospira, Bacteroides, and Prevotella; while chronic SF (Supplemental Figure 5 B) resulted in increases in the families Ruminococcaceae and Turicibacteraceae and genus Turicibacter, and Clostridium. Gneiss analysis of proximal colon population indicated acute SF (Supplemental Figure 5 C) induced depletions in the family Lachnospiraceae and the genus Lactobacillus and expansions to the genus Parabacteroides, while ANCOM analysis detected no significant changes in the acute SF study. However, ANCOM analysis of chronic SF in the proximal colon-identified increases in families Turicibacteraceae, Clostridiaceae, Erysipelotrichaceae and genus Turicibacter and 02d06, (which is in the Clostridiaceae family) and a decrease in the family Bamesiellaceae (Table 1), while Gneiss balance trees illustrate overriding, aberrant changes to large populations of Bacteroides and Prevotella (Supplemental Figure 5 D).

Effect of sleep fragmentation on microbial adhesion, penetration and invasion

Intestinal macromolecular permeability and mucosal bacterial adhesion, penetration and invasion were evaluated by measuring total culturable bacteria (aerobic and anaerobic). The results of the acute study, presented in Supplemental Table 4, showed that the only significantly altered parameter was extraintestinal microbial invasion in the spleen, which was increased with SF. In the chronic SF studies, there were no significant alterations of microbial invasion in the spleen or any other tissues tested. However, intestinal bacterial adhesion and penetration of microbes into the surrounding tissue was significantly decreased in both the distal ileum and cecum (Supplemental Tables 5).

Alterations to intestinal structures with sleep fragmentation

Changes in intestinal structural were evaluated by investigation of the epithelial crypt-villus architecture and are presented in Supplemental Table 6 (acute study) and Supplemental Table 7 (chronic study). Acute SF did not result in significant changes in the depth, width, or number of crypts. However, chronic SF did induce architectural changes and are presented in Figure 5. In the distal ileum (A & B), chronic SF significantly increased \( p = .023 \) crypt depth. In addition, chronic SF significantly increased the number of villi per mm of intestine in both the cecum \( p = .004 \) (C & D) and proximal colon \( p = .032 \) (E & F). In addition, tight junction integrity of the three intestinal regions was gauged by tissue staining with occludin (an integral plasma-membrane protein). No significant changes in occludin were noted in any region with either acute or chronic SF (data not shown).

Immune response, mucosal inflammation, bacteria-derived endotoxin load, and hypothalamic-pituitary-adrenal (HPA) axis dysregulation

Cytokine profiles of SPF rats altered by either acute or chronic SF (Supplemental Table 8) were constructed from 10 different proinflammatory cytokine measurements of serum levels (IFN-Gamma, IL-1 Beta, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-Alpha). Only one of these factors, KC/GRO, was significantly increased \( p = .037 \) with chronic SF. Mucosal inflammation (evaluated by MPO activity) and bacteria-derived endotoxin load (serum level of LPS binding protein) were not significantly altered by either acute or chronic SF (data not shown). HPA axis activity was assessed by serum measurements of CRF, ACTH and corticosterone. Neither acute nor chronic SF significantly altered any of these levels in SPF rats; with the exception, ACTH level was significantly decreased with chronic SF (Supplemental Table 9).

Discussion and conclusions

Our data show that within the first week of sleep interruption in rats, dysbiosis of gut microbiota occurred; and when SF was prolonged, aberrations to microbial communities and in host response to SF increased. In fact, a larger number of significantly altered taxa were detected in the distal ileum. Importantly, the earliest signs of SF-induced gut dysbiosis were only evident in the ileum. It is well
known that each gut location is anatomically and functionally diverse; and previous metagenomic studies have demonstrated that microbiota composition and intestinal barrier function are dependent on location. Yet, most studies of sleep disturbances have focused on microbial characterization of the colonic or fecal communities alone. While previous experiments by our laboratory have shown

Figure 5. Chronic sleep fragmentation induced changes in architectural structure. SPF rodents with (a) normal sleep had a smaller average crypt depth in the distal ileum than SPF rodents that had fragmented sleep (b). Chronic sleep fragmentation also resulted in an increased concentration of crypts in both the (d) cecum and (f) proximal colon as compared to their normal sleep counterparts (c and e), respectively (normal sleep, n = 12; fragmented sleep, n = 10).
that colonic and fecal community profiles in SPF Sprague Dawley rats are similar (Supplemental Figure 6), our regionally distinctive microbiota data emphasizes the importance of assessing microbiota changes throughout the gut.

It is significant to point out that during the period of SF, animals were maintained in germ-free isolators with sterile bedding, food and water to prevent introduction of foreign bacteria, which could potentially skew microbiota profiles. Accurate community profiling is imperative in detecting the aberrations in alpha- and beta-diversity that signal microbial dysbiosis in order to avoid ambiguous or misleading results. Perturbation of gut microbiota is typically associated with a decrease in species diversity, though this is not always the case.\(^{42-44}\) In this current study, SF resulted in differential, location-dependent variations in the diversity metrics of richness and evenness. The overall trend (both acute and chronic studies) of alpha diversity in the distal ileum was an increase in species evenness with SF, which suggests an increase in low-abundant species and/or a decrease in high-abundant species; and an increase in richness, which demonstrates an increase in detectable species. Together, this suggests the major reason is an increase in the low-abundant species. Furthermore, if these microbes are pathogenic, it would provide a mechanistic basis for the adverse effects of SF. Proximal colon communities maintained a similar number of detectable microbiota with SF, but exhibited decreased evenness (not significant). This also implies either an increase in high-abundant species and/or a decrease in low-abundant species that could lead to perturbation of the metabolic output of the microbiota community. In the cecum, SF did not affect species evenness. However, acute SF richness decreased as low-abundant species became undetectable; while chronic SF significantly increased richness, suggesting ultralow-abundant species became detectable. Further studies into the Gneiss and ANCOM results may provide insights into whether these microbes are pathogenic or if they have other unique roles in the overall metabolic function of the gut microbiota.

Beta-diversity was assessed by both Bray-Curtis dissimilarity and weighted normalized Unifrac methodology (which takes into account phylogenetic relatedness as well as relative OTU abundance) in order to determine SF-induced changes in microbiota composition. Our analysis with Unifrac distance matrices revealed that only acute SF significantly altered beta-diversity in the distal ileum. Meanwhile, Bray-Curtis dissimilarity showed that chronic SF resulted in significant aberrations to all three intestinal regions investigated. These results indicate that the microbial communities that are altered in the ileum under acute SF are more phylogenetically distinct from one another; and that chronic SF likely induced changes to larger, closely related microbiota, such as the Prevotella and Bacteroides populations that were identified in the Gneiss balance analysis. These results are consistent with the changes in the richness and evenness as discussed above.

Our results are supported by previous metagenomic studies of sleep disturbances as significant alterations to microbiota were recapitulated. To illustrate, in a previous study by Poroyko et al., 4 weeks of chronic SF in mice led to gut microbiota dysbiosis, including preferential growth of Lachnospiraceae and Ruminococcaceae and inhibition of Lactobacillaceae families.\(^{19}\) Similarly, our data show bacterial expansion of Ruminococcaceae and contraction of Lactobacillaceae families under both acute and chronic conditions. However, we found that Lachnospiraceae populations either increased or decreased depending on the intestinal region from which the microbiota were harvested. As many bacteria from the Lachnospiraceae family are associated with contributing to intestinal inflammation and metabolic dysfunction, knowing the intestinal location of the dysbiosis may have important therapeutic implications for targeted therapies.\(^{15,46}\)

The small intestine is a highly specialized organ for energy acquisition as it serves as the primary site for nutrient and mineral absorption, while also playing essential roles in drug uptake, immune response and metabolism.\(^{47,48}\) The intestines are lined with rapid self-renewing crypt-villi units, which project into the lumen to increase the absorptive area of intestinal walls, providing highly dynamic structures that can quickly respond to physiological needs.\(^{49}\) While the effect of altered crypt depth is context-dependent, increased crypt depth may be the result of crypt hypertrophy or hyperplasia (considered to be a reparative process). In the current study, immunohistochemistry studies revealed that while no significant changes
to intestinal architecture were detected with acute SF, chronic SF resulted in multiple aberrations. In the distal ileum, crypt depth increased with SF resulting in a reduced villus/crypt ratio. Enlarged crypt depth is indicative of increased turnover of intestinal epithelium in response to tissue damage. In addition, structural changes were noted in both the cecum and proximal colon as the number of crypts increased with chronic SF. Currently, not much is known regarding the effect of increased crypts. Crypt fission and regulation of intestinal homeostasis is believed to be controlled by the Wnt signaling pathway, but further investigation is needed to understand the underlying biochemical signaling dynamics induced by chronic SF that led to significant increases in crypt production. One caveat to our data is that even though all animals were the same age at the beginning of the experiment, the chronic SF study animals were aged to 15 weeks, while the acute SF group was aged to 10 weeks, due to the length of SF. This age difference is an important consideration when comparing acute and chronic data directly as various measurements (such as crypt depth, HPA axis hormones, and the presence of proinflammatory cytokines) change with age.

The intestinal mucosal barrier functions to contain undesirable luminal contents within the intestinal tracts to prevent uncontrolled translocation of luminal contents through the intestine into the body, protecting mucosal tissues and the circulatory system from exposure to pathogenic microorganisms, pro-inflammatory molecules and toxins. Previously, it has been shown that sleep deprivation in rats promoted extraintestinal microbial invasion of anaerobic microbes in the mesenteric lymph nodes, liver and spleen after 5 days without sleep. In these current SF studies, we found a similar increased presence of bacteria in the spleen following acute SF. No significant microbial invasions were noted in any other tissue tested. The acute SF data showed varying degrees of increased microbial invasion (as well as microbial adhesion and penetration), but a large standard deviation made these changes not statistically significant. The result of chronic SF was somewhat unexpected as microbial adhesion and penetration were decreased in both the distal ileum and in the cecum. In addition, no microbial invasion of surround tissues was observed with chronic SF. It is possible that microbial invasion may have increased in other organs that were not studied; however, the results from the LBP and occludin assays suggest that the gut does not becomes leaky after SF under the SF condition employed in this study.

It is known that total sleep deprivation leads to impaired immune function in humans and animals via pro-inflammatory response including elevated IL-1, IL-6 and TNF levels. In this study, we wanted to determine if an interrupted sleep pattern would lead to similar compromised immunity. After analyzing rat blood serum from both acute and chronic SF experiments for nine different cytokines/chemokines, only KC/GRO was found to be significantly increased, but only with chronic SF. The serum-level measurements suggest that unlike sleep deprivation, SF does not promote systemic inflammation in SPF rats, which is consistent with our findings of no changes in microbial extraintestinal translocation and invasion, bacteria-derived endotoxin load, or mucosal inflammation.

The hypothalamic-pituitary-adrenal (HPA) axis, a key component of neuroendocrine stress response, aides in sleep-wake cycle regulation. It is known that sleep deprivation and sleep disorders can induce hyperactivation of the HPA axis. In the current study, SF resulted in minimal changes in the HPA axis, where chronic SF led to significantly reduced levels of ACTH. However, it is important to note that the lack of changes could have been influenced by the time of day of sample acquisition as some HPA axis hormones are strongly influenced by circadian rhythm. This circadian effect might also be responsible for the significant differences between the control groups in the acute and chronic experiments, as shown in Supplemental Table 9.

In conclusion, the present study investigated the consequences of SF, examining the composition of gut microbial communities and perturbation of host functions following either acute or chronic sleep disruption, as well as assessing the structural and functional integrity of the gastrointestinal tract. Importantly, unique perturbations to the ileal microbiota that register even after acute SF suggests that analysis of fecal material alone may not be appropriate.
when assessing dysbiosis of the gut microbiome. Additionally, we showed that alterations to gut micro-
biota and intestinal architecture increased with prolonged SF, without increases in inflammation or
intestinal permeability. Together, these results emphasize that a regional approach when evaluating SF-
induced microbiota dysbiosis may be key for the development of targeted interventional therapies.

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Disclosure of potential conflicts of interest

All authors declare no potential conflicts of interest.

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References

1. Lowe CJ, Safati A, Hall PA. The neurocognitive con-
sequences of sleep restriction: A meta-analytic review. Neurosci Biobehav Rev. 2017;80:586–604. doi:10.1016/j.neubiorev.2017.07.010.
2. Lo JC, Groeger J, Santhi N, Arbon E, Lazar A, Hasan S, von Schantz M, Archer S, Dijk D. Effects of partial and
acute total sleep deprivation on performance across cognitive domains, individuals and circadian phase. PLoS One. 2012;7(9):e45987. doi:10.1371/journal.
pone.0045987.
3. Luyster FS, Strollo PJ, Zee PC, Walsh JK. Sleep: a health imperative. Sleep. 2012;35:727–734. doi:10.5665/sleep.1846.
4. André C, Tomadesso C, Flores R, Branger P, Rehel S, Mezenge F, Landeau B, Sayette V, Eustache F, Chetelat G, Rauchs G. Brain and cognitive correlates of sleep frag-
mentation in elderly subjects with and without cognitive deficits. Alzheimer’s Dement Diagnosis Assess Dis Monit. 2019;11:142–150.
5. Iacovides S, George K, Kamerman P, Baker FC. Sleep fragmentation hypersensitizes healthy young women to deep and superficial experimental pain. J Pain. 2017;18:844–854. doi:10.1016/j.jpain.2017.02.046.
6. Alkadhi K, Zagaar M, Alhaider I, Salim S, Aleisa A. Neurobiological consequences of sleep deprivation. Curr Neuropharmacol. 2013;11:231–249. doi:10.2174/1570159X11311030001.
7. Koo DL, Nam H, Thomas RJ, Yun C-H. Sleep distur-
bances as a risk factor for stroke. J Stroke. 2018;20:12–32. doi:10.5853/jos.2017.02887.
8. Reutrakul S, Van Cauter E. Sleep influences on obesity, insulin resistance, and risk of type 2 diabetes. Metabolism. 2018 Jul;84:56–66. doi:10.1016/j.metabol.2018.02.010.
9. Zilber-Rosenberg I, Rosenberg E. Role of microorgan-
isms in the evolution of animals and plants: the holo-
genome theory of evolution. FEMS Microbiol Rev. 2008;32:723–735. doi:10.1111/j.1574-6976.2008.00123.x.
10. Jandhyala SM, Talukdar R, Subramaniam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroenterol. 2015. doi:10.3748/
wjg.v21.i29.8787.
11. Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S. The evolution of the host microbiome as an ecosystem on a leash. Nature. 2017;548:43–51. doi:10.1038/nature23292.
12. Simon JC, Marchesi JR, Mogul C, Selosse MA. Host-
microbiota interactions: from holobiont theory to analysis. Microbiome. 2019;7. doi:10.1186/s40168-019-0619-4.
13. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet. 2012;13:260–270. doi:10.1038/nrg3182.
14. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol. 2015;28:203–209.
15. Koren O, Spor A, Felin J, Fak F, Stombaugh J, Tremaroli V, Behre C, Knight R, Fagerberg B, Ley R, Backhed F. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci. 2011;108:4592–4598. doi:10.1073/pnas.101383107.
16. Li Y, Yao H, Fan F, Zhang B. The role of microbiome in insomnia, circadian disturbance and depression. Front Psychiatry. 2018;9. doi:10.3389/fpsyt.2018.00669.
17. Hur KY, Lee M-S. Gut microbiota and metabolic disorders. Diabetes Metab J. 2015;39:198. doi:10.4093/dmj.2015.39.3.198.
18. Ho JM, Barf RP, Opp MR. Effects of sleep disruption and high fat intake on glucose metabolism in mice. Psychoneuroendocrinology. 2016;68:47–56. doi:10.1016/j.psyneuen.2016.02.024.
19. Poroyko VA, Carreras A, Khalyfa A, Khalyfa AA, Leone V, Peris E, Almendros I, Gilleles-Hilél A, Qiao Z, Hubert N, et al. Chronic sleep disruption alters gut microbiota, induces systemic and adipose tissue inflammation and insulin resistance in mice. Sci Rep. 2016;6. doi:10.1038/srep35405.

20. Zhang SL, Bai L, Goel N, Bailey A, Jang C, Bushman F, Meerlo P, Dinges D, Sehgal A. Human and rat gut microbiome composition is maintained following sleep restriction. Proc Natl Acad Sci. 2017;114(8):1564–1571.

21. Anderson JR, Carroll I, Aazarate-Peril MA, Rochette AD, Heinberg LJ, Peat C, Steffen K, Manderino LM, Mitchell J, Gunstad J, et al. A preliminary examination of gut microbiota, sleep, and cognitive flexibility in healthy older adults. Sleep Med. 2017;38:104–107. doi:10.1016/j.sleep.2017.07.018.

22. Benedict C, Vogel H, Jonas W, Woting A, Blaut M, Schürmann A, Cedernaes J. Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. Mol Metab. 2016;5:1175–1186. doi:10.1016/j.molmet.2016.10.003.

23. Eckburg PB, Bik E, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Microbiology: diversity of the human intestinal microbial flora. Science. 2005;308:1635–1638. doi:10.1126/science.1110591.

24. NRC. Guide for the care and use of laboratory animals: eight edition. Washington (DC): The National Academies Press. 2011.

25. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EC, Fierer N, Pena AG, Goodrich JK, Gordon JW, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–336. doi:10.1038/nmeth.f.303.

26. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Zech Xu Z, Kightley EP, Thompson LR, Hyde ER, Gonzalez A, et al. Deblur rapidly resolves single-nucleotide community sequence patterns. mSystems. 2017;2. doi:10.1128/mSystems.00191-16.

27. Cock PJA, Fields CJ, Goto N, Heuer ML, Rice PM. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 2010;38:1767–1771.

28. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–5072. doi:10.1128/AEM.03006-05.

29. Pielou EC. The measurement of diversity in different types of biological collections. J Theor Biol. 1966;13:131–144. doi:10.1016/0022-5193(66)90013-0.

30. Magurra AE. Measuring biological diversity. Oxford: Blackwell Publishing. 2004.

31. Lozupone CA, Hamady M, Kelley ST, Knight R. Quantitative and qualitative diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol. 2007;73:1576–1585. doi:10.1128/AEM.01996-06.

32. Vázquez-Baeza Y, Pirrung M, Gonzalez A, Knight R. EMPeror: a tool for visualizing high-throughput microbial community data. Gigascience. 2013;2. doi:10.1186/2047-217X-2-16.

33. Morton JT, Sanders J, Quinn RA, McDonald D, Gonzalez A, Vázquez-Baeza Y, Navas-Molina JA, Song SJ, Metcalf JL, Hyde ER, et al. Balance trees reveal microbial niche differentiation. mSystems. 2017;2. doi:10.1128/mSystems.00162-16.

34. Mandal S, Van Treuren W, White RA, Eggborh M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb Ecol Heal Dis. 2015;26:27663. doi:10.3402/mehd.v26.27663.

35. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Conserv. 1992;61:1–10. doi:10.1016/0006-3207(92)91201-3.

36. Yang H, Huang X, Fang S, Xin W, Huang L, Chen C. Uncovering the composition of microbial community structure and metagenomics among three gut locations in pigs with distinct fatness. Sci Rep. 2016;6:27427. doi:10.1038/srep27427.

37. Hamilton MK, Boudry G, Lemay DG, Raybould HE. Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. Am J Physiol Liver Physiol. 2015;308:840–851.

38. Dobranowski PA, Tang C, Sauvé JP, Menzies SC, Sly LM. Compositional changes to the ileal microbiome precede the onset of spontaneous ileitis in SHIP deficient mice. Gut Microbes. 2019;10:578–598. doi:10.1080/19490976.2018.1560767.

39. Thompson RS, Roller R, Mika A, Greenwood BN, Knight R, Chichlowski M, Berg BM, Fleschner M. Dietary prebiotics and bioactive milk fractions improve NREM sleep, enhance REM sleep rebound and attenuate the stress-induced decrease in diurnal temperature and gut microbiota alpha diversity. Front Behav Neurosci. 2017;10. doi:10.3389/fnbeh.2016.00240.

40. Voigt RM, Summa KC, Forsyth CB, Green SJ, Engen P, Naqib A, Vitaterna MH, Turek FW, Keshavarzian A. The Circadian Clock Mutation Promotes Intestinal Dysbiosis. Alcohol Clin Exp Res. 2016;40:335–347. doi:10.1111/acer.12943.

41. Durgan DJ, Ganesh BP, Cope JL, Ajami NJ, Phillips SC, Petrosino JF, Hollister EB, Bryan RM. Role of the gut microbiome in obstructive sleep apnea-induced hypertension. Hypertension. 2016;67:469–474. doi:10.1161/HYPERTENSIONAHA.115.06672.

42. Falony G, Joosens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, Kurilshikov A, Bonder MJ, Valles-Colomer M,
Vandeputte D, et al. Population-level analysis of gut microbiome variation. Science. 2016;352:560–564. doi:10.1126/science.aad3503.

43. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, Youn E, Summanen PH, Granpeesheh D, Dixon D, et al. Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe. 2010;16:444–453. doi:10.1016/j.anaerobe.2010.06.008.

44. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, Wang W, Tang W, Tan Z, Shi J, et al. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behavior Immunity. 2015;48:186–194. doi:10.1016/j.bbi.2015.03.016.

45. Nakamichi Y, Sato T, Ohteki T. Commensal Gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. Mucosal Immunol. 2015;8:152–160. doi:10.1038/mi.2014.53.

46. Kameyama K, Itoh K. Intestinal colonization by a lachnospiraceae bacterium contributes to the development of diabetes in obese mice. Microbes Environ. 2014;29:427–430. doi:10.1264/jsme2.ME14054.

47. Santaolalla R, Abreu MT. Innate immunity in the small intestine. Curr Opin Gastroenterol. 2012;28:124–129. doi:10.1097/MOG.0b013e3283506559.

48. Murakami T. Absorption sites of orally administered drugs in the small intestine. Expert Opin Drug Discov. 2017;12:1219–1232. doi:10.1080/17460441.2017.1378176.

49. Clevers H. X. The intestinal crypt, a prototype stem cell compartment. Cell. 2013;154:274–284. doi:10.1016/j.cell.2013.07.004.

50. Liu L, Fu C, Yan M, Xie H, Li S, Yu Q, He S, He J. Resveratrol modulates intestinal morphology and HSP70/90, NF-kB and EGF expression in the jejunal mucosa of black-boned chickens on exposure to circular heat stress. Food Funct. 2016;7:1329–1338.

51. Sabino M, Cappelli K, Capomaccio S, Pascucci L, Biasato I, Verini-Supplizi A, Valiani A, Trabalza-Marinucci M. Dietary supplementation with olive mill mill wastewaters induces modifications on chicken jejunum epithelial cell transcriptome and modulates jejunum morphology. BMC Genomics. 2018;19. doi:10.1186/s12864-018-4962-9.

52. Yang G, Wang H, Kang Y, Zhu M-J. Grape seed extract improves epithelial structure and suppresses inflammation in ileum of IL-10-deficient mice. Food Funct. 2014;5:2558–2563. doi:10.1039/C4FO00451E.

53. Hirata A, Utikal J, Yamashita S, Aoki H, Watanabe A, Yamamoto T, Okano H, Bardeesy N, Kunisada T, Ushijima T, et al. Dose-dependent roles for canonical Wnt signalling in de novo crypt formation and cell cycle properties of the colonic epithelium. J Cell Sci. 2013;126:e1–e1. doi:10.1242/jcs.130047.

54. Clarke RM. The effects of age on mucosal morphology and epithelial cell production in rat small intestine. J Anat. 1977;123:805–811.

55. Michaud M, Balardy L, Moulis G, Gaudin C, Peyrot C, Vellas B, Cesari M, Nourhashemi F. Proinflammatory cytokines, aging, and age-related diseases. J Am Med Dir Assoc. 2013;14:877–882. doi:10.1016/j.jamda.2013.05.009.

56. Gupta D, Morley J. Hypothalamic-pituitary-adrenal (HPA) axis and aging. Compr Physiol. 2014;4:1495–1510.

57. Tenk J, Rostás I, Füredi N, Mikó A, Szőcs M, Pétervári E, et al. Age-related changes in central effects of corticotropin-releasing factor (CRF) suggest a role for this mediator in aging anorexia and cachexia. GeroScience. 2017;39:61–72. doi:10.1007/s11357-017-9962-1.

58. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. Exp Mol Med. 2017;49:e338–e338. doi:10.1038/emm.2017.20.

59. Everson CA, Toth LA. Systemic bacterial invasion induced by sleep deprivation. Am J Physiol Integr Comp Physiol. 2000;278(4):905–916.