RELATIONSHIPS BETWEEN CIRCADIAN RHYTHMS, TIMING OF EATING BEHAVIORS, AND THE HUMAN GASTROINTESTINAL MICROBIOTA

BY

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THESIS

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ABSTRACT

In the obesity-prone environment in which we live, no avenue for potentially health-promoting intervention should be ignored. One such avenue that has gained recent attention is the modulation of the gastrointestinal microbiota. Microbiota interventions have come into the spotlight because of the proposed relationships between the microbial community's composition, function, and human health. One of the most common strategies for modulating the microbial community for potential health benefit is by dietary modifications, although antibiotics, microbial transplant, probiotics, and even exercise can also impact the gastrointestinal microbiome. With the emergence of evidence that timing of eating can impact health, it follows that the connection of eating behaviors to the gastrointestinal microbiota should be explored further. The objective of this research was to assess the links between circadian rhythms, timing of eating, and the human gastrointestinal microbiota. To accomplish this goal, a thorough review of the current literature was first conducted. Second, a cross-section of healthy, adult subjects was examined to determine the relative abundances of bacterial genera and concentrations of bacterial metabolites in fecal samples collected throughout the day. These variables were additionally assessed in relation to the subjects' eating habits, including eating frequency, consumption of energy earlier in the day, and overnight fast duration. This study found strong evidence in the existing literature for the impact of circadian rhythms and eating behaviors on the gastrointestinal microbiota and health. Additionally, this work presents the results of a large, cross-sectional clinical study which found an association between time of day, microbiota composition and function, and eating behaviors. The results presented herein propose that this connection not only exists, but also could hold relevance for human health, with application to health-promoting interventions.

This work is dedicated to my parents, John and Anna Kaczmarek. Without you, I would be nothing but a spot on the sidewalk.

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CHAPTER 1: COMPLEX INTERACTIONS OF CIRCADIAN RHYTHMS, EATING BEHAVIORS, AND THE GASTROINTESTINAL MICROBIOTA AND THEIR POTENTIAL IMPACT ON HEALTH[1]

ABSTRACT

Human health is intricately intertwined with the composition and function of the trillions of microorganisms that make up the gastrointestinal (GI) microbiome. The GI microbiome is essentially a microbial organ providing metabolic, immunologic, and protective functions for the host. Habitual diet, changes in macronutrient composition, and consumption of nondigestible dietary fibers have all been shown to impact the human GI microbiome. Intriguingly, the impact of diet on the microbiome may not only be related to what we eat but also to when we eat. Emerging preclinical research suggests that gut microbes experience diurnal rhythms, and the health effects of eating patterns, such as time-restricted feeding and meal frequency, may be related to the GI microbiome. Herein, we review the complex connections between circadian rhythms, eating behaviors, the GI microbiome, and health, and point to the need for additional translational research in this area.

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INTRODUCTION

The impact of diet on the gastrointestinal (GI) microbial community composition and function and human health is a rapidly evolving research domain. Exploring this area is essential in the present day as the cost and prevalence of chronic diseases continue to rise to staggering figures while standard, efficacious treatments such as dietary and physical activity modifications are infrequently prescribed and even less frequently followed (Milani & Lavie, 2015). The trillions of microbes that make up the GI microbiome form a microbial organ with a collective gene set 150 times larger than the human genome, providing metabolic, immunologic and protective functions for the host (Qin et al., 2010). In addition to contributing to immunological development and metabolic function, the GI microbiome also influences nervous system development and function (Mu, Yang, & Zhu, 2016). Furthermore, the composition and function of the GI microbiome has been shown to be linked to a growing list of metabolic diseases including obesity, diabetes, and cardiovascular disease (Albenberg & Wu, 2014; De Vos & De Vos, 2012).

Diet is an important mediator of the human GI microbiota —habitual intake (Wu et al., 2011), rapid changes in dietary fat and fiber composition (David et al., 2014), and consumption of dietary fibers (H. D. Holscher, Bauer, et al., 2015; H. D. Holscher, Caporaso, et al., 2015; Hannah D. Holscher, 2017), and other nondigestible food components have all been shown to impact both the composition and function of these resident microbes (Albenberg & Wu, 2014; Sonnenburg & Bäckhed, 2016). Intriguingly, the impact of diet on the microbiome may not only be related to what we eat but also to when we eat. Host-symbiont bidirectional communication occurs via signaling along the gut-microbiota-brain axis by a variety of bacterial metabolites

which have been shown to impact centrally mediated feeding behaviors such as appetite (Frost et al., 2014; Perry et al., 2016; Sandhu et al., 2017).

Increasingly, preclinical research has demonstrated that the bacteria in the GI tract vary over the course of a day, exhibiting diurnal rhythms in relative taxa abundances, proximity to the colonic epithelium, and metabolism (Thaiss, Levy, et al., 2016; Zarrinpar, Chaix, Yooseph, & Panda, 2014). Time of eating is considered a potential modulator of circadian rhythms in both bacterial abundance and function (R.M. Voigt, Forsyth, Green, Engen, & Keshavarzian, 2016). Furthermore, the gut microbiome appears to have a reciprocal relationship with the human body's circadian clock and eating patterns (Figure 1.1). Emerging research suggests that some of the observed health effects related to eating patterns, such as time-restricted feeding (TRF) and meal frequency, may also be related to the GI microbiome. Herein, we review preclinical and clinical research on circadian misalignment, eating behaviors, and the GI microbiome.

CIRCADIAN RHYTHMS

Most of the life on earth experiences a daily 24-hour light/dark cycle created by the earth's rotation in relation to the sun, and as a result, perform a 24-hour cycle of feeding and fasting (Hastings, Reddy, & Maywood, 2003). Circadian rhythms are cycles of gene expression, metabolism, and behaviors created by the internal clock that governs a multitude of metabolic functions such as hepatic lipid metabolism, cardiovascular function, obesity regulation, and glucose homeostasis (Bass & Takahashi, 2010; Huang, Ramsey, Marcheva, & Bass, 2011). Circadian rhythms are regulated in humans in two ways: 1) by light via the suprachiasmatic nucleus in the brain, and 2) by clock proteins present in nearly every cell that provide a transcriptional rhythm based on a 24-hour day (Zarrinpar, Chaix, & Panda, 2016). The

suprachiasmatic nucleus also regulates the circadian release of digestive peptides including vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) (Zarrinpar et al., 2016). Almost all human cells have circadian regulatory genes, including *Clock, Bmal1, RORα, Cry, Per, and Rev-erbα* (Zarrinpar et al., 2016). In mice, 45% of transcripts have 24-hour oscillations (Zarrinpar et al., 2016). The homeostatic sleep/wake cycles of the central nervous system, combined with pituitary gland activity, exert significant influences on the endocrine system (Zarrinpar et al., 2016).

Health Implications of Misaligned Circadian Rhythms

The natural state of most life on earth is to spend one phase of a 24-hour day (either light or dark) in an active and feeding state, and the other in a resting and fasting state (Hastings et al., 2003). Humans naturally spend the light phase in the active and feed state, while resting and fasting primarily occur during the dark phase. However, with the development of artificial light, humans have deviated from the original pattern of eating only during the light window of the day. Furthermore, individuals who work night shifts experience an almost complete reversal of food intake, with intake occurring primarily during the night, and rest and fasting occurring during daylight hours. Dietary intake that is misaligned to the natural rhythms of the circadian clock has been shown to negatively impact human health. Specifically, disruptions to the normal sleep/wake cycle in relation to the night/day cycle of the earth, as seen in shift work, are associated with a 40-60% increased risk for obesity and metabolic syndrome (Karlsson, Knutsson, & Lindahl, 2001; F. Wang et al., 2014).

Misaligned circadian rhythms refer to the disruption of the normal schedule of feeding and fasting. Circadian rhythms can be misaligned through environmental conditions such as shift

work or jet lag, or in the case of preclinical research, the creation of knockout models through genetic manipulation of relevant genes. The importance of cellular clock mechanisms to health has been demonstrated in knockout mice, whereby *Bmal1* knockout mice and *Rev-erbα and Rev-erbβ* double knockout mice display dysmetabolism in glucose and lipid homeostasis, respectively (Zarrinpar et al., 2016). Manipulation of environmental conditions such as restricting the food access of wild-type mice to only the light phase (when mice are normally not active or eating) has been shown to result in a 23% increase in weight gain and an 8% higher body fat percentage compared to mice who had access to food during their normal active phase (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). Thaiss et al. reported that circadian-disrupted mice fed a high-fat diet had 17% greater body weight compared to non-disrupted mice on a similar diet (Thaiss et al., 2014). Antibiotic-treated mice were resistant to these detrimental metabolic changes, suggesting the connection between misaligned rhythms and the microbiome (Thaiss et al., 2014).

Adding dim light to an animal's habitual dark phase has also been shown to disrupt circadian rhythms, and thus metabolism. Mice housed in dim light conditions have been shown to increase energy consumption during the light phase to 55% of total intake, compared to 36% in mice kept in standard light/dark phase conditions. Body mass and insulin resistance were also greater in both dim light and constant light situations when compared to standard light/dark, despite the use of isocaloric diets and matched physical activity levels (Fonken et al., 2010). Chronic sleep fragmentation, a model of obstructive sleep apnea, also affects both the murine microbiome and health (Poroyko et al., 2016). Poroyko et al. demonstrated that mice exposed to chronic sleep fragmentation by tactile stimulation every two minutes during the sleep phase showed increased food intake and decreased colonic barrier function, and that transplantation of

the Firmicutes-enriched microbiome of these animals into germ-free animals resulted in enhanced inflammatory response and insulin resistance in the recipient animals (Poroyko et al., 2016). Recent work has also examined sleep restriction in both mice and a small group of humans (S. L. Zhang et al., 2017). Following five days of restricting sleep to four hours per night, the authors found minor changes in the murine microbiota, but no changes among humans (n=11). They concluded that, while weight and behavioral alertness were impacted in humans, the microbiota was resistant to sleep restriction-induced change. However, the small sample size of human participants, and the acute nature of the study may have not been adequately powered to detect changes in the highly individualistic human microbiota (Faul, Erdfelder, Lang, & Buchner, 2007).

Results from preclinical circadian rhythm misalignment studies are further supported by clinical studies that reveal associations between specific single-nucleotide polymorphisms (SNPs) in the *Clock* gene and risk for metabolic syndrome (Scott, Carter, & Grant, 2008). There are three known polymorphisms of this gene, relatively equally distributed throughout the population, and haplotype is significantly associated with presence of metabolic syndrome (Scott et al., 2008). The functional role of these SNPs has not yet been elucidated (Zarrinpar et al., 2016). Furthermore, it has also been demonstrated in shift workers, both observationally and in a laboratory setting, that working at times other than daylight hours negatively impacts health outcomes, including a 66% elevated risk of obesity and 57% elevated risk of metabolic syndrome (F. Wang et al., 2014). Shift workers also have been shown to have 12-16% reduced energy expenditure (McHill et al., 2014), and they are more likely to experience dyslipidemia, including elevated blood triglycerides and reduced high-density lipoprotein cholesterol (Karlsson et al., 2001). In one experiment, human participants experiencing out-of-phase treatment through

a 12-hour reversal from their habitual schedule had 17% lower leptin concentrations compared to their values under circadian alignment. Furthermore, insulin, glucose, and mean arterial pressure were 22%, 6%, and 3% higher, respectively, under misalignment than under alignment (Scheer, Hilton, Mantzoros, & Shea, 2009). A summary of clinical findings of misaligned circadian rhythms can be found in Table 1.1 (McHill et al., 2014; Scheer et al., 2009; Thaiss et al., 2014; F. Wang et al., 2014; Zarrinpar et al., 2016; S. L. Zhang et al., 2017).

Impact of Misaligned Circadian Rhythms and Diet on the Microbiota

There is increasing evidence for a connection between the microbiota, diet, circadian rhythms, and internal clock mechanisms (Figure 1.2). One method to explore interactions between these factors is to provide continuous intravenous nutritional support, which eliminates effects of feeding time or even intestinal presence of food on the GI microbiome. Interestingly, mice given continuous parenteral nutrition have been shown to have significant changes in microbial community structure, but the microbiota did not completely lose diurnal variation (Leone et al., 2015). Specifically, while beta diversity, a measure of dissimilarity, or distance, between samples, clustered by treatment when comparing enteral to parenteral nutrition, within each treatment group samples clustered by time of day. Additionally, relative abundances of different phyla varied between treatments, with Verrucomicrobia dramatically blooming at the expense of the Firmicutes in the parenteral group. Bacteroidetes also had a cyclical abundance pattern over the course of the day, increasing during the light phase and decreasing during the dark, changes that were independent of the study treatment provided.

Research has also been conducted to investigate interactions of environmental circadian rhythm disruption and diet on the GI microbiome in murine models (Robin M. Voigt et al.,

2014). Continuous circadian rhythm disruption was induced by reversing the 12-hour light-dark cycle every week for 12 weeks in mice receiving chow or high-fat/high-sugar diet. Mice in the misalignment group experienced intestinal hyperpermeability and disrupted circadian gene expression in intestinal cells. Cycle shifting resulted in significant weight gain in mice consuming chow, but there were no significant changes in GI microbial composition in these animals. There were, however, significant differences in microbial community composition between high-fat shifted and high-fat non-shifted groups. The high-fat shifted mice had decreased alpha diversity, a measure of the number of different microbial species (richness) within a sample and their relative abundances (evenness), and a higher ratio of Firmicutes/Bacteriodetes compared to the other high-fat diet mice who were not exposed to a light/dark cycle shift. This specific microbial ratio has been associated with obesity or ill health in both rodent models and humans (Robin M. Voigt et al., 2014). These results suggest that it may be a combination of both a high-fat diet and circadian disruption that drives microbial dysbiosis in mice.

A "jet lag" model has also been utilized to explore the effects of shorter (e.g. ≤ 12 hours) circadian cycle disruptions. In one study, mice exposed to an 8-hour time light shift every three days for four weeks experienced a loss of rhythmicity in oscillating GI bacterial taxa (Thaiss et al., 2014). Microbiome community composition was impacted after four weeks of this jet lag model intervention, and dysbiosis was even more pronounced by 16 weeks. Fecal transplant of jet-lagged mouse microbiome into germ-free mice resulted in weight gain and glucose intolerance in the recipient animals (Thaiss et al., 2014).

Interestingly, there is evidence of bacteria containing clock genes (Kondo, 2007) and regulating the behavior of their host in a circadian manner (Heath-Heckman et al., 2013). For

example, *Enterobacter aerogenes* is purported to contain an endogenous circadian clock that synchronizes with the human host through melatonin secreted into the GI tract (Paulose, Wright, Patel, & Cassone, 2016). In mice, reprogramming of the hepatic circadian clock following a high-fat diet intervention has been attributed to microbiota-driven induction and activation of the transcription factor PPARγ (M. Murakami et al., 2016), and both liver and intestinal circadian genes are affected by unconjugated bile acids, known products of microbial metabolism (Govindarajan et al., 2016). The absence of a microbiota, both in germ-free and antibiotic-induced murine models, has been shown to alter intestinal epithelial cell transcription of nuclear receptors and clock components such as *Rev-erbα, RORα, Bmal1, Cry1, Per1*, and *Per2* (Mukherji, Kobiita, Ye, & Chambon, 2013). Additionally, these microbiota-deficient mice have decreased insulin levels and elevated blood glucose, triglycerides, and free fatty acid concentrations, as a result of intestinal corticosterone overproduction (Mukherji et al., 2013). Circadian regulation within ileal and colonic epithelial cells was found to be completely disrupted in animals without a microbiota (Mukherji et al., 2013). The researchers theorized that cues are released from the microbiota in a continuous fashion, but toll-like receptors translate this information into rhythmic signals (Mukherji et al., 2013). Conversely, Leone et al. asserted that circadian shifts in bacterial composition result in corresponding shifts in concentration of bacterial metabolites such as butyrate, which peaked during fasting, and hydrogen sulfide, which peaked during feeding (Leone et al., 2015). Fecal butyrate was shown to cycle in mice on a standard, not high-fat, diet. Hydrogen sulfide exhibited cyclical behavior in the ceca of mice on a high-fat, but not normal, diet. It has been previously demonstrated *in vitro* that these metabolites can directly impact the cycling of hepatic clock genes *Per2* and *Bmal1* (Leone et al., 2015). Taken together, the negative consequences observed following circadian disruption may be

related to inflammatory processes, due to alterations in intestinal barrier function, increased abundances of proinflammatory bacteria, and etiologies of circadian-disruption-related diseases (R.M. Voigt et al., 2016).

There is emerging evidence that the circadian clock impacts eating time among humans (Thaiss et al., 2014). It has also been established that habitual diet and dietary alterations affect the GI microbial composition (Albenberg & Wu, 2014). Given these relationships, it is purported that changes to the clock and/or feeding time may impact the human gut microbiome. However, to date, there is only preliminary data on time-shift-induced microbiota dysbiosis in humans. It has been demonstrated in two subjects that jet lag induced by flying ahead eight time zones resulted in significant changes in microbial abundances, including a higher relative abundance of Firmicutes (Thaiss et al., 2014). Observed microbial changes in these jet-lagged individuals resolved within two weeks after landing (Thaiss et al., 2014). Fecal transplant from jet-lagged human subjects into germ-free mice resulted in weight gain that was 37% greater and peak blood glucose concentrations that were 35% higher during an oral glucose challenge compared to mice who received samples from the same individuals taken before jet lag occurred (Thaiss et al., 2014). Although the sample size is small in this study, the similarities to results from animal trials are promising.

TIME-RESTRICTED FEEDING

TRF is defined as a specific window of time during which a person or animal can consume as much food as desired, followed by a subsequent period where food access is denied (Chaix, Zarrinpar, Miu, & Panda, 2014). A common theory for the observed benefits of TRF is that it mimics natural eating patterns based on circadian rhythms, the way human ancestors ate

before artificial lighting and high-energy foods became available 24 hours a day (Fonken et al., 2010; Zarrinpar et al., 2016). TRF results in food being consumed during the light phase when the body is in the active state, and not consumed during the dark phase when the body is ready to rest and repair. Thus the body receives the energy it needs when it is metabolically expecting and prepared for it. There is evidence to suggest that there are protective effects on weight, blood lipids, and glucose homeostasis associated with eating only within a specific window of the day, e.g. TRF (Gill & Panda, 2015; Rothschild, Hoddy, Jambazian, & Varady, 2014).

Health Implications of Time-Restricted Feeding

Much of the research on TRF has been conducted in animal models, commonly through the use of diet-induced-obesity rodent models involving a high-fat diet intervention. When mice are given *ad libitum* access to normal chow, intake occurs almost entirely during the dark phase, and the mice consume an adequate amount of energy for their needs and maintain normal body weight (Friedman & Halaas, 1998; Kohsaka et al., 2007). However, when mice are given *ad libitum* access to high-fat chow, the tendency to eat only during the dark phase disappears (Hatori et al., 2012; Kohsaka et al., 2007; Zarrinpar et al., 2014). Contrary to their natural rhythm, the mice eat around the clock, which results in obesity and metabolic dysfunction. Introducing TRF in the context of a high-fat diet has been shown to reverse many detrimental metabolic consequences (Chaix et al., 2014; Hatori et al., 2012). Mice provided 8-hour access to a high-fat diet, for example, were protected against obesity, hyperinsulinemia, hepatic steatosis, and inflammation, despite consuming an equivalent number of calories from identical diets as the animals in the 24-hour access treatment arm (Hatori et al., 2012). TRF has also been linked to 26-62% lower fat mass, 60% lower postprandial glucose concentrations, and a 93% reduction in

insulin resistance in mice undergoing a six month TRF intervention compared to an *ad libitum* fed control (Chaix et al., 2014).

In humans, TRF has been shown to result in modest weight reductions of 1-3% in individuals that consistently consumed food only during a 10-12h feeding window, small to moderate (5-31%) improvements in plasma lipid measures (LDL, HDL, TG, and total cholesterol) when intake is restricted to 7-8h and 10-12h windows, and improved insulin sensitivity and fasting blood glucose concentrations across interventions ranging from 4 to 10-12h intake windows (Rothschild et al., 2014). Recently, a study in healthy, overweight adults demonstrated that decreasing eating window from approximately 14 hours per day to between 10 and 11 hours for a 16-week period resulted in an average weight loss of 3.27 kg (Gill & Panda, 2015). Clinical studies of TRF are summarized in Table 1.2 (Gill & Panda, 2015; Rothschild et al., 2014; Thaiss et al., 2014).

Impact of Time-Restricted Feeding on the Microbiota

In addition to the connection between food consumption and bacterial abundances, restricting the time of food access has also been shown to affect the GI microbial community structure in mice. In healthy mice fed a standard chow diet *ad libitum*, 17% of bacterial operational taxonomic units (OTUs) showed cyclical behavior, with 20-83% of bacterial sequences at a single point in time belonging to cyclical OTUs (Zarrinpar et al., 2014). Cyclical behavior among bacteria may be a result of microbial adaptation to the availability of food in the intestine at different points in the day (Thaiss et al., 2014). Firmicutes were found to peak during the normal murine feeding phase (e.g. dark), and Bacteroidetes peaked during the normal fasting phase (e.g. light) (Zarrinpar et al., 2014). Interestingly, a reduced Bacteroidetes/Firmicutes ratio

is associated with increased body weight and obesity in rodent models and among humans. Fecal transplants in gnotobiotic mice suggest that these perturbations increase energy harvest and weight gain (De Vos & De Vos, 2012). Another major phylum that displayed cyclical behavior was Verrucomicrobia, which followed the same pattern as Bacteroidetes of peaking in relative abundance during the fasting phase. Interestingly, this phylum contains the species *Akkermansia muciniphila*, which has been associated with positive health outcomes such as improved glucose homeostasis and decreased inflammation (Dao et al., 2016; Schneeberger et al., 2015; Shin et al., 2014).

Preclinical research demonstrates that feeding pattern alterations also disrupt the cyclical nature of OTUs. Mice fed a high-fat diet *ad libitum* alter their daytime fast/nighttime feed behaviors (Hatori et al., 2012; Kohsaka et al., 2007; Zarrinpar et al., 2014). Alongside this eating pattern degradation, these mice lose much of their normal OTU cycling (Zarrinpar et al., 2014). While TRF of a high-fat diet may be beneficial metabolically, it did not completely restore OTU cycling (Zarrinpar et al., 2014). This is of note as others have reported that microbiome alterations can persist for longer than dietary interventions and even longer than the metabolic consequences of dysbiosis (Thaiss, Itav, et al., 2016). TRF did, however, decrease the relative abundances of several presumed obesogenic microbes such as *Lactobacillus* and *Lactococcus* species, and increase the abundances of presumed obesity-protective bacteria such as *Oscillibacter* and other Ruminococcaceae species (Zarrinpar et al., 2014).

Mice lacking a circadian clock, through knockout of the *Per1* and *Per2* genes, given *ad libitum* food access eat irregularly and have lower alpha diversity (Thaiss et al., 2014). These circadian clock-absent mice also showed a distinct microbial community and lacked microbial cycling. When placed on a TRF regime, in either the light or dark phase, the cycling of the

microbiota was restored. Several bacteria, including *Bacteroides* and *Lactobacillus reuteri*, showed cycling in these *Per1, Per2* deficient animals under TRF, but the time of peaks and troughs was reversed in animals under TRF in the dark phase compared to TRF in the light phase. This further confirms the impact of feeding times on GI microbial composition within murine models.

To the best of our knowledge, there are no published studies on the effect of TRF on the human GI microbiota. Given the preclinical findings related to relationships between the microbiota and TRF, it is probable that TRF interventions in humans would impact the microbiota. However, additional TRF research is needed to determine if this behavioral modification impacts the human GI microbiome and overall health.

Health Implications of Eating Frequency

There is some observational evidence that differences in eating frequency are linked to varying health effects in human subjects (Table 1.3) (Chen, Wang, & Cheskin, 2016; Jenkins et al., 1989; Kant, 2014; Mekary, Giovannucci, Willett, van Dam, & Hu, 2012; Metzner, Lamphiear, Wheeler, & Larkin, 1977; Raynor, Goff, Poole, & Chen, 2015). Results from a study of nearly 2000 adults revealed an inverse relationship between adiposity and number of eating occasions after controlling for energy intake per kilogram of ideal body weight (Metzner et al., 1977). Furthermore, analyses of the 1988-1992 NHANES III cohort followed through 2006 revealed a lower hazard ratio (0.68) for cardiovascular disease-related mortality for those eating greater than 6 meals per day compared to eating four times per day (Chen et al., 2016). This association was stronger for those consuming greater than 2,500 calories per day. Another large epidemiological study (n=29,206) reported that men who skipped breakfast had a 21% higher

risk of diabetes, and men who ate one to two meals per day had a 25% higher risk compared to those that consumed three meals per day (Mekary et al., 2012).

Intervention trials focused on the impact of eating occasions on metabolic health are lacking. One small crossover study (n=7) investigated serum markers of metabolic health following two isocaloric interventions: 1) a snacking pattern consisting of 17 small meals eaten 1-hour apart and 2) a three meal pattern (Jenkins et al., 1989). The researchers reported that participants in the snacking pattern had 8-15% reductions in total cholesterol, LDL, apolipoprotein B, and serum insulin concentrations compared to the three meal pattern. A potential confounding aspect of this study was that the eating time window differed between the two treatments—during the three-meal condition, participants consumed their treatments during an 11-hour window while the hourly snacking pattern involved a 17-hour eating window. As discussed earlier, restricting food intake to a smaller time interval is associated with improvements in metabolic health. Intriguingly, the snacking pattern improved metabolic health despite the longer eating duration.

Raynor et al. conducted a systematic review of human and animal studies on eating frequency and weight status and concluded that the relationship between eating frequency and weight is unclear (Raynor et al., 2015). Over 60% of the studies reviewed found no effect of eating frequency on consumption/intake, body weight, or BMI. Inconsistent findings related to eating frequency and body weight may be due to reporting bias. It has been suggested that underreporting of food and eating occasions in adults with overweight and obesity may result in an erroneous connection between fewer meals and higher weight (Bellisle, McDevitt, & Prentice, 1997; Kant, 2014). A review by Kant of four prospective cohort studies and 12 controlled trials of eating frequency and body weight revealed mixed results among the cohort studies with one

reporting a benefit, two reporting a detriment, and one showing no effect; the majority of the randomized controlled trials found no relationship between eating frequency and weight loss. Only one study reported a significant difference (loss of 0.6kg) in subjects consuming one meal per day over eight weeks compared to three meals per day over eight weeks (gain of 0.8kg) (Kant, 2014).

The connection between the number of eating occasions and health is not fully understood. Although beneficial clinical results have been reported in the areas of adiposity, insulin, blood lipids, and risk of diabetes and cardiovascular-related death, effects on weight status are less clear. Plausible mechanisms underlying the inverse relationships between more frequent eating occasions and lower glucose, insulin, total cholesterol, and LDL cholesterol concentrations include a lower glycemic load as food is spread throughout the day, slower stomach emptying from smaller meals leading to a lower need for insulin, decreased insulin leading to decreased stimulation of enzymes for cholesterol synthesis, and increased LDL receptors because of the lower circulating cholesterol (Palmer, Capra, & Baines, 2009).

Impact of Eating Frequency on the Microbiota

Although there is considerable research on eating frequency and health, the impact of eating frequency on the GI microbiome has only recently been explored. In horses, the cecal microbiota is impacted by feeding frequency, with higher frequency being associated with increased relative abundance of the genus *YRC22* and decreased relative abundances of *Prevotella, Lactobacillus, Streptococcus, Coprococcus,* and *Phascolarctobacterium* (Venable et al., 2017). Additional research is necessary to determine if changes in eating patterns affect taxa associated with glucose response, lipid metabolism, and adiposity in humans.

Independent of eating frequency, certain bacterial taxa are associated with improved glucose, lipids, and adiposity. For example, *Akkermansia muciniphila*, a mucin-degrading intestinal bacterium has been associated with improved glucose homeostasis (Shin et al., 2014), and inversely correlated with inflammation in animal models (Schneeberger et al., 2015). In humans, an increased abundance of *Akkermansia* was found to inversely relate to fasting glucose, waist-to-hip ratio, and subcutaneous adipocyte diameter (Dao et al., 2016).

LIMITATIONS

This review is limited by the novelty of the field it explores. Recent advances in microbiome investigation technologies have only lately made this research possible. Additionally, performing circadian rhythm or food timing interventions on humans can be challenging. Self-reported behaviors are subject to bias and error, and in-house experiments are costly and difficult to execute. Furthermore, human studies involving eating pattern alterations, e.g. time-restricted feeding, must be carefully monitored to assess and control energy intake because humans generally decrease energy intake when given a reduced eating window, while animals will not.

Limitations of rodent models in microbiota research include the anatomy of the GI tract as well as coprophagy. Rodents are cecal fermenters, meaning most of the bacterial fermentation takes place in the cecum. In humans, most bacterial fermentation occurs in the large intestine. Additionally, the distribution of goblet (mucin-producing) cells is consistent throughout the human colon but decreases along the length of the mouse colon, which could affect the distribution of mucin-degrading bacteria (Nguyen et al., 2015). Furthermore, rodents and mice practice coprophagy, or consuming their fecal material as well as fecal material of other animals.

Lastly, rodent studies frequently examine the bacterial content of the cecum, in addition to fecal samples, while human studies employ fecal samples to characterize the microbiota.

In addition to differences in physiology, the use of undefined animal diets limits comparisons among rodent studies as well as translation to clinical populations. Chow varies in composition and sources of nutrients due to price and availability of ingredients while refined diets, such as the AIN diets, have defined nutrient composition. The high-fat diets utilized in animal research are infrequently representative of the proportion and composition of fat in human populations. High-fat chow typically contains up to 60% of total energy from fat with 24% of total energy from saturated fat. Alternatively, NHANES data from 2009-2012 show a mean intake of 33% total energy from fat and 10.6% of total energy from saturated fat among men and women over 20 years old (National Center for Health Statistics, 2016).

Despite these limitations, the murine model is still a powerful tool in microbiota research. Mice have a different core microbiome than humans, but many of the species are similar and in relatively similar abundances. Interventions also tend to show similar shifts in the microbiota of both mice and humans for many conditions, although it may take longer for diet to affect shifts in humans than in mice (Nguyen et al., 2015). Colonization of germ-free mice with fecal transplants from humans overcomes some of these challenges, but the cross-talk between host and symbiont is not identical. Overall, the mouse is a valuable model allowing isolation of variables that would be impossible in humans. While bearing in mind the drawbacks to the translation of results, we can still draw helpful conclusions from these studies.

FUTURE DIRECTIONS AND CONCLUSIONS

Disrupted circadian rhythm research suggests a reciprocal relationship between the microbiome and the internal clock. Animal evidence supporting the detrimental health effects of disrupting the normal circadian rhythm is robust (Arble et al., 2009; Thaiss et al., 2014; Zarrinpar et al., 2016). Human evidence is preliminary but promising. In both models, the microbiome has been suggested as a potential mediator between circadian misalignment and negative health consequences. Further work is needed with interventions rather than observational studies to establish a causal link between misaligned rhythms and the microbiome, investigating not only microbial composition but also the functional capacity of the microbiome and/or metabolomics.

Results from TRF studies indicate that restricting the time of food access may be protective against weight gain, insulin resistance, and dyslipidemia (Chaix et al., 2014; Hatori et al., 2012). These results also indicate that there is a connection between the microbiome and metabolic health as results demonstrate an absence of diet-induced obesity in germ-free mice and a lack of microbial cycling in mice without an internal clock. To date, evidence from human studies has not been robust, including small sample sizes and methodological limitations inherent to human subjects research, such as the inability to conduct germ-free experimentation.

There is extensive research on the connection between eating frequency and health, but almost no published research on the connection between eating frequency and the microbiome. Additional adequately-powered, well-controlled randomized trials investigating the impact of eating frequencies on the GI microbiome as a primary outcome are necessary to translate pre-clinical research findings to human populations. In general, additional well-designed, randomized controlled trials of eating behaviors such as circadian rhythm alignment, TRF, and eating frequency with the microbiome as a primary outcome will vastly increase our knowledge

and strengthen the evidence for using “when we eat” as a novel intervention to prevent or treat disease through GI microbiota manipulation.

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TABLES AND FIGURES

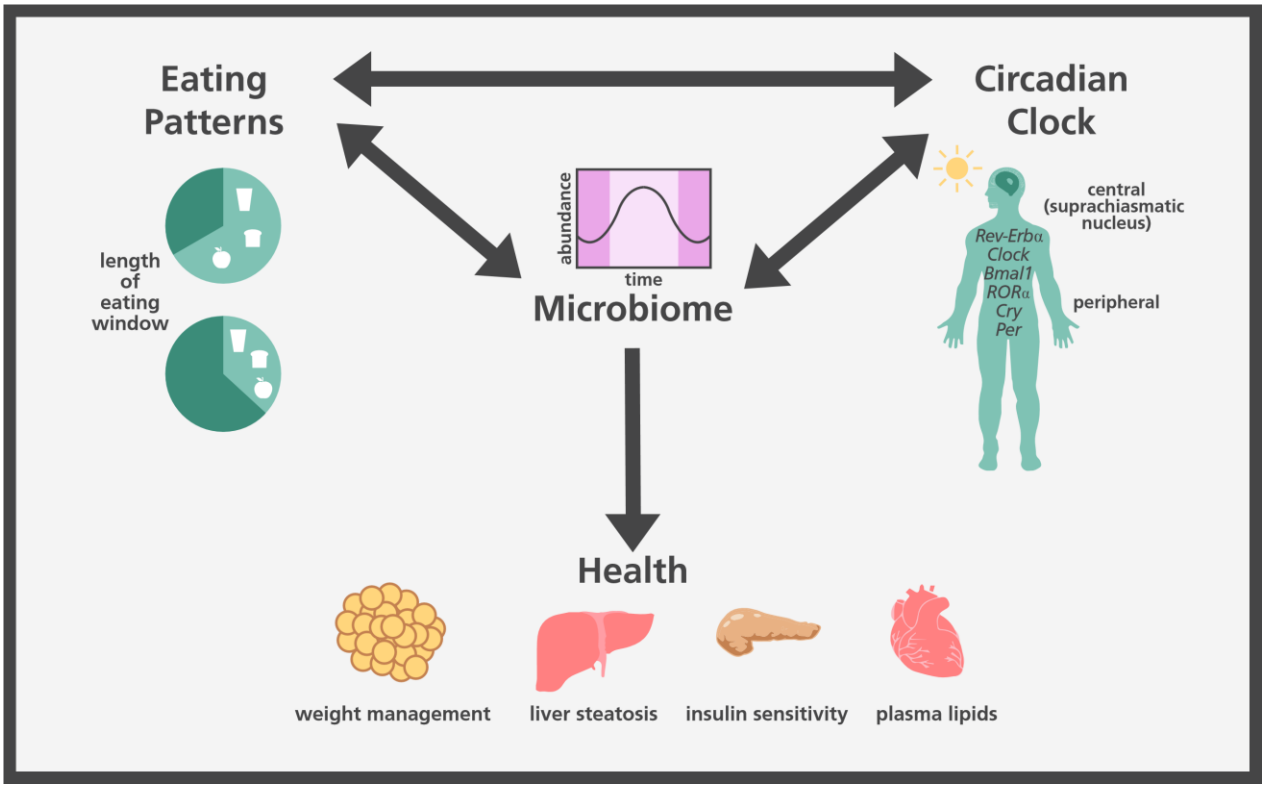

Figure 1.1: Connections between the internal clock, eating patterns, the microbiome, and health.

Table 1.1: Summary of Human Studies of Misaligned Circadian Rhythms

Relationship	Reference
SNPs of *Clock* gene → ↑ or ↓ risk of metabolic syndrome	Zarrinpar et al. (2016)
Shift work → ↑ risk of obesity and metabolic syndrome	Wang et al. (2014)
Shift work → ↓ daily energy expenditure	McHill et al. (2014)
Shift work → ↓ leptin ↑ insulin ↑ glucose ↑ mean arterial pressure	Scheer et al. (2009)
Sleep restriction → ↑ BMI, ↓ alertness, no change in microbiota	Zhang et al. (2017)
Jet lag → ↑ relative abundance of Firmicutes → ↑ weight gain and blood glucose in mice receiving transplants from these humans	Thaiss et al. (2014)

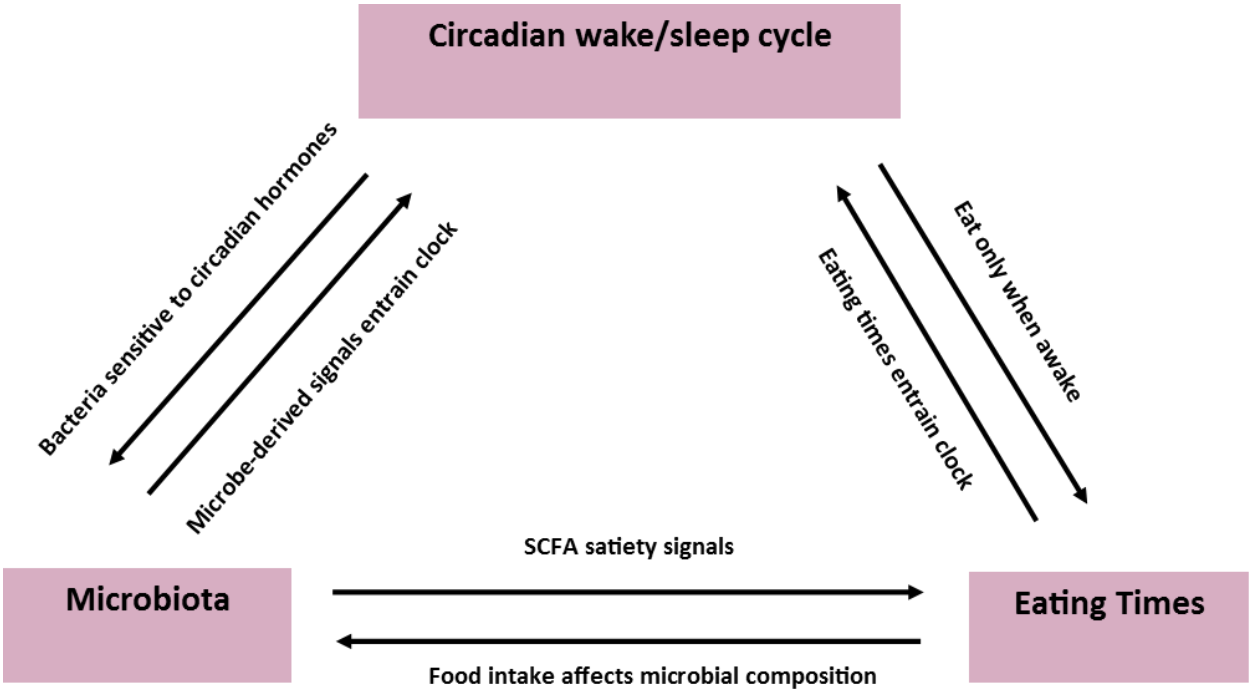

Figure 1.2: Integration of circadian homeostasis with eating patterns and the microbiota

Table 1.2: Summary of Human Studies of Time-Restricted Feeding

Relationship	Reference
4 hr feeding window → ↑ insulin sensitivity	Rothschild et al. (2014)
7-8 hr feeding window → ↑ insulin sensitivity ↑ HDL cholesterol ↓ LDL cholesterol ↓ triglycerides ↓ total cholesterol	Rothschild et al. (2014)
10-12 hr feeding window → ↓ weight ↑ insulin sensitivity ↑ HDL cholesterol ↓ LDL cholesterol ↓ triglycerides ↓ total cholesterol	Rothschild et al. (2014)
10-11 hr window vs. 14 hr window → ↓ weight	Gill et al. (2015)
Human microbiome displays cyclical behavior, likely as a result of feeding times	Thaiss et al. (2014)

Table 1.3: Summary of Human Studies of Eating Frequency

Relationship	Reference
↑ eating frequency → ↓ adiposity	Metzner et al. (1977)
> 6 meals/day vs. 4 meals/day → ↓ CVD mortality	Chen et al. (2016)
No breakfast → ↑ Type 2 Diabetes Mellitus risk	Mekary et al. (2012)
1-2 meals/day vs. 3 meals/day → ↑ Type 2 Diabetes Mellitus risk	Mekary et al. (2012)
17 meals/day vs. 3 meals/day → ↓ total cholesterol ↓ LDL cholesterol ↓ apolipoprotein B ↓ insulin	Jenkins et al. (1989)
1 meal/day vs. 3 meals/day → ↑ weight loss	Kant (2014)
Vast majority of weight maintenance research → no relationship	Raynor et al. (2015)

CHAPTER 2: TIME OF DAY AND EATING BEHAVIORS ARE ASSOCIATED WITH THE COMPOSITION AND FUNCTION OF THE HUMAN GASTROINTESTINAL MICROBIOTA[2]

ABSTRACT

Background: Preclinical research has demonstrated that the gastrointestinal (GI) microbiota exhibits circadian rhythms and that timing of food consumption can impact the composition and function of gut microbes. However, there is a dearth of knowledge on these relationships in humans.

Objective: We aimed to determine if human GI microbes and bacterial metabolites were associated with time of day or behavioral factors, including eating frequency, percent of energy consumed early in the day, and overnight fast duration.

Design: We analyzed 77 fecal samples collected from 28 healthy adult men and women. Fecal DNA was extracted and sequenced to determine the relative abundances of bacterial operational taxonomic units (OTUs). Gas chromatography mass spectroscopy was utilized to assess short-chain fatty acid concentrations. Eating frequency, percentage of energy consumed before 2 pm, and overnight fast duration were determined from dietary records. Data were analyzed by linear mixed models or generalized linear mixed models, which controlled for fiber intake, sex, age, BMI, and repeated sampling within each participant. Each OTU and metabolite was tested as the outcome in a separate model.

[2.] The final, definitive version of this paper has been published in the American Journal of Clinical Nutrition, 106, 5, November 2017 by the American Society for Nutrition. Free Access article. © 2017 The American Society for Nutrition. Kaczmarek JL, Musaad SMA, Holscher HD. Time of day and eating behaviors are associated with the composition and function of the human gastrointestinal microbiota. *Amer J Clin Nutr*. 2017;106(5):1220-1231. doi:10.3945/ajcn.117.156380.

Results: Acetate, propionate, and butyrate concentrations decreased throughout the day (*P=0.006, 0.04, 0.002,* respectively). Thirty-five percent of bacterial OTUs were associated with time. Additionally, relationships were observed between gut microbes and eating behaviors including eating frequency, early energy consumption, and overnight fast duration. Conclusions: These results indicate that the human GI microbiota composition and function varies throughout the day, which may be related to the circadian biology of the human body, the microbial community itself, or human eating behaviors. Behavioral factors, including timing of eating and overnight fast duration, were also predictive of bacterial abundances. Longitudinal intervention studies are needed to determine causality of these biological and behavioral relationships.

INTRODUCTION

The composition and function of the human gastrointestinal (GI) microbiota is increasingly linked to metabolic health (Albenberg & Wu, 2014; De Vos & De Vos, 2012), and there is keen interest in developing evidence-based strategies to modulate the GI microbiota for health benefit. Clinical research findings indicate that diet and consumption of fibers and prebiotics impact the gut microbiota (David et al., 2014; Hannah D. Holscher, 2017; Wu et al., 2011). Intriguingly, preclinical research suggests that GI microbes are influenced by circadian rhythms (Kaczmarek, Thompson, & Holscher, 2017; Kondo, 2007; Paulose et al., 2016). Circadian rhythms are cycles of gene expression, metabolism, and behaviors created by an internal clock to maximize an organism's metabolic efficiency (Rutter, Reick, & McKnight, 2002). The underlying transcriptional/translational feedback loops will proceed without

environmental input (e.g., pure biology), but they can also be affected by environmental factors such as light and food.

Cyclical variations in GI bacteria are likely related to both biological circadian factors, as shown in the absence of enteral feeding (Leone et al., 2015), and to eating behaviors that result in cyclical abundance of food in the intestines (Thaiss et al., 2014; Zarrinpar et al., 2014). Furthermore, there may be compounding effects of diet and circadian rhythms on the GI microbiota, gut barrier function, and health (Robin M. Voigt et al., 2014, 2016). However, at present, much of the literature is based on preclinical findings. Indeed, there is a dearth of knowledge on these relationships in humans.

Robust circadian rhythms can be developed by aligning phase and duration of feeding and fasting patterns with the environmental light-dark cycle. This entrainment means that peripheral clocks, which are affected by the presence of food, and the central clock, which is affected by the presence of light, are in sync. Importantly, synchronized circadian rhythms are associated with human health (Gill & Panda, 2015; F. Wang et al., 2014). Behavioral patterns, such as time-restricted feeding, and eating frequency may also have health benefits in humans—time restricted feeding positively affects body weight, blood lipids, and glucose homeostasis (Gill & Panda, 2015; Rothschild et al., 2014), and greater eating frequency may be associated with improvements in metabolic health, although conflicting evidence exists (Chen et al., 2016; Jenkins et al., 1989; Metzner et al., 1977). Consuming a larger proportion of energy early in the day compliments human circadian rhythms; for example, glucose tolerance and diet-induced thermogenesis are higher in the morning than evening (Jarrett, Baker, Keen, & Oakley, 1972; Romon, Edme, Boulenguez, Lescroart, & Frimat, 1993).

The aim of this study was to examine two closely intertwined elements: time, as it relates to biological circadian rhythms, and behavior, as it relates to time of eating, on the human GI microbiota composition and function. Given the rhythmic nature of the GI microbiota in preclinical studies, we hypothesized that human GI microbial abundances and metabolites vary throughout the day and are impacted by behavioral patterns of eating timing.

SUBJECTS AND METHODS

Participants

The study described herein was a secondary analysis of samples and data collected from the control period (0 g supplemental fiber) of a previously completed trial of agave inulin consumption in healthy adults (*n*=28; females = 14) (H. D. Holscher, Bauer, et al., 2015). The inclusion criteria for the primary study were that participants 1) be between the ages of 20 and 40 years; 2) have BMI > 18.5 kg/m^2 and < 29.5 kg/m^2; 3) have no current or historical metabolic or GI diseases; 4) avoid medications known to affect GI function; 5) had not taken antibiotics for at least the past 8 weeks; 6) limit alcohol consumption to $\leq$ 2 servings/d (e.g., < 28 g ethanol/d); 7) avoid taking prebiotics or probiotics; 8) consume a moderate fiber diet, consistent with US average of 12-19 g/d; 9) maintain consistent vitamin/mineral supplementation as at baseline; 10) maintain current level of physical activity; 11) record detailed dietary and stool information daily; and 12) meet with study personnel weekly. Female participants were excluded if they had menstrual cycles < 27 d or > 29 d in length, or if they were pregnant or lactating. Before study initiation, all participants voluntarily signed a written informed consent as approved by the University of Illinois Institutional Review Board. This study was conducted from January 2013 to May 2013 and was registered with Clinicaltrials.gov as NCT01925560.

Fecal samples

In the primary study protocol, participants consumed 0, 5.0, or 7.5 g agave inulin/d in a randomized order for 21 d with 7-d washouts between periods. They provided up to three fecal samples, each within 15 min of defecation, during days 16–20 of each of the three periods (maximum 9 total samples per participant). Herein, the fecal samples from the 0 g supplemental fiber control period (maximum 3 total fecal samples per participant) were utilized for this secondary analysis, which included 77 total fecal samples from 28 study participants. Additional analyses were conducted on the data from all three treatment periods (0, 5.0, or 7.5 g agave inulin/d) to determine if there was an interaction of time and fiber treatment on the composition and function of the gut microbiota; those results are available as Appendix A: Supplemental Tables. Fecal samples were transported to the laboratory with Commode Specimen Collection Systems (Sage Products, Cary, IL) on ice packs within coolers. Upon arrival, time of defecation was confirmed to be less than 15 minutes from delivery to the laboratory, and the time that the sample arrived for processing was recorded by a laboratory technician. Samples were manually homogenized, a pH measurement was taken (Denver Instrument, Bohemia, NY), and then samples were aliquoted for individual assays.

Short-chain fatty acids

The fecal aliquot for SCFAs (acetate, propionate, butyrate) was immediately acidified with 2N-HCl (10% wt:vol) and frozen at −20°C until analysis. A separate aliquot was utilized for dry matter measurement according to the methods of the Association of Official Analytical Chemists (1984) (Association of Official Analytical Chemists, 1984). Fecal SCFA concentrations were analyzed by gas chromatography mass spectroscopy as previously described and normalized on a dry matter basis (Vester Boler et al., 2011).

Microbial analysis

The samples for microbial analysis were flash-frozen in liquid nitrogen and stored at −80°C until analysis. Fecal bacterial DNA was extracted according to the manufacturer's instructions by using the PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). After extraction, the V4 region of the 16S bacterial rRNA gene was amplified using a Fluidigm Access Array system prior to high-throughput sequencing on an Illumina HiSeq. Sequencing was performed at the W. M. Keck Center for Biotechnology at the University of Illinois. High-quality (quality value > 25) data derived from the sequencing process were analyzed with QIIME 1.8.0 and 1.9.1 (Caporaso et al., 2010). Briefly, sequences were clustered into operational taxonomic units (OTUs) by using closed-reference OTU picking against the Greengenes 13_8 reference OTU database (97% similarity threshold). After quality filtering, alpha and beta diversity were calculated at an even sampling depth of 63,467 sequences per sample (Bokulich et al., 2012; Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011).

Dietary data

Study participants were educated on dietary recording methods by a registered dietitian prior to study initiation. Dietary intake was recorded in a 7-day diet record during the study, and participants met with dietetic interns weekly to review their dietary records and clarify ambiguities. Dietary record data were entered and analyzed using Nutrition Data Systems for Research software, 2015 edition (University of Minnesota, Minneapolis, MN). The number of eating occasions on the day before fecal sample collection was calculated by counting every food or beverage event reported by the participants in the 24 hour calendar day that contained at least 50 kcal (K. Murakami & Livingstone, 2015). Overnight fast duration was calculated as the time from the last kilocalorie consumption the previous night to the time of the first kilocalorie

consumption on the day of sample collection. Water and other non-caloric beverages were not considered as breaking the fast, but occasions of low energy (<50 kcal) consumption that would not be counted in the eating occasions calculation were considered here. Early energy consumption was calculated as the percent of total kilocalories consumed at or before 14:00 on the day before fecal sample collection. The time of 14:00 was chosen because it encompassed what would be considered "lunch" by almost all of the participants.

Statistics

Statistical analysis was performed in SAS 9.4 (SAS Institute, Inc., Cary, NC). A probability of $P \leq 0.05$ was accepted as statistically significant and was not adjusted for multiple testing since this is a preliminary study (Bender & Lange, 2001). In order to be as comprehensive as possible, genus-level OTUs that were present in at least 50% of the fecal samples were analyzed. Although the majority of preclinical literature in this field utilizes the JTK_cycle algorithm to detect cyclical features (Hughes, Hogenesch, & Kornacker, 2010), design differences between this clinical study and the preclinical studies necessitated different statistical methods. Firstly, these samples were only collected during the waking hours, and thus use of an algorithm that is designed for 24-hour data collection would not provide robust results. Secondly, the human GI microbiota has been shown to be impacted by age (Yatsunenko et al., 2012), sex (Dominianni et al., 2015), BMI (Turnbaugh et al., 2006, 2009), and dietary fiber intake (Hannah D. Holscher, 2017). This warranted statistical control of these variables within the models to allow for detection of associations beyond these variables. Before utilizing the statistical modeling described below, the methodology was confirmed to successfully replicate the majority of the findings from a preclinical circadian study of the murine cecal microbiota (Zarrinpar et al., 2014).

Mixed modeling was utilized to model normally (PROC MIXED) and non-normally (PROC GLIMMIX) distributed outcomes including person as a repeated effect. Each bacterial abundance and bacterial metabolite (SCFA) outcome was tested separately. SCFAs and bacterial genera distributions were examined in order to specify the best fitting model. Model fit was assessed using the ratio of the Chi-Square to its degrees of freedom. This ratio was used to assess residual variability that is not explained by the model (Schabenberger, 2005). Values <2 were deemed to indicate appropriate model fit (Hooper, Coughlan, & Mullen, 2008).

Three statistical models were utilized for data analysis. The first model was designed to examine the association of sample time (considered as a biological factor) with bacterial OTU relative abundances and SCFA concentrations. This model controlled for sex, age, BMI, and total dietary fiber per 1000 kcal and considered the within-subject correlation. The second model examined behavioral factors including eating frequency, overnight fast duration, and percent energy consumed before 14:00, while controlling for the same covariates as model one and accounting for the within-subject correlation. The third, full model included the biological variable (time) and the behavioral variables (eating frequency, overnight fast, and percent energy before 14:00) together as well as the covariates (sex, age, BMI, and fiber) and the within-subject correlation, to determine which factors contributed most strongly to existing relationships, and whether the inclusion of these factors in the same model would strengthen or weaken these relationships.

For the results presented, the estimate (beta coefficient) is the percent change in the predicted value of the outcome variable for each one-unit change in the predictor variable, if all the other predictors remain constant. It is important to note when interpreting these estimates that "one unit" is defined differently for each predictor. One unit of time or overnight fast duration is

one hour. One unit of eating frequency is one eating occasion. For energy consumption prior to 14:00, one unit is 1% of energy.

RESULTS

Fecal samples were collected between 7:32 A.M. and 10:00 P.M., with a mean time of 11:36 A.M. Additional descriptive data on the participant characteristics and the distributions of behavioral variables are presented in Table 2.1. In model 1, the biological model, there were significant relationships between time of day and relative abundance of bacteria, as well as time of day and bacterial metabolites (Table 2.2). Acetate, propionate, and butyrate concentrations decreased with clock time, e.g. the concentrations of these bacterial fermentative end products decreased throughout the day (Figure 2.1). Among microbes, the relative abundances of *Roseburia*, *Veillonella*, *Haemophilus*, and an unspecified genus within the S24-7 family decreased with clock time. Alternatively, the relative abundances of *Adlercreutzia*, *Eggerthella*, *Anaerotruncus*, *Oscillospira*, *Ruminococcus*, *Holdemania*, *Desulfovibrio*, *Escherichia*, and an unspecified genus within the Enterobacteriaceae family increased with clock time. Significant results are also presented in Figure 2.2. Collectively, the genera that were associated with time represent 24% of the OTUs examined for a total of 7% of the bacterial community composition.

In the behavioral model (Table 2.3), the relative abundance of *Coprobacillus* increased with greater eating frequency. The relative abundances of *Actinomyces*, *Eggerthella*, *Anaerotruncus*, *Dialister*, *Veillonella*, and unspecified genera within the Barnesiellaceae and Ruminococcaceae families decreased with greater eating frequency. *Oscillospira*, *Megamonas*, *Coprobacillus*, *Holdemania*, and an unspecified genus within the Erysipelotrichaceae family had higher relative abundance as a greater percentage of daily energy was consumed prior to 14:00. Alternatively, *Turicibacter*, *Coprococcus*, *Lachnospira*, *Roseburia*, *Veillonella*, and

Haemophilus had lower relative abundance as a greater percentage of energy was consumed prior to 14:00. *Turicibacter* decreased with longer overnight fast duration. There were no significant behavioral relationships observed with SCFAs in this model.

In the full model, which included both time and behavioral factors, most of the results from the preceding models remained, and new relationships emerged (Table 2.4). Specifically, new associations included an increase in the relative abundances of *Bifidobacterium*, *Butyricimonas*, *Sutterella*, *Bilophila*, and an unspecified genus within the Rikenellaceae family with clock time, and a reduction of the relative abundances of *Collinsella*, *Streptococcus*, and *Eubacterium* with clock time. *Roseburia* (P=0.17), the unspecified genus within S24-7 (P=0.07), and the unspecified Enterobacteriaceae genus (P=0.16) were no longer significantly related to clock time when eating behaviors were included in the model. Overall, in this full model, 35% of OTUs, or 12% of the total bacterial community composition, were associated with time.

Behavioral relationships were similar in the full model. All eating frequency relationships remained significant except the unspecified Barnesiellaceae genus, which had decreased with higher eating frequency in the behavioral model, only tended (P=0.05) to be related to eating frequency when time was included in the model. All early energy consumption relationships remained except that the positive relationship between *Roseburia* and greater energy consumed before 14:00 became a trend (P=0.06) when time was included in the model. New relationships emerged with overnight fast duration when time was also included in the model, whereby the relative abundance of *Coprococcus* increased with longer overnight fast duration, while *Holdemania* decreased with longer overnight fast duration. Finally, propionate was present at higher concentrations with longer overnight fast duration.

DISCUSSION

The relationships between the human GI microbiota and health and disease make it a promising target for lifestyle interventions. In parallel, research has demonstrated the importance of circadian rhythms to normal metabolic homeostasis (Hastings et al., 2003), and the presence of these rhythms in the bacterial community of the murine GI tract (Kaczmarek et al., 2017; Zarrinpar et al., 2014). Herein, we report for the first time a connection between time, eating behaviors, and human GI microbiota composition and function.

Our data reveal that the bacterial fermentative end-products, acetate, propionate, and butyrate, decrease over the course of the day. Butyrate and propionate have previously been shown to behave rhythmically in murine models (Leone et al., 2015). In our study, butyrate concentrations decreased by 2-6 μmol/g per hour, which represents a 5-12% decrease per hour. Leone et al. reported changes of 58 μmol/g per hour, which represents a change of 22% per hour in the ceca of mice. The larger effect in mice compared to humans may be related to sample collection and analysis methods. For example, the murine study utilized cecal contents immediately harvested from euthanized mice, while our study utilized fecal samples collected from humans within 15 minutes of passing and concentrations were reported on a dry matter basis.

We also report that certain members of the human GI microbiota changed with time. *Eggerthella*, *Anaerotruncus*, and *Desulfovibrio* have the largest positive estimates, increasing by more than 20% of their relative abundances every hour throughout the day. However, these are low abundance bacteria, together constituting less than 1% of the microbiota community. The two most abundant genera associated with time were *Roseburia* and *Ruminococcus*, which represent 2% and 3% of the bacterial community in our participants, respectively. Changes in

microbial abundances and metabolites are likely related. We reported a relationship between *Desulfovibrio* and time. Although *Desulfovibrio* has not previously been shown to cycle, hydrogen sulfide, a metabolite produced by *Desulfovibrio*, displayed cyclical fluctuations in mice (Leone et al., 2015). In addition, we report that *Roseburia* and *Eubacterium* decreased throughout the day. Both genera produce butyrate (Louis & Flint, 2009), and our results likewise revealed that butyrate concentration decreased throughout the day.

In one murine study, 17% of OTUs were cyclical, with 20-83% of reads at any one time belonging to OTUs that cycled (Zarrinpar et al., 2014). Genera found to cycle in murine models, which were also present at detectable levels in the human participants in our study, include *Bacteroides* (Liang, Bushman, & FitzGerald, 2015; Thaiss et al., 2014), *Lactococcus* (Leone et al., 2015; Zarrinpar et al., 2014), *Lactobacillus* (Thaiss et al., 2014; Zarrinpar et al., 2014), *Oscillospira* (Leone et al., 2015; Liang et al., 2015), *Ruminococcus* (Leone et al., 2015), S24-7 (Liang et al., 2015), *Turicibacter* (Liang et al., 2015), *Sutterella* (Liang et al., 2015), *Akkermansia* (Zarrinpar et al., 2014), and *Bifidobacterium* (Zarrinpar et al., 2014). Additionally, total bacterial load, number of mucosal-associated bacteria, and Firmicutes peak during feeding, while the other major phyla – Actinobacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia – peak during fasting in murine models (Liang et al., 2015; Thaiss, Levy, et al., 2016; Zarrinpar et al., 2014). In the only human microbiota-focused circadian study to date, which included two individuals, oscillations were found in 10% of OTUs, including *Parabacteroides*, *Lachnospira*, and *Bulleida* (Thaiss et al., 2014). Herein, we reported that up to 12% of sequences in the human GI microbiota belong to an OTU that was associated with time. Of these, *Bifidobacterium*, S24-7, *Oscillospira*, *Ruminococcus*, and *Sutterella* replicated preclinical findings (Leone et al., 2015; Liang et al., 2015; Zarrinpar et al., 2014). Similar to previous clinical findings, we reported that

Lachnospira tended (*P*=0.07) to be associated with time and was related to early energy consumption in our full model, which suggests that both biological and behavioral factors influence the cyclical behaviors of this microbe in the human GI tract. *Parabacteroides* and *Bulleida* were present in < 50% of participants, and thus were not assessed in the current study. Thus, our results that human GI bacteria fluctuate in abundance throughout the day are supported by changes in metabolite concentrations throughout the day, preclinical findings (Leone et al., 2015; Liang et al., 2015; Zarrinpar et al., 2014), and the results reported in a small (n=2) human study (Thaiss et al., 2014).

Associations between time and bacterial abundances may be related to specific bacterial traits, such as bile resistance. For example, *Oscillospira* and *Bilophila*, which increased throughout the day in our cohort, are bile tolerant, and thus may have a competitive advantage during waking hours, when more bile is secreted due to food ingestion (Devkota et al., 2012; Konikoff & Gophna, 2016). Alternatively, oscillations may be related to factors independent of the presence of food in the GI tract—the murine microbiota has shown circadian variation even when parenteral nutrition is the only source of nutrition (Leone et al., 2015). Other factors independent of food intake could include hormonal signals from the host. For example, *Enterobacter aerogenes* is impacted by melatonin, a circadian hormone (Paulose et al., 2016).

Few studies exist on behavioral elements of eating timing and the GI microbiota—one study in horses reported that increased meal frequency was associated with increased relative abundance of the genus *YRC22*, within the family Paraprevotellaceae, and decreased relative abundances of *Prevotella*, *Lactobacillus*, *Streptococcus*, *Coprococcus,* and *Phascolarctobacterium* (Venable et al., 2017). Although we also reported associations with eating frequency amongst phylogenetically diverse microbes, none of the microbes that were

associated with feeding frequency in equine cecum were the same as those in the human GI tract. With regard to overnight fast duration, we reported that propionate concentrations and *Coprococcus*, a microbe that produces propionate (Reichardt et al., 2014), increased with increasing overnight fast duration. Contrary to previous literature, *Akkermansia* and overnight fast duration were not related (Remely et al., 2015; Sonoyama et al., 2009). Two possible explanations for this discrepancy include, 1) as a mucosa-associated genus, *Akkermansia* measurements in stool (humans) may vary from that in the cecum (murine) (Reunanen et al., 2015; Vandeputte et al., 2016), and 2) the type and/or amount of fiber in the diet of the study participants may have been adequate to keep the abundance of *Akkermansia* stable throughout the study (Desai et al., 2016).

This study was limited by the fact that it was secondary data analysis and relied on self-report dietary records for behavioral factors. Intervention studies that modify biological and behavioral factors and assess the GI microbiota as a primary outcome are necessary to determine causality. As with any observational study, we must also consider the possibility that the directionality of the associations is reversed. For example, microbes may signal via the gut-brain axis and influence appetite in a way that drives certain eating behaviors, rather than the behaviors themselves impacting the microbial composition. Indeed, microbial metabolites have been shown to impact hyperphagia in rodents (Frost et al., 2014; Perry et al., 2016), and propionate reduced appetite in overweight adults (Chambers et al., 2015). Furthermore, our data cover half of the circadian cycle, specifically the awake/feeding phase. Assessments over a 24-hour period are needed to establish that human GI microbes and metabolites that increase during the day demonstrate circadian rhythms, e.g. correspondingly decreasing at night.

Despite some limitations, the study has several strengths. It is the first of its kind to examine the biological and behavioral influences of time on the human GI microbiota, and many of our findings replicate and extend those reported in preclinical studies. We utilized a robust statistical model that controlled for many of the factors known to be associated with the GI microbiota (e.g. age (Yatsunenko et al., 2012), BMI (Turnbaugh et al., 2006), sex (Dominianni et al., 2015), and dietary fiber intake (Hannah D. Holscher, 2017).) Through the use of models that independently assessed biology and behavior, followed by a combination of both time and behavioral factors, we are able to discern how the variables may be interrelated. Interestingly, several bacteria associated with time were also affected by timing of eating, underscoring the potential relevance of how eating behavior may modulate circadian variation in the human GI microbiome.

The relationships reported between the human gut microbiota and time of day highlights several important points in the expanding area of microbiome research. Firstly, associations between time and human GI microbiota are modest compared to preclinical studies. However, as these relationships may be of relevance, time of defecation should be recorded, and considered as a potential covariate in analyses. Although this study did not examine health outcomes, the connections between the GI microbiota and host health are too well documented to ignore. Thus, circadian variation within the microbiome, and the potential for eating behaviors to modify this variation, should be further studied as an avenue for health interventions. Future directions include the need for adequately powered, randomized controlled trials of timing interventions with the GI microbiota as a primary outcome. These trials should employ interventions of various eating window lengths, and with varying eating frequencies, preferably with participants serving as their own controls to minimize inter-individual variation.

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TABLES AND FIGURES

Table 2.1: Descriptive Characteristics of Sample

Variable	Mean	Median	SD	Range
Age, y	28	27	4	21–36
BMI, kg/m^2	24.3	24.3	2.2	20.2–28.9
Sample clock time (24 h)	1136	1035	0307	0732–2200
Eating frequency, number of occasions	4.6	4.0	1.5	2–10
Early energy consumption (before 1400), %	47.4	46.7	14.7	9.6–84.0
Overnight-fast duration, h	11.8	11.5	2.4	7.0–21.0

[1] n = 28 participants (14 women) and 77 fecal samples.

Table 2.2: Associations of Metabolite Concentrations and Bacterial Operational Taxonomic Unit Relative Abundances with Time[1]

			Biological model, time (hours)		
	Participants, *n*	Fecal samples, *n*	Estimate ± SEE	95% CI	*P*
Short-chain fatty acids, μmol/g					
Acetate	23	63	−5.4 ± 1.88	−9.08, −1.72	<0.01*
Propionate	23	63	−4.61 ± 2.19	−8.9, −0.32	0.04*
Butyrate	23	63	−7.28 ± 2.27	−11.73, −2.83	<0.01*
Archaea, % of sequences					
Methanobrevibacter	23	64	9.96 ± 6.37	−2.53, 22.45	0.12
Actinobacteria, % of sequences					
Actinomyces	23	64	1.31 ± 4.98	−8.45, 11.07	0.79
Bifidobacterium	23	64	7.39 ± 4.65	−1.72, 16.5	0.12
Coriobacteriaceae unspecified genus	23	64	−9.01 ± 6.12	−21.01, 2.99	0.15
Adlercreutzia	23	64	12.01 ± 5	2.21, 21.81	0.02*
Collinsella	23	64	−6.06 ± 4.93	−15.72, 3.6	0.22
Eggerthella	23	64	27.95 ± 5.41	17.35, 38.55	<0.01*
Bacteroidetes, % of sequences					
Bacteroides	23	64	2.48 ± 1.27	−0.01, 4.97	0.06
Parabacteroides	23	64	0.27 ± 2.94	−5.49, 6.03	0.93
Rikenellaceae unspecified genus	23	64	2.68 ± 3.35	−3.89, 9.25	0.43
S24-7 unspecified genus	23	64	−19.29 ± 8.48	−35.91, −2.67	0.03*
Barnesiellaceae unspecified genus	23	64	6.13 ± 7.25	−8.08, 20.34	0.40
Butyricimonas	23	42	8.59 ± 4.62	−0.47, 17.65	0.07
Odoribacter	23	64	−4.84 ± 3.26	−11.23, 1.55	0.14
Firmicutes, % of sequences					
Granulicatella	23	64	0.32 ± 5.34	−10.15, 10.79	0.95
Lactococcus	23	64	−5.8 ± 11.35	−28.05, 16.45	0.61
Streptococcus	23	64	−4.08 ± 4.66	−13.21, 5.05	0.38
Turicibacter	23	64	0.3 ± 8.41	−16.18, 16.78	0.97
Clostridiales unspecified genus	23	64	0.26 ± 2.32	−4.29, 4.81	0.91
Christensenellaceae unspecified genus	23	64	−0.99 ± 6.33	−13.4, 11.42	0.88
Clostridiaceae unspecified genus	23	64	0.97 ± 4.15	−7.16, 9.1	0.82
Clostridium	23	63	−2.97 ± 3.12	−9.09, 3.15	0.34
Lachnospiraceae unspecified genus	23	64	0.34 ± 1.3	−2.21, 2.89	0.79
Anaerostipes	23	64	0.85 ± 3.69	−6.38, 8.08	0.82
Blautia	23	64	3.92 ± 2.33	−0.65, 8.49	0.10
Coprococcus	23	64	−2.01 ± 3.37	−8.62, 4.6	0.55
Dorea	23	64	6.53 ± 3.27	0.12, 12.94	0.05
Lachnobacterium	23	64	12.52 ± 10.46	−7.98, 33.02	0.24
Lachnospira	23	64	2.64 ± 4.11	−5.42, 10.7	0.52
Roseburia	23	64	−7.03 ± 3.39	−13.67, −0.39	0.04*
Ruminococcaceae unspecified genus	23	64	−2.05 ± 1.67	−5.32, 1.22	0.23
Anaerotruncus	23	64	23.52 ± 4.06	15.56, 31.48	<0.01*
Faecalibacterium	23	64	0.39 ± 2.17	−3.86, 4.64	0.86
Oscillospira	23	64	6.38 ± 2.07	2.32, 10.44	<0.01*
Ruminococcus	23	64	6.05 ± 2.83	0.5, 11.6	0.04*
Acidaminococcus	23	34	−18.75 ± 25.76	−69.24, 31.74	0.47
Dialister	23	64	−0.04 ± 6.35	−12.49, 12.41	1.00
Megamonas	23	41	0.68 ± 22.78	−43.97, 45.33	0.98
Megasphaera	23	42	12.64 ± 20.03	−26.62, 51.9	0.53
Phascolarctobacterium	23	64	−0.03 ± 4.48	−8.81, 8.75	0.99
Veillonella	23	64	−25.38 ± 9.84	−44.67, −6.09	0.01*
Mogibacteriaceae unspecified genus	23	64	1.54 ± 2.98	−4.3, 7.38	0.61
Erysipelotrichaceae unspecified genus	23	64	7.71 ± 4.42	−0.95, 16.37	0.09
Coprobacillus	23	64	2.45 ± 6.8	−10.88, 15.78	0.72
Holdemania	23	64	9.39 ± 3.21	3.1, 15.68	<0.01*
Eubacterium	23	61	−7.48 ± 5.25	−17.77, 2.81	0.16
Proteobacteria, % of sequences					
Sutterella	23	64	3.16 ± 1.76	−0.29, 6.61	0.08
Bilophila	23	64	3.26 ± 3.54	−3.68, 10.2	0.36
Desulfovibrio	23	64	20.33 ± 8.14	4.38, 36.28	0.02*
Enterobacteriaceae unspecified genus	23	64	15.16 ± 7.2	1.05, 29.27	0.04*

Table 2.2 (continued) [1]

			Biological model, time (hours)		
	Participants, *n*	Fecal samples, *n*	Estimate ± SEE	95% CI	*P*
Escherichia	23	64	17.74 ± 6.16	5.67, 29.81	<0.01*
Haemophilus	23	64	−19.76 ± 9.44	−38.26, −1.26	0.04*
Verrucomicrobia, % of sequences					
Akkermansia	23	64	12.8 ± 7.36	−1.63, 27.23	0.09

[1] Results of linear mixed-model analysis were adjusted for repeated sampling, age, BMI, sex, and normalized total fiber intake. Estimates represent the percentage change in the predicted value of the outcome variable for each 1-unit change in time (hours) if all of the other predictors remain constant. This analysis represents a linear relation between microbes or metabolites and time during the awake/feeding phase of the circadian cycle. A negative estimate indicates that the highest values were seen earlier and decreased throughout the day. A positive estimate indicates that the values increased throughout the day and were highest later. *Significant (P <0.05). OTU, operational taxonomic unit.

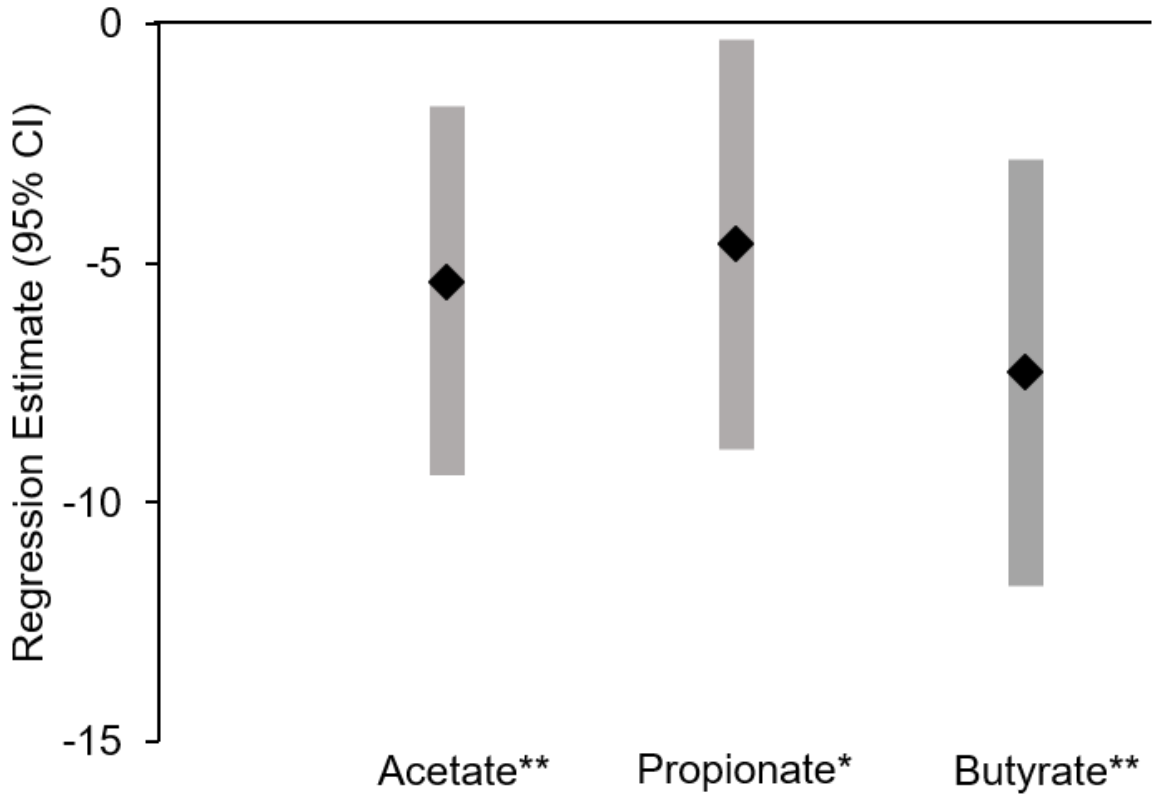

Figure 2.1: Associations of short-chain fatty acids with time in the biological model. Results of linear mixed model analysis adjusted for age, BMI, sex, normalized total fiber intake, and repeated sampling. Estimate represents the percent change in the predicted value of the outcome variable for each one-unit change in time (hours), if all the other predictors remain constant. Black box indicates the estimate; gray area indicates the 95% confidence interval. A negative estimate indicates that the highest concentrations were seen earlier, and decreased throughout the day. * <0.05, ** <0.01; BMI, body mass index.

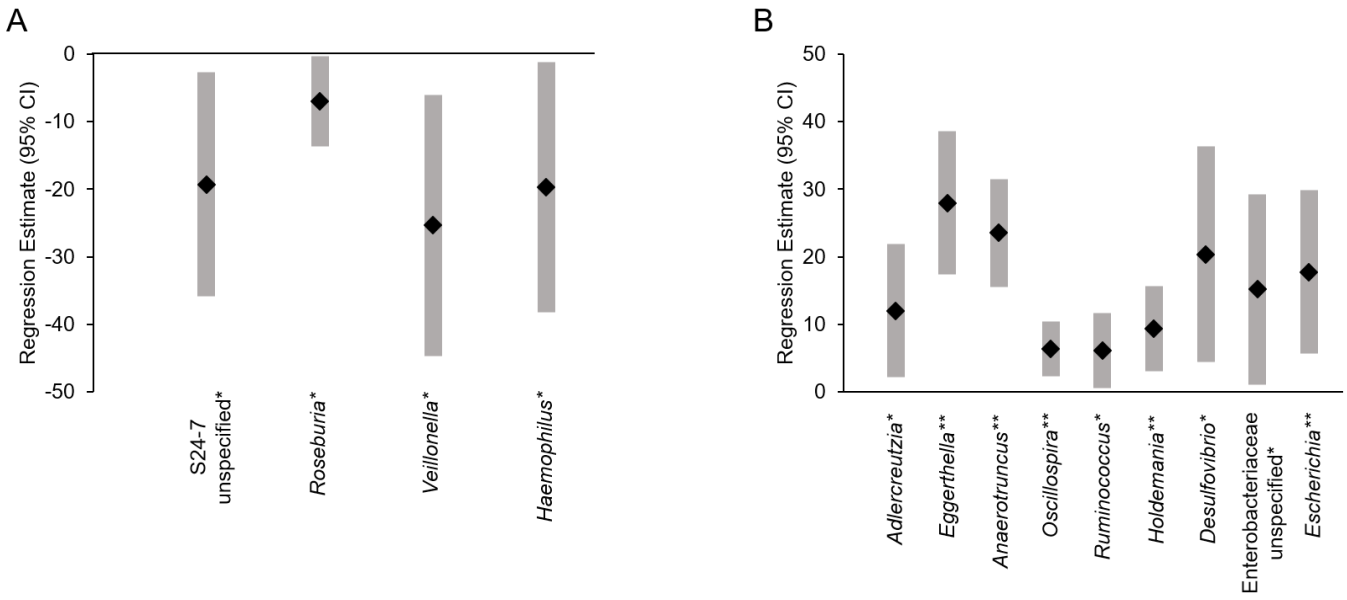

Figure 2.2: Associations of bacterial operational taxonomic units with time in the biological model. Results of linear mixed model analysis adjusted for age, BMI, sex, normalized total fiber intake, and repeated sampling. Estimate represents the percent change in the predicted value of the outcome variable for each one-unit change in time (hours), if all the other predictors remain constant. Black box indicates the estimate; gray area indicates the 95% confidence interval. A negative estimate indicates that the highest relative abundances of the bacteria were seen earlier, and decreased throughout the day. A positive estimate indicates that the relative abundances of the respective bacteria increased throughout the day, and were highest later. A) OTUs that significantly decreased throughout the day. B) OTUs that significantly increased throughout the day. * <0.05, ** <0.01; BMI, body mass index; OTU, operational taxonomic unit.

Table 2.3: Associations of Metabolite Concentrations and Bacterial Operational Taxonomic Unit Relative Abundances with Behavioral Patterns[1]

			Behavioral model									
			Eating frequency (occasions)			Early energy consumption (% of energy)			Overnight-fast duration (hours)			
	Participants, *n*	Samples, *n*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	›
Short-chain fatty acids, μmol/g												
Acetate	22	58	2.94 ± 4.92	−6.7, 12.58	0.55	−0.71 ± 0.46	−1.61, 0.19	0.13	2.09 ± 3.08	−3.95, 8.13	0.50	0
Propionate	22	58	4.37 ± 5.68	−6.76, 15.5	0.45	−0.1 ± 0.53	−1.14, 0.94	0.86	4.25 ± 3.56	−2.73, 11.23	0.24	4
Butyrate	22	58	1 ± 6.22	−11.19, 13.19	0.87	−0.78 ± 0.58	−1.92, 0.36	0.18	−0.28 ± 3.9	−7.92, 7.36	0.94	4
Archaea, % of sequences												
Methanobrevibacter	22	59	1.45 ± 15.69	−29.3, 32.2	0.93	−1.81 ± 1.48	−4.71, 1.09	0.23	11.39 ± 9.15	−6.54, 29.32	0.22	2
Actinobacteria, % of sequences												
Actinomyces	22	59	−32.17 ± 14.52	−60.63, −3.71	0.03*	0.62 ± 1.05	−1.44, 2.68	0.56	−1.94 ± 6.5	−14.68, 10.8	0.77	7
Bifidobacterium	22	59	−10.77 ± 11.96	−34.21, 12.67	0.37	2.05 ± 1.11	−0.13, 4.23	0.07	−0.18 ± 7.52	−14.92, 14.56	0.98	8
Coriobacteriaceae unspecified genus	22	59	15.32 ± 10.6	−5.46, 36.1	0.15	0.98 ± 1	−0.98, 2.94	0.33	−13.86 ± 8.82	−31.15, 3.43	0.12	2
Adlercreutzia	22	59	4.32 ± 14.9	−24.88, 33.52	0.77	0.87 ± 1.52	−2.11, 3.85	0.57	−8.89 ± 10.54	−29.55, 11.77	0.40	0
Collinsella	22	59	8.69 ± 13.87	−18.5, 35.88	0.53	−0.37 ± 0.98	−2.29, 1.55	0.71	5.68 ± 7.76	−9.53, 20.89	0.47	7
Eggerthella	22	59	−64.92 ± 21.53	−107.12, −22.72	<0.01*	0.42 ± 1.5	−2.52, 3.36	0.78	−0.89 ± 7.01	−14.63, 12.85	0.90	0
Bacteroidetes, % of sequences												
Bacteroides	22	59	−2.52 ± 3.42	−9.22, 4.18	0.46	−0.13 ± 0.22	−0.56, 0.3	0.55	1.84 ± 1.35	−0.81, 4.49	0.18	8
Parabacteroides	22	59	0.64 ± 7.99	−15.02, 16.3	0.94	−0.56 ± 0.52	−1.58, 0.46	0.29	2.69 ± 3.32	−3.82, 9.2	0.42	2
Rikenellaceae unspecified genus	22	59	−10.9 ± 7.26	−25.13, 3.33	0.14	−0.2 ± 0.68	−1.53, 1.13	0.77	1.02 ± 4.56	−7.92, 9.96	0.82	2
S24-7 unspecified genus	22	59	−0.74 ± 24.64	−49.03, 47.55	0.98	−2.55 ± 2.34	−7.14, 2.04	0.28	8.27 ± 14.04	−19.25, 35.79	0.56	6
Barnesiellaceae unspecified genus	22	59	−54.6 ± 26.15	−105.85, −3.35	0.04*	0.95 ± 1.6	−2.19, 4.09	0.56	−10.81 ± 10.83	−32.04, 10.42	0.32	2
Butyricimonas	22	38	−20.09 ± 11.4	−42.43, 2.25	0.09	0.68 ± 1.06	−1.4, 2.76	0.53	−3.06 ± 7.19	−17.15, 11.03	0.67	7
Odoribacter	22	59	0.33 ± 7.95	−15.25, 15.91	0.97	−0.17 ± 0.75	−1.64, 1.3	0.83	−3.51 ± 5.18	−13.66, 6.64	0.50	0
Firmicutes, % of sequences												
Granulicatella	22	59	9.39 ± 11.9	−13.93, 32.71	0.43	1.03 ± 1.22	−1.36, 3.42	0.41	2.65 ± 7.38	−11.81, 17.11	0.72	2
Lactococcus	22	59	−0.53 ± 22.97	−45.55, 44.49	0.98	−3.48 ± 2.32	−8.03, 1.07	0.14	8.5 ± 14.11	−19.16, 36.16	0.55	5
Streptococcus	22	59	−14.07 ± 11.4	−36.41, 8.27	0.22	0.09 ± 1.06	−1.99, 2.17	0.93	−0.64 ± 7.17	−14.69, 13.41	0.93	3
Turicibacter	22	59	−31.48 ± 20.02	−70.72, 7.76	0.12	−3.11 ± 1.31	−5.68, −0.54	0.02*	−29.28 ± 14.16	−57.03, −1.53	0.04*	4*
Clostridiales unspecified genus	22	59	0.74 ± 5.92	−10.86, 12.34	0.90	0.12 ± 0.55	−0.96, 1.2	0.83	−0.07 ± 3.72	−7.36, 7.22	0.98	8
Christensenellaceae unspecified genus	22	59	0.15 ± 12.87	−25.08, 25.38	0.99	−0.51 ± 1.54	−3.53, 2.51	0.74	−9.54 ± 9.82	−28.79, 9.71	0.34	4
Clostridiaceae unspecified genus	22	59	4.35 ± 10.34	−15.92, 24.62	0.68	0.49 ± 0.96	−1.39, 2.37	0.61	−6.44 ± 6.5	−19.18, 6.3	0.33	3
Clostridium	22	58	12.43 ± 7.97	−3.19, 28.05	0.13	−1.38 ± 0.74	−2.83, 0.07	0.07	3.84 ± 5.03	−6.02, 13.7	0.45	5
Lachnospiraceae unspecified genus	22	59	0.56 ± 3.37	−6.05, 7.17	0.87	0.31 ± 0.22	−0.12, 0.74	0.16	−2.52 ± 1.39	−5.24, 0.2	0.08	8
Anaerostipes	22	59	−14.62 ± 9.13	−32.51, 3.27	0.12	0.47 ± 0.85	−1.2, 2.14	0.59	−4.45 ± 5.74	−15.7, 6.8	0.44	4
Blautia	22	59	−8.45 ± 5.5	−19.23, 2.33	0.13	0.2 ± 0.51	−0.8, 1.2	0.70	−0.05 ± 3.46	−6.83, 6.73	0.99	9
Coprococcus	22	59	0.02 ± 7.57	−14.82, 14.86	1.00	−1.69 ± 0.71	−3.08, −0.3	0.02*	8.99 ± 4.76	−0.34, 18.32	0.06	6
Dorea	22	59	2.82 ± 8.26	−13.37, 19.01	0.73	0.64 ± 0.77	−0.87, 2.15	0.41	1.38 ± 5.2	−8.81, 11.57	0.79	9
Lachnobacterium	22	59	−52.67 ± 30.19	−111.84, 6.5	0.09	−2.29 ± 2.2	−6.6, 2.02	0.30	−6.49 ± 13.74	−33.42, 20.44	0.64	4
Lachnospira	22	59	6.89 ± 9.88	−12.47, 26.25	0.49	−1.94 ± 0.92	−3.74, −0.14	0.04*	2.23 ± 6.22	−9.96, 14.42	0.72	2
Roseburia	22	59	12.09 ± 8.27	−4.12, 28.3	0.15	−1.55 ± 0.77	−3.06, −0.04	<0.05*	−3.7 ± 5.2	−13.89, 6.49	0.48	8
Ruminococcaceae unspecified genus	22	59	−8.42 ± 4	−16.26, −0.58	0.04*	−0.13 ± 0.37	−0.86, 0.6	0.73	−3.72 ± 2.52	−8.66, 1.22	0.15	5
Anaerotruncus	22	59	−44.25 ± 16.05	−75.71, −12.79	<0.01*	2.1 ± 1.07	0, 4.2	0.05	0.86 ± 5.96	−10.82, 12.54	0.89	9
Faecalibacterium	22	59	0.85 ± 4.81	−8.58, 10.28	0.86	−0.22 ± 0.45	−1.1, 0.66	0.63	−1.31 ± 3.02	−7.23, 4.61	0.67	7

Table 2.3 (continued)

			Behavioral model								
			Eating frequency (occasions)			Early energy consumption (% of energy)			Overnight-fast duration (hours)		
	Participants, *n*	Samples, *n*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*
Oscillospira	22	59	−3.04 ± 5.01	−12.86, 6.78	0.55	1.59 ± 0.47	0.67, 2.51	<0.01*	−3.4 ± 3.15	−9.57, 2.77	0.29
Ruminococcus	22	59	0.56 ± 7.89	−14.9, 16.02	0.94	−0.18 ± 0.74	−1.63, 1.27	0.81	1.86 ± 4.96	−7.86, 11.58	0.71
Acidaminococcus	22	31	27.44 ± 75.57	−120.68, 175.56	0.72	5.69 ± 6.28	−6.62, 18	0.37	−32.8 ± 34.34	−100.11, 34.51	0.35
Dialister	22	59	−34.97 ± 15.83	−66, −3.94	0.03*	−1.92 ± 1.16	−4.19, 0.35	0.10	−3.55 ± 8.33	−19.88, 12.78	0.67
Megamonas	22	37	78.85 ± 47.3	−13.86, 171.56	0.11	14.88 ± 4.47	6.12, 23.64	<0.01*	8.58 ± 31.45	−53.06, 70.22	0.79
Megasphaera	22	39	−3.8 ± 52.98	−107.64, 100.04	0.94	1.69 ± 4.8	−7.72, 11.1	0.73	−3.37 ± 27.7	−57.66, 50.92	0.90
Phascolarctobacterium	22	59	14.19 ± 10.29	−5.98, 34.36	0.17	0.62 ± 1.06	−1.46, 2.7	0.56	6.42 ± 6.59	−6.5, 19.34	0.33
Veillonella	22	59	−51.72 ± 20.89	−92.66, −10.78	0.02*	−6.61 ± 1.61	−9.77, −3.45	<0.01*	−3.19 ± 10.75	−24.26, 17.88	0.77
Mogibacteriaceae unspecified genus	22	59	−3.66 ± 7.57	−18.5, 11.18	0.63	−0.2 ± 0.66	−1.49, 1.09	0.77	4.61 ± 4.43	−4.07, 13.29	0.30
Erysipelotrichaceae unspecified genus	22	59	−8.46 ± 10.64	−29.31, 12.39	0.43	2.6 ± 0.99	0.66, 4.54	0.01*	−8.34 ± 6.69	−21.45, 4.77	0.22
Coprobacillus	22	59	24.69 ± 12.18	0.82, 48.56	<0.05*	3.15 ± 1.49	0.23, 6.07	0.04*	−7.91 ± 10.47	−28.43, 12.61	0.45
Holdemania	22	59	−11.69 ± 8.52	−28.39, 5.01	0.18	1.78 ± 0.76	0.29, 3.27	0.02*	−9.28 ± 5.26	−19.59, 1.03	0.08
Eubacterium	22	57	6.72 ± 13.45	−19.64, 33.08	0.62	0.06 ± 1.24	−2.37, 2.49	0.96	3.65 ± 8.41	−12.83, 20.13	0.67
Proteobacteria, % of sequences											
Sutterella	22	59	−5.23 ± 4.89	−14.81, 4.35	0.29	0.12 ± 0.32	−0.51, 0.75	0.72	−1.34 ± 2.06	−5.38, 2.7	0.52
Bilophila	22	59	12.55 ± 7.84	−2.82, 27.92	0.12	1.19 ± 0.81	−0.4, 2.78	0.15	−6.14 ± 5.89	−17.68, 5.4	0.30
Desulfovibrio	22	59	19.52 ± 20.3	−20.27, 59.31	0.34	3.35 ± 1.84	−0.26, 6.96	0.08	−14.17 ± 14.33	−42.26, 13.92	0.33
Enterobacteriaceae unspecified genus	22	59	12.38 ± 15.95	−18.88, 43.64	0.44	0.23 ± 1.74	−3.18, 3.64	0.90	17.46 ± 8.7	0.41, 34.51	0.05
Escherichia	22	59	15.24 ± 14.98	−14.12, 44.6	0.31	0.87 ± 1.56	−2.19, 3.93	0.58	13.78 ± 7.67	−1.25, 28.81	0.08
Haemophilus	22	59	−32.97 ± 20.98	−74.09, 8.15	0.12	−4.16 ± 1.41	−6.92, −1.4	<0.01*	−28.35 ± 14.55	−56.87, 0.17	0.06
Verrucomicrobia, % of sequences											
Akkermansia	22	59	2.4 ± 18.23	−33.33, 38.13	0.90	0.6 ± 1.85	−3.03, 4.23	0.75	0.42 ± 11.79	−22.69, 23.53	0.97

[1] Results of linear mixed-model analysis were adjusted for repeated sampling, age, BMI, sex, and normalized total fiber intake. Estimates represent the percentage of change in the predicted value of the outcome variable for each 1-unit change in the predictor variable if all of the other predictors remain constant. One unit of eating frequency is 1 eating occasion. One unit of energy consumption is 1% of daily energy intake. One unit of overnight-fast duration is 1 h. This analysis represents a linear relationship between microbes or metabolites and behaviors during the awake/feeding phase of the circadian cycle. A negative estimate indicates an inverse relationship between the outcome and the predictor (e.g., relative abundance of the bacterium was higher when eating frequency was lower). A positive estimate indicates a positive relationship between the outcome and predictor (e.g., relative abundance of the bacterium was higher when eating frequency was higher). *Significant (P <0.05). OTU, operational taxonomic unit.

Table 2.4: Associations of Metabolite Concentrations and Bacterial Operational Taxonomic Unit Relative Abundances with Time and Behavioral Patterns in the Full Model[1]

			Full model											
			Time (hours)			Eating frequency (occasions)			Early energy consumption (% of energy)			Overnight-fast duration (hours)		
	Participants, *n*	Samples, *n*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*
Short-chain fatty acids, μmol/g														
Acetate	22	58	−8.35 ± 2.02	−12.31, −4.39	<0.01*	−0.4 ± 4.35	−8.93, 8.13	0.93	−0.58 ± 0.4	−1.36, 0.2	0.15	5.4 ± 2.8	−0.09, 10.89	0.06
Propionate	22	58	−8.29 ± 2.43	−13.05, −3.53	<0.01*	1.06 ± 5.25	−9.23, 11.35	0.84	0.03 ± 0.48	−0.91, 0.97	0.95	7.53 ± 3.37	0.92, 14.14	0.03*
Butyrate	22	58	−9.76 ± 2.62	−14.9, −4.62	<0.01*	−2.9 ± 5.64	−13.95, 8.15	0.61	−0.63 ± 0.52	−1.65, 0.39	0.23	3.58 ± 3.62	−3.52, 10.68	0.33
Archaea, % of sequences														
Methanobrevibacter	22	59	7.84 ± 7.53	−6.92, 22.6	0.30	4.53 ± 16.09	−27.01, 36.07	0.78	−1.89 ± 1.47	−4.77, 0.99	0.20	8.21 ± 9.56	−10.53, 26.95	0.39
Actinobacteria, % of sequences														
Actinomyces	22	59	−2.82 ± 5.24	−13.09, 7.45	0.59	−33.21 ± 14.68	−61.98, −4.44	0.03*	0.67 ± 1.05	−1.39, 2.73	0.53	−0.65 ± 6.92	−14.21, 12.91	0.93
Bifidobacterium	22	59	11.62 ± 5.5	0.84, 22.4	0.04*	−4.73 ± 11.82	−27.9, 18.44	0.69	2.18 ± 1.09	0.04, 4.32	0.05	−4.71 ± 7.63	−19.66, 10.24	0.54
Coriobacteriaceae unspecified genus	22	59	−5.82 ± 6.31	−18.19, 6.55	0.36	13.13 ± 11.1	−8.63, 34.89	0.25	0.87 ± 1.03	−1.15, 2.89	0.41	−11.66 ± 9.35	−29.99, 6.67	0.22
Adlercreutzia	22	59	15.72 ± 5.78	4.39, 27.05	<0.01*	10.51 ± 13.36	−15.68, 36.7	0.44	0.47 ± 1.39	−2.25, 3.19	0.73	−13.04 ± 9.34	−31.35, 5.27	0.17
Collinsella	22	59	−10.06 ± 4.98	−19.82, −0.3	<0.05*	7.11 ± 13.26	−18.88, 33.1	0.59	−0.22 ± 0.94	−2.06, 1.62	0.82	8.84 ± 7.51	−5.88, 23.56	0.24
Eggerthella	22	59	24.81 ± 5.52	13.99, 35.63	<0.01*	−49.46 ± 16.03	−80.88, −18.04	<0.01*	−0.2 ± 1.25	−2.65, 2.25	0.87	−10.63 ± 5.94	−22.27, 1.01	0.08
Bacteroidetes, % of sequences														
Bacteroides	22	59	2.46 ± 1.67	−0.81, 5.73	0.15	−2.91 ± 3.4	−9.57, 3.75	0.40	−0.11 ± 0.21	−0.52, 0.3	0.62	0.92 ± 1.52	−2.06, 3.9	0.55
Parabacteroides	22	59	2.81 ± 3.93	−4.89, 10.51	0.48	0.08 ± 8.04	−15.68, 15.84	0.99	−0.55 ± 0.52	−1.57, 0.47	0.30	1.56 ± 3.72	−5.73, 8.85	0.68
Rikenellaceae unspecified genus	22	59	8.87 ± 3.74	1.54, 16.2	0.02*	−8.46 ± 8.04	−24.22, 7.3	0.30	0.01 ± 0.74	−1.44, 1.46	0.99	−3.26 ± 5.2	−13.45, 6.93	0.53
S24-7 unspecified genus	22	59	−21.27 ± 11.32	−43.46, 0.92	0.07	−10.49 ± 24.77	−59.04, 38.06	0.67	−1.99 ± 2.14	−6.18, 2.2	0.36	12.86 ± 14.96	−16.46, 42.18	0.39
Barnesiellaceae unspecified genus	22	59	6.5 ± 7.62	−8.44, 21.44	0.40	−50.84 ± 25.84	−101.49, −0.19	0.05	0.96 ± 1.61	−2.2, 4.12	0.55	−13.22 ± 11.17	−35.11, 8.67	0.24
Butyricimonas	22	38	15.25 ± 6.05	3.39, 27.11	0.02*	−12.65 ± 11.7	−35.58, 10.28	0.29	1.23 ± 1.06	−0.85, 3.31	0.26	−7.73 ± 7.32	−22.08, 6.62	0.30
Odoribacter	22	59	−4.4 ± 3.93	−12.1, 3.3	0.27	−1.27 ± 7.99	−16.93, 14.39	0.87	−0.13 ± 0.74	−1.58, 1.32	0.86	−1.73 ± 5.39	−12.29, 8.83	0.75
Firmicutes, % of sequences														
Granulicatella	22	59	−2.56 ± 5.77	−13.87, 8.75	0.66	8.53 ± 12.15	−15.28, 32.34	0.49	1.1 ± 1.24	−1.33, 3.53	0.38	3.63 ± 7.81	−11.68, 18.94	0.64
Lactococcus	22	59	−2.93 ± 11.69	−25.84, 19.98	0.80	−0.99 ± 23.39	−46.83, 44.85	0.97	−3.43 ± 2.38	−8.09, 1.23	0.16	9.95 ± 15.56	−20.55, 40.45	0.53
Streptococcus	22	59	−10.19 ± 4.76	−19.52, −0.86	0.04*	−16.81 ± 10.24	−36.88, 3.26	0.11	0.48 ± 0.94	−1.36, 2.32	0.61	4.86 ± 6.61	−8.1, 17.82	0.47
Turicibacter	22	59	6.75 ± 7.56	−8.07, 21.57	0.38	−28.69 ± 20.01	−67.91, 10.53	0.16	−3.15 ± 1.3	−5.7, −0.6	0.02*	−32.66 ± 14.9	−61.86, −3.46	0.03*
Clostridiales unspecified genus	22	59	−0.1 ± 2.83	−5.65, 5.45	0.97	0.7 ± 6.08	−11.22, 12.62	0.91	0.12 ± 0.56	−0.98, 1.22	0.83	−0.03 ± 3.92	−7.71, 7.65	0.99
Christensenellaceae unspecified genus	22	59	1.99 ± 7.37	−12.46, 16.44	0.79	0.81 ± 13.18	−25.02, 26.64	0.95	−0.55 ± 1.56	−3.61, 2.51	0.72	−10.41 ± 10.37	−30.74, 9.92	0.32
Clostridiaceae unspecified genus	22	59	1.57 ± 4.94	−8.11, 11.25	0.75	4.96 ± 10.6	−15.82, 25.74	0.64	0.47 ± 0.97	−1.43, 2.37	0.63	−7.06 ± 6.85	−20.49, 6.37	0.31
Clostridium	22	58	−3.88 ± 3.88	−11.48, 3.72	0.32	12.14 ± 8.16	−3.85, 28.13	0.14	−1.4 ± 0.75	−2.87, 0.07	0.07	5.5 ± 5.26	−4.81, 15.81	0.30
Lachnospiraceae unspecified genus	22	59	0.41 ± 1.75	−3.02, 3.84	0.82	0.35 ± 3.51	−6.53, 7.23	0.92	0.3 ± 0.22	−0.13, 0.73	0.18	−2.67 ± 1.59	−5.79, 0.45	0.10
Anaerostipes	22	59	−2.11 ± 4.35	−10.64, 6.42	0.63	−15.44 ± 9.36	−33.79, 2.91	0.11	0.49 ± 0.86	−1.2, 2.18	0.57	−3.61 ± 6.04	−15.45, 8.23	0.55
Blautia	22	59	1.75 ± 2.63	−3.4, 6.9	0.51	−7.53 ± 5.65	−18.6, 3.54	0.19	0.22 ± 0.52	−0.8, 1.24	0.68	−0.89 ± 3.65	−8.04, 6.26	0.81
Coprococcus	22	59	−6.33 ± 3.5	−13.19, 0.53	0.08	−2.43 ± 7.53	−17.19, 12.33	0.75	−1.6 ± 0.69	−2.95, −0.25	0.03*	11.51 ± 4.86	1.98, 21.04	0.02*
Dorea	22	59	5.22 ± 3.88	−2.38, 12.82	0.18	4.84 ± 8.34	−11.51, 21.19	0.56	0.57 ± 0.77	−0.94, 2.08	0.46	−0.7 ± 5.38	−11.24, 9.84	0.90
Lachnobacterium	22	59	9.51 ± 9.97	−10.03, 29.05	0.34	−47.04 ± 30.03	−105.9, 11.82	0.12	−2.46 ± 2.25	−6.87, 1.95	0.28	−10 ± 14.23	−37.89, 17.89	0.49
Lachnospira	22	59	8.27 ± 4.44	−0.43, 16.97	0.07	10.27 ± 9.53	−8.41, 28.95	0.29	−2.29 ± 0.88	−4.01, −0.57	0.01*	−1.52 ± 6.16	−13.59, 10.55	0.81
Roseburia	22	59	−5.41 ± 3.88	−13.01, 2.19	0.17	9.99 ± 8.33	−6.34, 26.32	0.24	−1.47 ± 0.77	−2.98, 0.04	0.06	−1.54 ± 5.38	−12.08, 9	0.78
Ruminococcaceae unspecified genus	22	59	0.56 ± 1.91	−3.18, 4.3	0.77	−8.28 ± 4.1	−16.32, −0.24	<0.05*	−0.14 ± 0.38	−0.88, 0.6	0.71	−3.95 ± 2.65	−9.14, 1.24	0.14

Table 2.4 (continued)[1]

			Full model											
			Time (hours)			Eating frequency (occasions)			Early energy consumption (% of energy)			Overnight-fast duration (hours)		
	Participants, *n*	Samples, *n*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*	Estimate ± SEE	95% CI	*P*
Anaerotruncus	22	59	17.95 ± 3.88	10.35, 25.55	<0.01*	−34.93 ± 13.57	−61.53, −8.33	0.01*	1.23 ± 0.99	−0.71, 3.17	0.22	−4.91 ± 5.49	−15.67, 5.85	0.38
Faecalibacterium	22	59	−0.79 ± 2.29	−5.28, 3.7	0.73	0.53 ± 4.93	−9.13, 10.19	0.92	−0.21 ± 0.45	−1.09, 0.67	0.64	−0.96 ± 3.18	−7.19, 5.27	0.76
Oscillospira	22	59	7.1 ± 2.2	2.79, 11.41	<0.01*	−0.55 ± 4.72	−9.8, 8.7	0.91	1.48 ± 0.43	0.64, 2.32	<0.01*	−5.75 ± 3.05	−11.73, 0.23	0.07
Ruminococcus	22	59	7.99 ± 3.52	1.09, 14.89	0.03*	2.4 ± 7.56	−12.42, 17.22	0.75	−0.35 ± 0.69	−1.7, 1	0.61	−0.93 ± 4.88	−10.49, 8.63	0.85
Acidaminococcus	22	31	−8.77 ± 32.96	−73.37, 55.83	0.79	23.16 ± 78.81	−131.31, 177.63	0.08	5.68 ± 6.41	−6.88, 18.24	0.39	−28.98 ± 37.87	−103.21, 45.25	0.45
Dialister	22	59	−2.04 ± 5.98	−13.76, 9.68	0.73	−35.52 ± 16.15	−67.17, −3.87	0.03*	−1.8 ± 1.22	−4.19, 0.59	0.15	−2.79 ± 8.75	−19.94, 14.36	0.75
Megamonas	22	37	−6.06 ± 26.36	−57.73, 45.61	0.82	75.44 ± 50.34	−23.23, 174.11	0.15	15.13 ± 4.67	5.98, 24.28	<0.01*	10.58 ± 33.14	−54.37, 75.53	0.75
Megasphaera	22	39	16.68 ± 24.94	−32.2, 65.56	0.51	1.95 ± 54.15	−104.18, 108.08	0.97	2.04 ± 4.87	−7.51, 11.59	0.68	−9.49 ± 29.41	−67.13, 48.15	0.75
Phascolarctobacterium	22	59	−0.18 ± 5.43	−10.82, 10.46	0.97	14.11 ± 10.65	−6.76, 34.98	0.19	0.62 ± 1.07	−1.48, 2.72	0.56	6.49 ± 6.95	−7.13, 20.11	0.36
Veillonella	22	59	−29.46 ± 8.3	−45.73, −13.19	<0.01*	−60 ± 21.49	−102.12, −17.88	<0.01*	−5.64 ± 1.53	−8.64, −2.64	<0.01*	7.22 ± 11.61	−15.54, 29.98	0.54
Mogibacteriaceae unspecified genus	22	59	6.14 ± 3.3	−0.33, 12.61	0.07	0.37 ± 7.8	−14.92, 15.66	0.96	−0.27 ± 0.65	−1.54, 1	0.68	2.72 ± 4.49	−6.08, 11.52	0.55
Erysipelotrichaceae unspecified genus	22	59	7.33 ± 4.98	−2.43, 17.09	0.15	−5.62 ± 10.7	−26.59, 15.35	0.60	2.5 ± 0.98	0.58, 4.42	0.01*	−11.26 ± 6.91	−24.8, 2.28	0.11
Coprobacillus	22	59	6.7 ± 7.15	−7.31, 20.71	0.35	27.32 ± 12.64	2.55, 52.09	0.04	3.11 ± 1.51	0.15, 6.07	<0.05*	−10.83 ± 11.01	−32.41, 10.75	0.33
Holdemania	22	59	11.61 ± 3.1	5.53, 17.69	<0.01*	−7.64 ± 7.52	−22.38, 7.1	0.31	1.52 ± 0.68	0.19, 2.85	0.03*	−14.38 ± 4.89	−23.96, −4.8	<0.01*
Eubacterium	22	57	−12.4 ± 6.04	−24.24, −0.56	<0.05*	2.32 ± 13.21	−23.57, 28.21	0.86	0.25 ± 1.2	−2.1, 2.6	0.84	8.77 ± 8.52	−7.93, 25.47	0.31
Proteobacteria, % of sequences														
Sutterella	22	59	5.45 ± 2.25	1.04, 9.86	0.02*	−6.35 ± 4.57	−15.31, 2.61	0.17	0.13 ± 0.3	−0.46, 0.72	0.66	−3.51 ± 2.13	−7.68, 0.66	0.11
Bilophila	22	59	8.71 ± 4.2	0.48, 16.94	0.04*	15.43 ± 7.89	−0.03, 30.89	0.06	1.19 ± 0.8	−0.38, 2.76	0.14	−10.65 ± 6.28	−22.96, 1.66	0.10
Desulfovibrio	22	59	24.44 ± 8.86	7.07, 41.81	<0.01*	29.37 ± 18.51	−6.91, 65.65	0.12	3.2 ± 1.68	−0.09, 6.49	0.06	−18.01 ± 13.07	−43.63, 7.61	0.17
Enterobacteriaceae unspecified genus	22	59	11.58 ± 8.15	−4.39, 27.55	0.16	17.11 ± 16.37	−14.98, 49.2	0.30	−0.05 ± 1.73	−3.44, 3.34	0.98	12.85 ± 9.06	−4.91, 30.61	0.16
Escherichia	22	59	15.87 ± 6.7	2.74, 29	0.02*	20.07 ± 15.39	−10.09, 50.23	0.20	0.67 ± 1.59	−2.45, 3.79	0.67	7.28 ± 7.96	−8.32, 22.88	0.36
Haemophilus	22	59	−20.11 ± 8.56	−36.89, −3.33	0.02*	−42.83 ± 22.01	−85.97, 0.31	0.06	−3.96 ± 1.39	−6.68, −1.24	<0.01*	−22.93 ± 14.47	−51.29, 5.43	0.12
Verrucomicrobia, % of sequences														
Akkermansia	22	59	12.68 ± 8.6	−4.18, 29.54	0.15	8.67 ± 19.14	−28.84, 46.18	0.65	0.3 ± 1.9	−3.42, 4.02	0.88	−3.66 ± 12.26	−27.69, 20.37	0.77

[1] Results of linear mixed-model analysis were adjusted for repeated sampling, age, BMI, sex, and normalized total fiber intake. Estimates represent the percentage change in the predicted value of the outcome variable for each 1-unit change in the predictor variable if all the other predictors remain constant. One unit of eating frequency is 1 eating occasion. One unit of energy consumption is 1% of daily energy intake. One unit of time or overnight-fast duration is 1 h. This analysis represents a linear relationship between microbes or metabolites and time or behaviors during the awake/feeding phase of the circadian cycle. A negative estimate for time indicates that the highest values were seen earlier and decreased throughout the day. A positive estimate for time indicates that the values increased throughout the day and were highest later. A negative estimate for eating behaviors indicates an inverse relationship between the outcome and the predictor (e.g., relative abundance of the bacterium was higher when eating frequency was lower). A positive estimate for eating behaviors indicates a positive relationship between the outcome and predictor (e.g., relative abundance of the bacterium was higher when eating frequency was higher). *Significant ($P < 0.05$). OTU, operational taxonomic unit.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

In summary, the circadian regulatory system of the human body, the timing of eating, and the gastrointestinal microbiota form a network of complex relationships with potential relevance for human health that is worthy of further exploration. This work has reviewed and summarized the current literature on these connections and presented new, observational research on the link between time of day and eating behaviors and the human gastrointestinal microbiota, identifying the potential circadian rhythmicity within the microbiota and the modulation of this rhythmicity by the timing of eating.

Since the writing of the works presented herein, several new research studies have been published, shedding further light on this topic area. The aim of this section is to update this work in the context of this new literature.

Work by Wang et al. published in *Science* in September 2017 identifies a mechanism by which the microbiota regulates fat storage in the body via the circadian transcription factor NFIL3 (Y. Wang et al., 2017). This factor oscillates diurnally in intestinal epithelial cells, and the amplitude of these oscillations is determined by signals from the intestinal microbiota, specifically, microbial-induced repression of REV-ERBα via STAT3. NFIL3, in turn, controls expression of a circadian metabolic program and regulates lipid absorption and export in intestinal epithelial cells. Their work indicated that the presence of both NFIL3 expression and a microbiota were necessary for high-fat diet-induced body fat accumulation, highlighting the central role this gene plays in the coordination of these systems (Y. Wang et al., 2017). They also described the pathway by which microbial signals interact with the circadian system and identify Gram-negative, flagellated species (such as *Salmonella* and *E. coli*) as being the microbiota community members capable of this selective activation.

A recent epidemiological study investigated the timing of eating and weight maintenance (Kahleova, Lloren, Mashchak, Hill, & Fraser, 2017). The Adventist Health Study 2 reported a relationship between meal frequency and timing and changes in BMI over a 7-year period in a sample of over 50,000 healthy adults (Age [Mean ± SD]: 58 ± 13, BMI: 27 ± 5). The researchers found that five eating behaviors were associated with a reduction in BMI per year: eating 1-2 meals/day rather than 3, abstaining from snacking, having a longer overnight fast, not skipping breakfast, and consuming breakfast or lunch as the largest meal rather than dinner. These results on early energy consumption and longer overnight fast are in agreement with previous research on timing of eating and health. Results regarding eating frequency add to a body of literature which is not yet at a consensus. While strong evidence, this study does have the limitation of only considering weight as an outcome, rather than other metabolic markers. Interestingly, their conclusion presents practical advice, recommending consumption of breakfast and lunch 5-6 hours apart, followed by an 18-19 hour overnight fast.

In February 2017, the American Heart Association (AHA) released evidence-based guidelines for meal timing and frequency to prevent heart disease (St-Onge et al., 2017). This work was a thorough review of available epidemiological and clinical intervention evidence. Findings included (1) breakfast consumption may be beneficial, (2) evidence is mixed regarding eating frequency, and more, longer-term trials with increased power are needed. Increasing eating frequency may not be effective at decreasing weight or improving cardiometabolic profile, but some epidemiological evidence indicates that increased eating frequency may be protective for cardiovascular health and prevention of diabetes mellitus, (3) intermittent fasting regimens may be beneficial for weight loss, lowering triglyceride concentrations, and lowering blood pressure, although the blood pressure effect may depend on weight loss, and, (4) although

clinical interventions are limited, consuming more energy later in the day may be detrimental to cardiovascular health.

The AHA additionally proposed definitions to be used in research on meal timing and frequency. They propose that studies of meal frequency focus on "eating occasions" rather than meals versus snacks, and that, based on the best predictability of variance in total energy intake, eating occasions be defined as containing at least 50 kcal and be separated by at least 15 minutes. This is also the definition utilized in this thesis. This report found that meal patterns have become increasingly more varied in recent years, and that the prevalence of meal skipping and snacking have increased. The authors proposed that an intentional approach to eating be adopted, requiring eating at regular, planned intervals to distributed total energy intake throughout the day to maintain a more healthful cardiometabolic profile. Interestingly, our laboratory has also established that regularity or variability in eating frequency is also associated with the human gastrointestinal microbiota (Benishay, Kaczmarek, & Holscher, 2017).

Finally, a publication by Collado et al. (2018) directly examined circadian rhythms in the salivary microbiota and the impact of meal timing on these rhythms via a randomized, crossover study. The authors reported significant diurnal rhythms in both diversity and bacterial relative abundance in the human salivary microbiota (Collado et al., 2018). Additionally, timing of meal consumption (early versus late) shifted these rhythms. They also collected one fecal sample per participant at the end of each week-long eating condition but did not report significant differences in the fecal microbiota. This may be due to small sample size (n=10). Also, the time of fecal sample collection was not reported and may have introduced additional variability to the data. The authors concluded that this intervention on eating behavior not only impacted the

bacterial community of the oral cavity but could also be relevant to inflammation and health. This study provides novel and encouraging support to the theory described in this thesis.

Clearly, the body of evidence in the area of circadian rhythms, meal timing, and the human gastrointestinal microbiota continues to grow. More research is needed to better understand the complexities of this system. Specifically, further studies of the kind by Collado et al. (2018) that employ a human sample and a meal timing intervention are needed to establish causality and strengthen a body of literature that is currently built largely on preclinical trials and clinical observational studies. Crossover studies are especially valuable because of the highly individualistic nature of the human gastrointestinal microbiota. Many clinical studies of meal timing such as manipulations of the length of the eating window or number of eating occasions in a day have been performed, but few with the gastrointestinal microbiota as a primary outcome. The best future studies will include these interventions with both health markers (weight, blood lipids, glucose tolerance) and microbial measures (bacterial abundance and metabolite concentrations) as primary outcomes to obtain a complete picture of this complex system.

In addition to clinical feeding trials, an integrated system of this kind requires specialized models to tease apart relationships. This is especially important considering the evidence that animals dependent on parenteral nutrition still show circadian rhythms in the gastrointestinal microbial community (Leone et al., 2015). The gastrointestinal tract and brain are in constant bidirectional communication via the gut-brain axis which includes nervous, immune, and neuroendocrine pathways (Sandhu et al., 2017). Is one – or several – of these pathways responsible for the cyclical behavior of bacteria in the intestine? Studies utilizing rodent models with a severed vagus nerve may begin to answer parts of this question. Additionally,

supplementation of melatonin could demonstrate hormonal sensitivity if circadian rhythms within the microbial community could be shifted by an intervention of this kind.

Utilizing a clinical sleep lab with controlled lighting would also add critical information to our knowledge base, taking advantage of the dependence of the master pacemaker, the suprachiasmatic nucleus, on the presence of light. Could bacterial rhythms be shifted by changes in lighting conditions without changing the timing of presence of food, indicating changes in neuronal, hormonal, or gene transcriptional signals? In animal models, lighting conditions have been shown to disrupt the circadian clock, timing of food intake, and other metabolic signals (Fonken et al., 2010), and in humans similar metabolic disturbances have been observed with simulated nightshift work (McHill et al., 2014). It would be helpful to measure short-term metabolic outcomes such as energy expenditure, satiety hormone levels, and glucose tolerance in conjunction with bacterial outcomes, metabolites, and intestinal gene transcription in both clinical and preclinical experiments of this kind.

Probiotics would also be an interesting intervention to further explore these relationships. If cycling bacteria are consistently confirmed in the human microbiota, can these bacteria be delivered to the gastrointestinal tract to improve circadian rhythms of gastrointestinal tract for health benefit? Also, does differing the timing of delivery of current probiotics alter their efficacy?

As improving health outcomes is the long-term goal of this research, determining causality between the microbial community and health outcomes is of paramount importance. This causation is best established by fecal transplantation into germ-free animals. In addition to allowing for isolation of a bacterial community, animal models show signs of chronic diseases in a matter of weeks or months, rather than years as is the case with humans. Germ-free pigs

especially may improve our understanding with regard to circadian relationships to the immune system and intestinal development, gene expression, and health, as their anatomy is more similar to humans than rodent models (Q. Zhang, Widmer, & Tzipori, 2013).

This is only a brief summary of suggestions for future directions, and much work remains to be done. In the future, we hope to be able to concisely present these findings as an evidence-based approach to preventing or treating chronic disease and improving human health.

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APPENDIX A: SUPPLEMENTAL TABLES

The supplementary file SupplementalTables.pdf includes Tables A.1-A.7.

These results represent analyses of an expanded data set from the original clinical study. In the primary study protocol, participants consumed 0, 5.0, or 7.5 g agave inulin/d in a randomized order for 21 d with 7-d washouts between periods. They provided up to three fecal samples during days 16–20 of each of the three periods (maximum 9 total samples per participant). All of the fecal samples from the 0 g supplemental fiber (e.g. control) and 7.5 g agave inulin periods, as well as a selection of the 5 g agave inulin period samples, were sequenced for use in analyses presented in the online supplementary material resulting in 189 total samples from 29 study participants (females = 15 (52%)).

These supplemental tables present results of linear mixed model analysis adjusted for repeated sampling, age, BMI, sex, and normalized total fiber intake, including supplemental treatment fiber. The estimate represents the percent change in the predicted value of the outcome variable for each one-unit change in the predictor variable, if all the other predictors remain constant. One unit of eating frequency is one eating occasion. One unit of energy consumption is 1% of daily energy intake. One unit of time or overnight fast duration is one hour. This analysis represents a linear relationship between microbes or metabolites and time or behaviors during the light/feeding phase of the circadian cycle. A negative estimate for time indicates that the highest values were seen earlier, and decreased throughout the day. A positive estimate for time indicates that the values increased throughout the day, and were highest later. A negative estimate for eating behaviors indicates an inverse relationship between the outcome and the predictor (e.g. relative abundance of the bacterium was higher when eating frequency was lower). A positive estimate for eating behaviors indicates a positive relationship between the outcome and predictor (e.g. relative abundance of the bacterium was higher when eating frequency was higher). The “Treatment Fiber” effect in Tables A.4-A.6 is a classification variable with values of 0, 5 or 7.5 in the regression model. Table A.7 also includes a “Treatment Fiber x Time” interaction factor in the regression model. A significant Treatment x Time interaction indicates that the effect of fiber treatment varies by time of day, so the effect of fiber treatment alone cannot be considered without accounting for time of day.