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# Prebiotic diet modulates disruptions in sleep, core body temperature, and locomotor activity produced by circadian disruption

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Prebiotic diet modulates disruptions in sleep, core body temperature, and locomotor activity produced by circadian disruption

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## ABSTRACT

Bacteria living in the gastrointestinal tract (gut microbiota) provide beneficial roles for the host including communication with the central nervous system. Recent evidence, for example, suggests that gut-brain signaling may modulate sleep patterns. Since sleep disorders are prevalent in military personnel due to chronic stressors including circadian disruption (CDR), this study was designed to explore a potential intervention known to be efficacious in preventing several negative physiological impacts of acute intense stressor exposure, including disturbed sleep and reduced alpha diversity of the gut microbiota. Prebiotic diets are rich in plant fibers that stimulate growth and activity of healthy promoting gut flora. The current study tested the hypothesis that a prebiotic diet would reduce the negative impacts of CDR on sleep. Male Sprague Dawley rats were implanted with biotelemetry devices to record sleep/wake activity, core body temperature (CBT), and locomotor activity (LA) via electroencephalographic (EEG) leads. The results were that rats fed a prebiotic diet compared to a calorically matched control diet were exhibited protection against CDR-evoked reductions in rapid eye movement (REM) sleep (bouts and percent time) during the light phase after four days of re-alignment. In contrast, the impact of CDR on CBT and LA was not impacted by the prebiotic diet.

### **INTRODUCTION**

The prevalence of sleep disturbances has become a priority to address in the medical and military sectors. Sleep disorders, such as insomnia, are commonly found during military deployment due to stress, anxiety, and shift work<sup>18</sup>. Military settings threaten normal sleep patterns because of environmental and work-related stressors; thus, uncovering the prevalence of sleep disturbances and mental health issues for active duty personnel is emerging as a necessary study<sup>21</sup>. Notably, repeated night stress has long-term consequences on the appropriate timing of physiological activities and has been theorized to prevent resetting of the circadian rhythm<sup>1</sup>. Shift workers, such as military personnel, have shorter sleep durations that produce a physiological stress response which generates a dysbiosis in the gut microbiota<sup>19,25,26</sup>.

In order to quantitatively measure sleep disturbances, changes in percent sleep as well as the number of bouts and episode duration are examined. Analyzing the amount of time spent in certain sleep stages is an important scientific assessment since early stages of neurodegeneration have been linked to sleep disorders, particularly with decreases in rapid eye movement (REM) sleep<sup>3</sup>. Curiously, alterations in the number of bouts for each sleep state depends on the previous sleep state from which an individual was transitioning<sup>10</sup>. While studies predict that short-term regulation of rodent sleep occurs due to changes in sleep intensity as opposed to episode duration, successive circadian cycles control sleep time in humans based on previous wake times<sup>14</sup>. Episode duration crucially depends on greater periods of wakefulness which instigate restorative sleep processes on the acquired sleep deficits<sup>14</sup>. By evaluating alterations in episode duration and bout number, assessment of overall changes in percent time spent during each sleep state can provide a platform from which to test potential sleep deficit interventions.

Circadian rhythms are 24-hour endogenous oscillations that regulate hormone levels and physiological processes such as core body temperature (CBT) and locomotor activity (LA). Such circadian physiological rhythms are disrupted by controllable and uncontrollable stressors<sup>22</sup>. Internal temporal coordination of physiological processes within organisms anticipate environmental changes<sup>11</sup>. Lack of synchrony in the timing of these processes creates health issues that are typically associated with shift work including sleep loss and impaired cognitive function<sup>24</sup>. The suprachiasmatic nuclei (SCN) of the hypothalamus has been shown to control

such circadian oscillations, but social stress masks SCN output and causes rhythmic disturbances in heart rate, CBT, and LA<sup>6,11</sup>. Acute stress impacts circadian rhythms; yet, behavioral control over the stress does not significantly prevent disruptions in CBT or LA<sup>22</sup>. Since circadian disruptions also produce dysbiosis in gut microbiota<sup>25,26</sup>, equating the degree of physiological impairments to diet is the focus of this research.

Diet greatly influences the composition of gut microbiota and the ability to metabolize food<sup>29</sup>. Gut bacteria are important for processes such as lipolysis and gluconeogenesis in the liver, and for maintaining circadian rhythmicity<sup>16,17</sup> through communication to the central nervous system. In addition, microbial ecology is dynamic, impacted by food composition and time of day<sup>17,28</sup>. Effectiveness of a prebiotic diet is measured not by the daily dose but by the overall changes in composition of fecal flora, increases in probiotic species such as *Lactobacillus* and *Bifidobacteria*, and changes in bacterial metabolites<sup>20</sup>.

The timing and average period of sleep cycles are controlled by circadian rhythms and correlates with CBT<sup>4</sup>. Mammals cycle from non-rapid eye movement (NREM) to REM sleep in an ultradian rhythm<sup>27</sup> where NREM sleep is associated with reduced neural activity and REM sleep has increased brain activity<sup>9</sup>. In humans, REM episodes occur nearly every 90 minutes and tend to last for 30 minutes while rats tend to have 12-minute sleep cycles which can be monitored via EEG activity<sup>9</sup>. With REM sleep deprivation, the hypothalamic-pituitary-adrenal (HPA) axis is activated resulting in greater secretion of stress-related glucocorticoids, which if chronically stimulated can confer negative health consequences on the host. Acute stressor exposure has been shown to reduce REM and NREM sleep directly following the stress and additional impairment of REM rebound was found with repeated fear<sup>5</sup>. Chronic stress significantly decreased the amount of time spent in REM sleep yet baseline REM levels were recovered by the seventh day of stress<sup>7</sup>.

Prebiotic diet increases stress-protective microbiome composition and produces stress resistance<sup>12,13,23</sup>. Specifically, prebiotic diet prevents acute stress-evoked disruptions in mood, REM sleep, and microbial diversity<sup>12,13,23</sup>. What remains unknown, however, is if dietary prebiotics are also efficacious in preventing the negative consequences of chronic stressors on physiological states. The following experiment was designed to test if a prebiotic diet can reduce the negative impacts of circadian disruption on sleep disturbances and related changes in physiological activities. This study tested the impact of two weeks of circadian disruption by examining sleep patterns, CBT, and LA during the first week of re-alignment. These data are only a section of what has been collected and what will be examined for the Fleshner lab's research for the Office of Naval Research.

#### **METHODS**

#### Animals

Juvenile male Sprague-Dawley 344 rats (n=84, Envigo) arrived on post-natal day (PND) 24 and were pair-housed in Nalgene Plexiglas cages (45 cm x 25.2 cm x 14.7 cm) at a controlled temperature (22°C). The rats were introduced to a control or test (prebiotic) diet upon arrival. The prebiotic diet contained galactooligosaccharides (GOS; 21.23 g/Kg), polydextrose (PDX; 6.58 g/Kg), lactoferrin (Lf; 1.86 g/Kg), and Whey protein concentrate milk fat globule membrane (MFGM: 15.9 g/Kg). The normal light/dark cycle (NLD) was maintained with a 12:12 h schedule (lights on 05:00-17:00). During the experiment, circadian disruption (CDR) was induced by inverting the light/dark cycle for a week. Animals had *ad libitum* access to food and water throughout the experiment. After surgery (PND 59), animals were single-housed to accurately record from biotelemetry devices. Weekly weights were obtained during the inactive cycle to assess food consumption.

#### **Experimental Design**

Project groups for this experiment were based on diet and application of a chronic stressor. Four experimental groups were used to examine if a prebiotic diet can protect against the effect of CDR on sleep patterns, CBT, and LA: control/NLD (n=20), control/CDR (n=21), prebiotic/NLD (n=20), prebiotic/CDR (n=23). The CDR rats underwent circadian disruption on week 1 and were reintroduced to a normal light/dark cycle on week 2 (Figure 1) while NLD rats maintained the same 12:12 h light/dark cycle throughout the duration of the experiment. All rats were on a NLD prior to the start of the experiment; thus, CDR rats experienced a two-week period of disruption. Biotelemetry devices acquired data for LA, CBT, wakefulness (WAKE), non-rapid eye movement (NREM), and rapid eye movement (REM). The data examined for this project

reflects sleep patterns, CBT, and LA during week 2 solely. Data were examined at two time points during week 2 to analyze behavior immediately after re-aligning the CDR groups back to the NLD (Days 8,9) and 4 days after re-aligning to the NLD (Day 12). These time points were decided based on evaluation of potential re-aligning patterns that is still being assessed in the lab.



Figure 1. Experimental timeline. Inverse light/dark cycle (Week 1); Normal light dark cycle (Week 2). Misalignment was measured on days 8,9. Re-alignment was measured on Day 12.

### **Biotelemetry Surgery**

F40-EET biotelemetry transmitters (Data Sciences International, St. Paul, MN) were surgically implanted following established protocols<sup>22,23</sup>. Rats were anesthetized with via an intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg). A 5 cm incision was made in the abdominal cavity for the EET transmitter to be suture in placed. The electromyography (EMG) leads were directed to the posterior neck to monitor skeletal muscle activity while electroencephalographic (EEG) leads were installed subcutaneously to record electrical activity of the brain. Administration of 1.0 mL of antibiotics was provided prior to animals waking from anesthesia. Self-ambulatory rats were then single-housed. Biotelemetry recordings were obtained following 10 days of recovery for the duration of the experiment.

#### **Data Acquisition and Analysis**

Use of the F40-EET transmitters provided real-time *in vivo* measurement of electrical activity, CBT, and LA. The biotelemetry recordings were obtained/analyzed using Dataquest ART Analysis Software (Data Sciences International, St. Paul, MN). NeuroScore software (Data Sciences International, St. Paul, MN) was used to analyze the sleep/wake patterns as indicated by EEG activity. The spectra used for analysis of individual NREM, REM, and WAKE followed published protocols and parameters<sup>23</sup>. WAKE was distinguished by having low amplitude EEG frequency followed by increases in LA and CBT. NREM sleep was established with increased EEG absolute amplitudes and decreased LA and CBT. REM sleep was identified by decreased amplitude EEG and decreased LA and CBT. Time spent in each sleep/wake category (% sleep), the number of episodes per category per hour (#), and average episode durations which indicated changes in sleep/wake states greater than 10 s per hour were calculated. Statistical analysis was performed using StatView (SAS Institute). Diurnal differences were determined by subtracting the average 12-h night values from the day values<sup>22</sup>. Data were analyzed using repeated measures ANOVA and two-way ANOVAs for averaged values. Data are presented as mean  $\pm$  standard error as determined using StatView. Average percent sleep/wake, number of bouts, and episode duration were examined separately for REM, NREM, and WAKE conditions over days 8,9 and day 12 during week 2 of the experimental procedure.

#### RESULTS

#### CDR alters percent sleep upon re-introduction to a NLD cycle

CDR decreased percent REM during the light phases and increased percent REM during the dark phase on days 8,9 regardless of diet type, and diet reduced the percent REM in NLD rats during the light phases on days 8,9 (Figures 2A,B). Percent NREM was decreased during the light phases and increased during the dark phase on days 8,9 for CDR rats regardless of diet type (Figures 2C,D). Percent WAKE was increased during the light phases and decreased during the dark phase on days 8,9 for CDR rats regardless of diet type (Figures 2E,F). These observations were supported statistically with reliable main effects of CDR (ps < 0.05) and reliable interactions of CDR and light/dark cycle phases (ps = 0.0001) for percent REM, NREM, and WAKE on days 8,9.



Misalignement Week 2 %Sleep - Experimental Days 8 & 9

Figure 2. Percent time spent in REM, NREM, and WAKE on experimental days 8,9 (37 hours) for each experimental group. (A) Average percent REM sleep. (B) Average diurnal percent REM. (C) Average percent NREM sleep. (D) Average diurnal percent NREM. (E) Average percent WAKE. (F) Average diurnal percent WAKE. \* denotes a significant effect of CDR at p < 0.05; # denotes a significant effect of diet at p < 0.05.

**Diet exhibited a trend in protecting % REM sleep decrease during light phase on day 12** CDR increased percent REM in the dark phase regardless of diet type and decreased percent REM for control diet rats during the light phase on day 12 (Figures 3A,B). There was a reliable interaction of diet on CDR during light/dark cycle phases (p = 0.022) on day 12 (Figures 3A,B). Percent NREM did not show significant differences in diet or stress on day 12 (Figures 3C,D). CDR reduced percent WAKE regardless of diet during the dark phase on day 12 (Figures 3E,F). There was a main effect of CDR for percent WAKE on day 12 (p = 0.014). These observations indicate a reliable interaction of CDR and light/dark cycle phases (ps < 0.05) for percent REM and WAKE on day 12.



Figure 3. Percent time spent in REM, NREM, and WAKE on experimental day 12 (25 hours) for each experimental group. (A) Average percent REM sleep. (B) Average diurnal percent REM. (C) Average percent NREM sleep. (D) Average diurnal percent NREM. (E) Average percent WAKE. (F) Average diurnal percent WAKE. \* denotes a significant CDR x diurnal effect at p < 0.05.

#### CDR alters number of bouts in each sleep stage on days 8,9

CDR decreased the number of REM bouts during the light phases and increased the number of REM bouts during the dark phase on days 8,9 regardless of diet type (Figures 4A,B). For control diet rats, CDR decreased the number of NREM bouts during the light phases; for prebiotic diet rats, CDR decreased the number of NREM bouts during the first light phase with a parallel increase during the dark phase on days 8,9 (Figures 4C,D). CDR decreased the number of WAKE bouts during the light phases on days 8,9 for control diet rats (Figures 4E,F). These observations were supported statistically with reliable main effects of CDR (ps < 0.05) for the number of REM bouts on days 8,9. There were reliable interactions of CDR and light/dark cycle phases (ps < 0.0001) for the number of REM, NREM, and WAKE bouts on days 8,9.



Figure 4. Number of bouts for REM, NREM, and WAKE on experimental days 8,9 (37 hours) for each experimental group. (A) Average number of bouts in REM sleep. (B) Average diurnal number of bouts in REM. (C) Average number of bouts in NREM sleep. (D) Average diurnal number of bouts in NREM. (E) Average number of bouts in WAKE. (F) Average diurnal number of bouts in WAKE. \* denotes a significant effect of CDR at p < 0.05.

# **Prebiotic diet protected the number REM bout decrease during light phase on day 12** For control diet rats, CDR decreased the number of REM bouts during the light phase with a parallel increase during the dark phase on day 12 (Figures 5A,B). There was a reliable interaction of diet on CDR during light/dark cycle phases (p = 0.004) on day 12 (Figures 5A,B). There was a reliable interaction of CDR and light/dark cycle phases (p < 0.0001) for the number of REM bouts on day 12 (Figures 5A,B). The number of NREM bouts did not show significant differences in diet or stress on day 12 (Figures 5C,D). The number of WAKE bouts did not show significant differences in diet or stress on day 12 (Figures 5E,F).



Re-alignment Week 2 Sleep Bout # - Experimental Day 12

Figure 5. Number of bouts for REM, NREM, and WAKE on experimental day 12 (25 hours) for each experimental group. (A) Average number of bouts in REM sleep. (B) Average diurnal number of bouts in REM. (C) Average number of bouts in NREM sleep. (D) Average diurnal number of bouts in NREM. (E) Average number of bouts in WAKE. (F) Average diurnal number of bouts in WAKE. \* denotes a significant CDR x diurnal effect at p < 0.05; # denotes a significant effect of diet at p < 0.05.

#### Average episode duration unaffected by diet on days 8,9

CDR decreased average REM episode duration during the light phases with a parallel increase during the dark phase on days 8,9 regardless of diet type (Figures 6A,B). CDR increased average NREM episode duration during the dark phase on days 8,9 regardless of diet type (Figures 6C,D). CDR increased average WAKE episode duration during the light phases with a parallel decrease during the dark phase on days 8,9 regardless of diet type (Figures 6E,F). These observations were supported statistically with a reliable main effect of CDR (p = 0.013) for average WAKE episode duration on days 8,9 (Figures E,F). There were reliable interactions of CDR and light/dark cycle phases (ps < 0.0001) for average REM, NREM, and WAKE episode duration on days 8,9.



Misalignement Neek 2 Sleep Avg. Eps. Duration - Experimental Days 8 & 9

Figure 6. Episode duration for REM, NREM, and WAKE on experimental days 8,9 (37 hours) for each experimental group. (A) Average episode duration in REM sleep. (B) Average diurnal episode duration in REM. (C) Average episode duration in NREM sleep. (D) Average diurnal episode duration in NREM. (E) Average episode duration in WAKE. (F) Average diurnal episode duration in WAKE. \* denotes a significant effect of CDR at p < 0.05.

### Average episode duration unaffected by diet on day 12

CDR increased average REM episode duration during the dark phase on day 12 regardless of diet type (Figures 7A,B). There was a reliable main effect of CDR (p = 0.048) for average REM episode duration on day 12 (Figures 7A,B). There were reliable interactions of CDR and light/dark cycle phases (p = 0.0009) for average REM episode duration on day 12 (Figures 7A,B). The average NREM episode duration did not show significant differences in diet or stress on day 12 (Figures 7C,D). The average WAKE episode duration did not show significant differences in diet or stress on day 12 (Figures 7E,F).



Re-alignment Week 2 Sleep Avg. Eps. Duration - Experimental Day 12

Figure 7. Episode duration for REM, NREM, and WAKE on experimental day 12 (25 hours) for each experimental group. (A) Average episode duration in REM sleep. (B) Average diurnal episode duration in REM. (C) Average episode duration in NREM sleep. (D) Average diurnal episode duration in NREM. (E) Average episode duration in WAKE. (F) Average diurnal episode duration in WAKE. \* denotes a significant CDR x diurnal effect at p < 0.05.

#### Core body temperature re-acclimated to a NLD cycle

CDR increased average CBT was increased during the light phases with a parallel decrease during the dark phase on days 8,9 regardless of diet type (Figures 8A,B). There was a reliable main effect of CDR (p < 0.0001) for average CBT on days 8,9 (Figures 8A,B). Average CBT did not show significant differences in diet or stress on day 12 (Figures 8C,D). There were reliable interactions of CDR and light/dark cycle phases for average CBT on days 8,9 (p < 0.0001) and on day 12 (p = 0.453).



Week 2 Core Body Temperature - Experimental Days 8,9 & 12

Figure 8. Core body temperature on experimental days 8,9 (37 hours) and Day 12 (25 hours) for each experimental group. (A) Average CBT on Days 8,9. (B) Average CBT on Days 8,9. (C) Average CBT on Day 12. (D) Average diurnal CBT on Day 12. \* denotes a significant effect of CDR at p < 0.05; # denotes a significant effect of diet at p < 0.05.

#### Locomotor activity was elevated during reintroduction of NLD

CDR increased LA during the light phases with a parallel decrease during the dark phase on days 8,9 regardless of diet type (Figures 9A,B). There were reliable interactions of CDR and light/dark cycle phases for average LA on days 8,9 (p < 0.0001) (Figures 9A,D). For control diet rats, CDR increased LA during the light and dark phase; for prebiotic diet rats, CDR increased LA during the light and dark phase; for prebiotic diet rats, CDR increased LA during the light on day 12 (Figures 9C,D). There was a reliable main effect of CDR for LA on days 8,9 (p < 0.0001) and on day 12 (p = 0.006).



#### Week 2 Locomotor Activity - Experimental Days 8,9 & 12

Figure 9. Locomotor activity on experimental days 8,9 (37 hours) and day 12 (25 hours) for each experimental group. (A) Average LA on days 8,9. (B) Average diurnal LA on days 8,9. (C) Average LA on day 12. (D) Average diurnal LA on day 12. \* denotes a significant main effect of CDR at p < 0.05.

### DISCUSSION

Sleep disturbances have a significant prevalence in military personnel and have an estimated effect on 30-40% of adults which costs the United States nearly \$14 billion per year<sup>18</sup>. Shift work in particular provides external environmental stressors that create an imbalance in proper timing of endogenous circadian rhythms. Chronic stressors, such as circadian disruption, challenge normal physiological oscillations thus impacting sleep quality and duration. Prebiotic diets can mitigate negative effects of stress on sleep: REM sleep in particular<sup>23</sup>. The use of dietary prebiotics, if it proves to be successful intervention for sleep disturbances caused by stress, can revolutionize the understanding of the gut-brain axis and potentially provide a relatively inexpensive method for circadian re-alignment.

This research looked at the second experimental week of a larger study where days 8,9 were the first 36 hours back on a NLD cycle for all rats following a week of CDR and day 12 was the fourth day of reintroduction to the NLD cycle. One strength of this study was that sleep could be explored during rhythm desynchrony (days 8,9) and after resynchrony (day 12). Analysis of EEG patterns found that CDR rats regardless of diet types had desynchronized percent time spent in REM, NREM, and WAKE as compared to NLD rats during the first 37 hours immediately following CDR. The prebiotic diet did reduce the overall decrease in time spent in REM sleep during the first 12 hours on day 8 that was typically induced by CDR as compared the difference in percent REM for the control diet rats. The rats still showed desynchrony for time spent in REM sleep during the light phase. This finding indicates that the prebiotic diet could protect against the CDR rats' decrease in amount of time spent in REM sleep during a NLD after four days of re-alignment. This effect was not seen during the dark phase on day 12 nor for time spent in NREM or WAKE.

The number of REM, NREM, and WAKE bouts had a similar trend as the percent time spent in REM sleep on days 8,9, though the number of REM bouts showed consistent effects of CDR diurnally regardless of diet types. This study found that the prebiotic diet protected against the decrease in the number of REM bouts during the light phase on day 12, but this effect was not seen during the dark phase. This effect of diet reflects that prebiotics protected against the

dampening effect of CDR on number of REM bouts after four days of re-alignment. The average episode duration was desynchronized most notably in REM and WAKE on days 8,9 for CDR rats regardless of diet type, but the average episode duration for CDR rats was not significantly different by day 12. There was no protective effect of diet on average episode duration.

CBT and LA were monitored in addition to sleep disturbances to further assess the effectiveness of dietary prebiotics on circadian disruption. This study found that CBT was desynchronized on days 8,9 for CDR rats regardless of diet type and it was resynchronized on day 12. LA was desynchronized on days 8,9 for CDR rats regardless of diet type, but LA was increased for control CDR rats during both the light and dark phase on day 12. Even though LA did not considerably increase for prebiotic CDR rats during the dark phase on day 12, there was no significant effect of diet to determine if this correlation was a prebiotic protective effect.

A prebiotic diet exhibited a trend in preventing reduced percent time in REM sleep and protected the number of REM bouts during the light phase on day 12; yet, average episode duration, core body temperature, and locomotor activity did not see changes induced by diet. These findings indicate that the number of times and overall percent time spent in REM sleep during a rat's normal sleep schedule (light phase) was brought back to control conditions through the use of a prebiotic diet after the first round of a chronic stressor. Further examination of dietary prebiotics after the first week of re-synchronization will be conducted to see if these findings change across time.

To assess the effect of a prebiotic diet on mitigating physiological impacts caused by a chronic stressor at the cellular level, analysis of neuromodulators that are directly related to sleep should be conducted. Examining the levels of serotonin receptors (5-HT<sub>1A</sub>) in the dorsal raphe nucleus (DRN) of the hypothalamus provides a method for detailing whether REM has been inhibited. Stress has been hypothesized to cause serotonin (5-HT) release from the DRN. This then projects to the arcuate nucleus to initiate synthesis of corticotropin-like intermediate peptide (CLIP), which is a POMC derivative that has been shown to produce long lasting REM episodes<sup>8</sup>. It is accepted that 5-HT predominantly inhibits REM sleep and stimulates wakefulness and 5-HT<sub>1A</sub> has inhibitory effects on target REM neurons in the laterodorsal and pedunculo-pontine

tegmental nuclei<sup>15</sup>. Interestingly, release of 5-HT in the DRN is a self-inhibitory process that prevents further 5-HT synthesis when DRN 5-HT<sub>1A</sub> autoreceptors are activated, contributing to increased REM sleep<sup>2,8</sup>. Examining the prevalence of 5-HT<sub>1A</sub> in the DRN of rats that experience circadian disruption can provide evidence for how stress impacts the presence of 5-HT and subsequent REM sleep patterns; having a prebiotic diet provides an additional metric of analysis of the stressor's impact on REM duration.

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