Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock

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In mammals, multiple physiological, metabolic, and behavioral processes are subject to circadian rhythms, adapting to changing light in the environment. Here we analyzed circadian rhythms in the fecal microbiota of mice using deep sequencing, and found that the absolute amount of fecal bacteria and the abundance of Bacteroidetes exhibited circadian rhythmicity, which was more pronounced in female mice. Disruption of the host circadian clock by deletion of *Bmal1*, a gene encoding a core molecular clock component, abolished rhythmicity in the fecal microbiota composition in both genders. *Bmal1* deletion also induced alterations in bacterial abundances in feces, with differential effects based on sex. Thus, although host behavior, such as time of feeding, is of recognized importance, here we show that sex interacts with the host circadian clock, and they collectively shape the circadian rhythmicity and composition of the fecal microbiota in mice.

microbiome | Bmal1 gene | circadian rhythm | gender differences

The composition of intestinal microbiota is influenced by host genetics (1), aging (2), antibiotic exposure (3), lifestyle (4), diet (5), pet ownership (6), and concomitant disease (7, 8). The impact of diet in shaping the composition of the microbiota has been well established in both humans and mice (9, 10). The type of food consumed and also the feeding behavior of the host influence the microbiota. For example, a 24-h fast increases the abundance of Bacteroidetes and reduces that of Firmicutes in mouse cecum, without altering the communal microbial diversity (11). Bacteroidetes are also dominant in the microbiota of the fasted Burmese python, whereas ingestion of a meal shifts the intestinal composition toward Firmicutes (12).

The rotation of the earth results in the oscillation of light during the 24-h cycle. Organisms adapted to this cycle by developing a circadian rhythm, an endogenous and entrainable mechanism that times daily events such as feeding, temperature, sleep-wakefulness, hormone secretion, and metabolic homeostasis (13, 14). In mammals, this rhythm is controlled by a master clock that resides in the suprachiasmatic nucleus of the hypothalamus. It responds to the changing light cycle and signals this information to peripheral clocks in most tissues (15). The core mammalian clock is comprised of activators BMAL1 and CLOCK as well as repressors PERIOD (PER) and CRYPTOCHROME (CRY), forming an interlocked regulatory loop (14).

Circadian rhythms also exist in fungi and cyanobacteria (16). For example, a pacemaker in cyanobacteria transduces the oscillating daylight signal to regulate gene expression and to time cell division (17, 18). Hence, the synchronization of endogenous circadian rhythms with the environment is crucial for the survival of the bacteria as well as metazoa.

Recent studies show that the intestinal microbiota undergo diurnal oscillation under the control of host feeding time, and that ablation of the host molecular clock *Per* genes causes dysbiosis (19, 20). Here, we report that microbial composition and its oscillation are influenced by the host clock, including the *Bmal1*-dependent forward limb of the signaling pathway. We also find that rhythmicity is conditioned by the sex of the host, being more pronounced in females than in males.

Results

Diurnal Oscillation of the Microbiota in C57BL/6 Mice. The circadian behavior in the fecal microbiota of wild-type mice was assessed by sampling fecal pellets from each mouse every 4 h for 48 h. Circadian rhythmicity of the fecal microbiota was tested by the JTK_CYCLE algorithm (21). The fecal bacterial load, as measured by the total 16S rRNA gene copy numbers, oscillated diurnally during the light–dark cycle both in the group as a whole (Fig. 1*A*) and when segregated by sex (Fig. 1*B*). The bacterial load peaked around 11:00 PM and gradually decreased toward the late dark phase until the lowest level was noted around 7:00 AM when the light was on. Male mice had more bacteria overall in feces than female mice; however, female mice showed more significant diurnal oscillation ($P = 2.8E\text{-}06$) than male mice ($P = 0.032$). The relative abundances of Bacteroidetes and Firmicutes, the two most abundant components of mammalian microbiota, varied during the light–dark cycle (Fig. 1*C*). The average abundance of Bacteroidetes was higher at 11:00 PM (66%) and 11:00 AM (60%) and lower at other times, whereas that of Firmicutes was higher at 3:00 AM (45%) and 7:00 AM (45%) and lowest at 11:00 PM (29%).

The microbiota composition can be analyzed by relative abundance or by absolute abundance. The former is commonly used, yet it may exaggerate or mask the actual microbial behavior. For these reasons, we present analysis of both. We observed diurnal oscillations in fecal microbial composition at the phylum level (Fig. 1 *D*–*I*). Both the relative abundance and the inferred absolute abundance of Bacteroidetes oscillate during the light–dark cycle in mice of combined genders (Fig. 1*D*), as

Significance

Recent studies of the murine microbiome have revealed circadian behavior and linked it to host feeding time, but the mechanism responsible for rhythmicity has not been fully clarified. Here we report that both the host circadian system and host gender influence the rhythmicity of the total load and taxonomic abundances in the fecal microbiota, and that regulation by the host clock is dominant. Disruption of the host circadian clock by deletion of *Bmal1* altered the fecal microbial composition in a gender-dependent fashion. Our analyses suggest the need to consider circadian factors and host gender in the design of microbiome studies, and highlight the importance of analyzing absolute abundance in understanding the microbiota.

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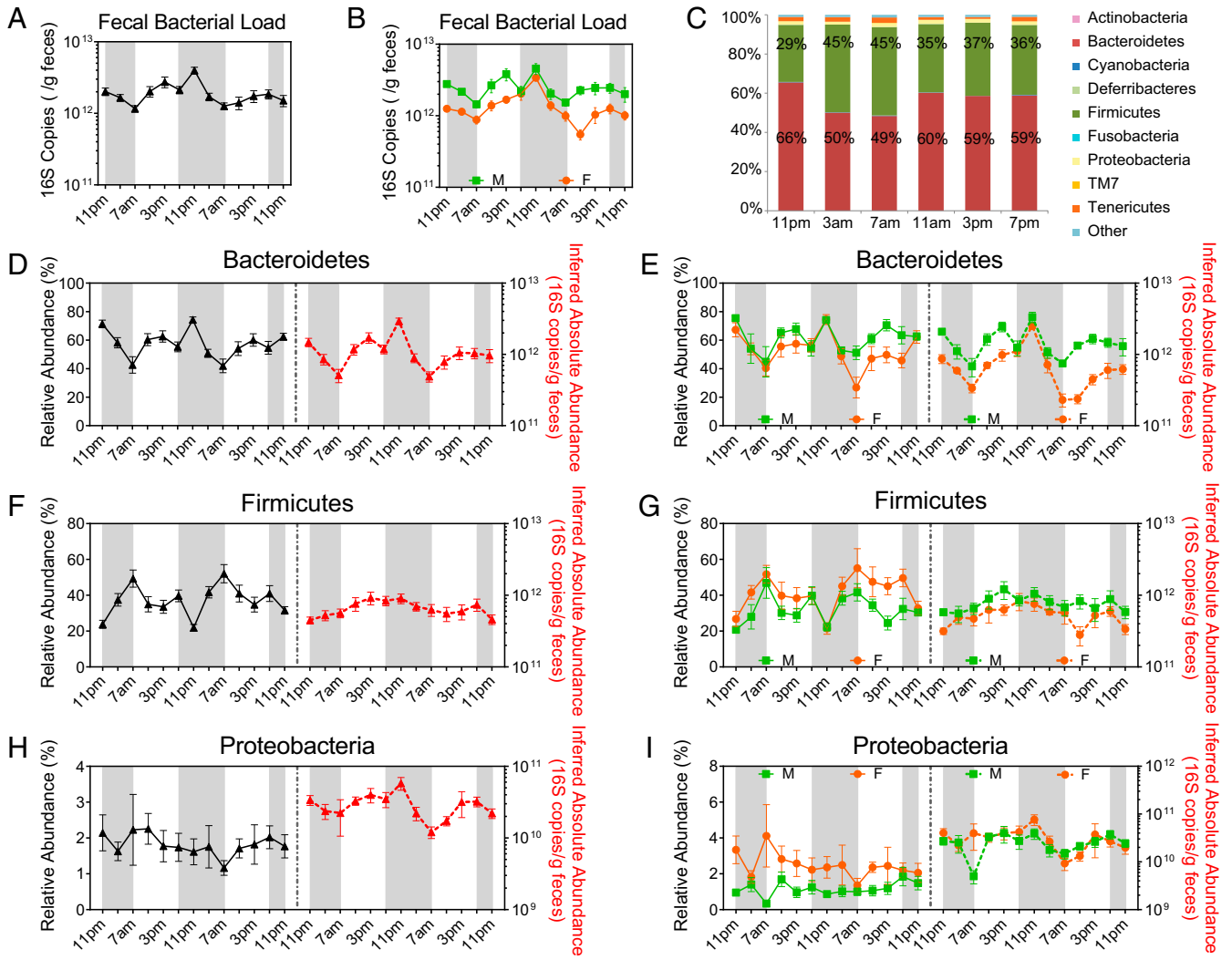

Fig. 1. Diurnal oscillation of intestinal microbiota composition in C57BL/6 mice. (*A* and *B*) Fecal bacterial load oscillates diurnally in mice both when combined (*A*) and separated by sex (*B*), as indicated by 16S rRNA copy numbers normalized to sample weight. JTK_ CYCLE revealed $P = 0.00012$ for mice of combined genders, $P = 0.032$ for male mice, and $P = 2.8E\text{-}06$ for female mice. (*C*) Bar graphs of average relative abundances of bacterial phyla in fecal microbial communities of combined genders at different time-of-day. Bacteroidetes (red) and Firmicutes (green) proportions are labeled. (*D–I*) The relative abundance (left *y* axis) and inferred absolute abundance (right *y* axis) of Bacteroidetes of combined genders (*D*, $p_r = 0.00012$ and $p_a = 1.7E\text{-}06$), male and female sex (*E*, $p_r = 0.025$ and $p_a = 0.0029$ for male mice; $p_r = 0.0006$ and $p_a = 9.5E\text{-}10$ for female mice); Firmicutes of combined genders (F, $p_r = 6.3E\text{-}05$ and $p_a = 1$), male and female sex (G, $p_r = 0.049$ and $p_a = 1$ for male mice; $p_r = 0.0011$ and $p_a = 1$ for female mice); and Proteobacteria of combined genders (H, $p_r = 1$ and $p_a = 0.00065$), male and female sex (*I*, $p_r = 0.69$ and $p_a = 0.0047$ for male mice; $p_r = 1$ and $p_a = 0.13$ for female mice). $N_{male} = 7$, $N_{female} = 7$. M, male; F, female. r, relative abundance; a, inferred absolute abundance. The dark phase is indicated by gray shading.

well as in either male or female mice (Fig. 1*E*). Bacteroidetes were most abundant at 11:00 PM and least abundant at 7:00 AM. The relative abundance of Firmicutes oscillates diurnally in mice of both combined (Fig. 1*F*) and separate (Fig. 1*G*) genders, exhibiting a contrasting pattern compared with Bacteroidetes. However, the inferred absolute abundance of Firmicutes did not oscillate in mice (Fig. 1 *F* and *G*). No diurnal oscillation was observed in the relative abundance of Proteobacteria, regardless of the sex (Fig. 1 *H* and *I*). In contrast, the inferred absolute abundance of Proteobacteria oscillates during the light–dark cycle (Fig. 1*H*), primarily due to the oscillation in male mice (Fig. 1*I*).

Examination of genus-level assignments (Fig. S1) revealed that both relative and inferred absolute abundances of *S24-7* spp., a major genus of Bacteroidetes, oscillate in the same pattern as Bacteroidetes. *Clostridiales* spp. and *Turicibacter*, genera of Firmicutes, exhibit significant oscillation, not only in their relative abundance, but also in their inferred absolute abundance. *Clostridiaceae* spp., a Firmicutes, oscillates diurnally in its inferred absolute abundance but not in its relative abundance. Several other abundant genera in Firmicutes, including *Ruminococcaceae* spp., *Clostridia* spp., *Lachnospiraceae* spp., and *Oscillospira*, as well as *Anaeroplasma*, a Tenericutes, oscillate in their relative abundance, but not in their inferred absolute abundance. The inferred absolute abundance of *Sutterella* oscillates diurnally, primarily driving the oscillation in Proteobacteria.

***Bmal1* Deletion Abolishes the Diurnal Oscillations of Microbiota Composition in Mice.** *Bmal1* is a nonredundant and essential component of the mammalian autoregulatory negative feedback loop that is responsible for generating molecular circadian rhythms (15, 22). Deletion of *Bmal1* disrupts the host circadian clock with loss of rhythmicity in animals in constant darkness. To investigate the effect of *Bmal1* deletion on the microbiota, we analyzed fecal pellets from *Bmal1* knock out (KO) mice and wild-type (WT) littermate controls every 4 h for 48 h.

The total bacterial load in WT and *Bmal1* KO mice showed a similar fluctuating pattern, although those in *Bmal1* KO mice were depressed relative to WT mice (Fig. 2*A*). In WT mice, the average abundance of Bacteroidetes was highest at 11:00 PM (76%) lowest at 7:00 AM (54%), and that of Firmicutes was highest at 7:00 AM (37%) and lowest at 11:00 PM (19%). In contrast, variability in the composition of the microbiota was suppressed in *Bmal1* KO mice (Fig. 2*B*).

JTK_CYCLE analysis revealed significant diurnal rhythmicity of the relative abundances of Bacteroidetes and Firmicutes in WT mice ($P = 0.0075$ and $P = 0.017$, respectively) but none in *Bmal1* KO mice ($P = 1$ for both) (Fig. 2). No oscillation was observed in the inferred absolute abundances of these two phyla in WT mice or *Bmal1* KO mice (Fig. 2), possibly due to an unexpectedly high 16S copy number at 11:00 AM of the second cycle. Proteobacteria did not oscillate in WT or *Bmal1* KO mice (Fig. 2*E*). At the genus level, both relative and inferred absolute abundances of *Bacteroides* and *Lacotobacillaceae* spp. oscillate significantly in WT mice, and this oscillation was abolished in *Bmal1* KO mice (Fig. S2). In contrast, the relative abundances of *S24-7* spp. and *Clostridia* spp. oscillate in WT mice but not in *Bmal1* KO mice, whereas their inferred absolute abundances failed to oscillate in either group (Fig. S2).

***Bmal1* Deletion Alters the Microbiota Composition.** The microbial compositions of WT and *Bmal1* KO mice were assessed using UniFrac, which is a phylogeny-based measure of the degree of similarity between microbial communities (23). Pairwise distances were determined for all pairs of samples based on either the taxonomic representation (unweighted), or the types and relative abundances (weighted). Principal Coordinate Analysis (PCoA) (24) based on the UniFrac distances (25) was used to visualize the differences among samples.

The microbial community composition in *Bmal1* KO mice and WT mice was different based on presence–absence analysis (Fig. 3*A*) (unweighted UniFrac, $P = 0.03$), although the abundance-weighted analysis did not alter significantly (weighted UniFrac, $P = 0.19$). The degree of difference between genotypes did not depend on the time of day when the samples were collected (unweighted UniFrac, $P = 0.97$).

Taxonomic abundances were influenced by *Bmal1*-deletion at multiple levels. The relative abundance of Bacteroidetes was comparable in WT and *Bmal1* KO mice (Fig. 3*B*). Several genera of it, including *Bacteroidales* spp., *Rikenellaceae* spp., and *Rikenella*, were increased in their relative abundances in *Bmal1* KO mice (Fig. S3). These increases were nearly evened out by the decrease of *S24-7* spp. and *Prevotella*, resulting in the unchanged proportion of Bacteroidetes. In contrast, the inferred absolute abundance of Bacteroidetes was reduced in *Bmal1* KO mice (Fig. 3*C*). The highly-abundant *S24-7* spp. and *Prevotella* was decreased, which overcame the increases of the absolute amount of *Bacteroidales* spp., *Rikenellaceae* spp., and *Rikenella* (Fig. S4), accounting for the overall decrease of Bacteroidetes absolute abundance.

Firmicutes were similarly influenced by *Bmal1* deletion. The unchanged proportion of Firmicutes (Fig. 3*B*) was driven by the increase of *Clostridiales* spp., *Clostridiaceae* spp., and *Peptococcaceae* spp. and the decreases of *Allobaculum* and *Lactobacillaceae* spp. (Fig. S3). The decreased absolute abundance was attributable to the decrease of *Allobaculum* and *Lactobacillaceae* spp., despite the small increase of *Peptococcaceae* spp. (Fig. S4).

Protebacteria were decreased in both relative and inferred absolute abundances in *Bmal1* KO mice (Fig. 3*B*), primarily driven by the reduction of *Helicobacter* and *Sutterella* (Fig. S3 and S4). The relative abundance of TM7 was increased (Fig. 3*B*) in *Bma1* KO mice, partially attributable to the increase of *F16* spp. (Fig. S3). Despite the increase of *F16* spp. absolute abundance in *Bmal1* KO mice (Fig. S4), TM7 were unchanged (Fig. 3*C*).

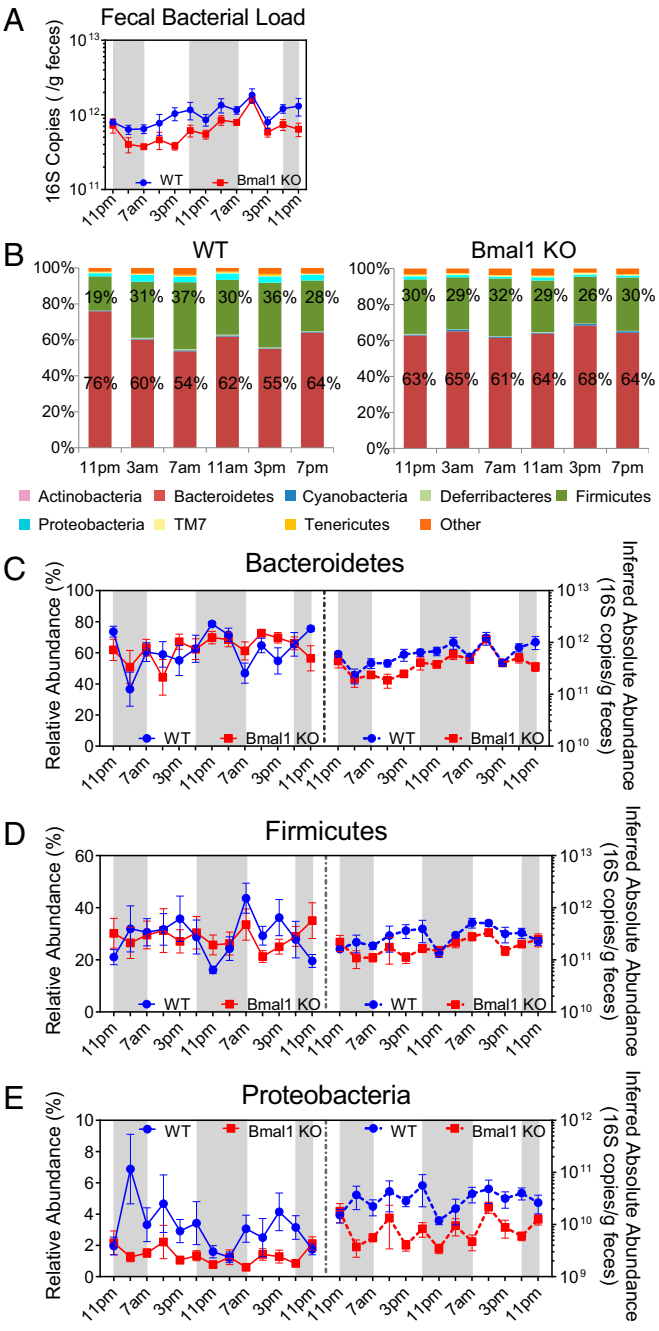

Fig. 2. Deletion of *Bmal1* abolishes the diurnal oscillation of intestinal microbiota composition in mice. (*A*) Fecal bacterial load in *Bmal1* KO mice (red) was slightly lower than that in WT mice (blue) but showed a similar fluctuating pattern during the light–dark cycle. (*B*) Bar graphs of average relative abundances of bacterial phyla in fecal microbial communities of WT (*Left*) and *Bmal1* KO (*Right*) mice at the indicated times of day. Bacteroidetes (red) and Firmicutes (green) proportions are labeled. (*C*–*E*) The relative abundance (left *y* axis) and inferred absolute abundance (right *y* axis) of Bacteroidetes (*C*) of WT ($p_r = 0.0075$ and $p_a = 0.39$) and *Bmal1* KO ($p_r = 1$ and $p_a = 1$) mice; Firmicutes (*D*) of WT ($p_r = 0.017$ and $p_a = 0.093$) and *Bmal1* KO ($p_r = 1$ and $p_a = 1$) mice; and Proteobacteria (*E*) of WT ($p_r = 0.52$ and $p_a = 1$) and *Bmal1* KO ($p_r = 1$ and $p_a = 1$) mice. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. r, relative abundance; a, inferred absolute abundance. The dark phase is indicated by gray shading.

***Bmal1* Deletion-Induced Changes in the Microbiota Are Sex-Dependent.** Principal Coordinate Analyses revealed sex differences in microbiota composition (Fig. 4*A*) ($P = 0.026$ for unweighted UniFrac; $P = 0.007$ for weighted UniFrac). The effect of *Bmal1* deletion was

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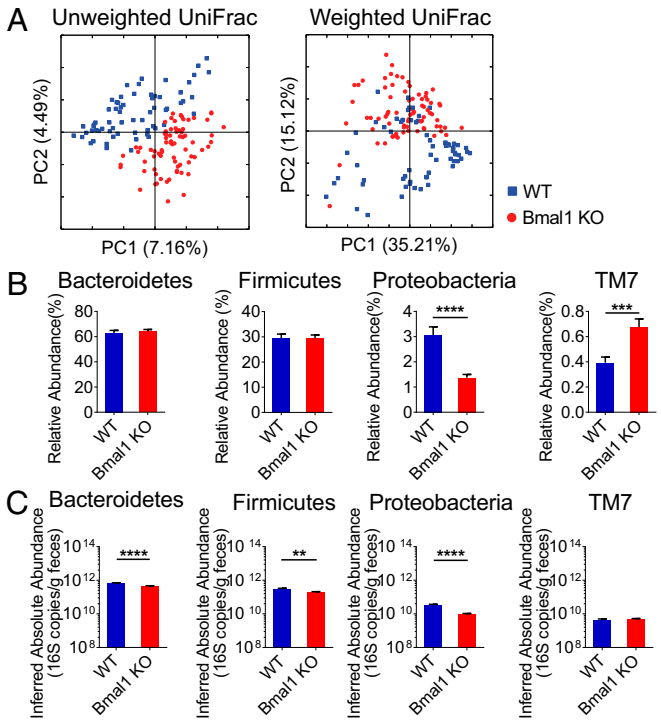

Fig. 3. *Bmal1* deletion alters bacterial abundances in fecal microbiota in mice. Fecal microbiota compositions from WT and Bmal1 KO mice at all time points across the day were compared. (*A*) Fecal microbiota composition from individual WT mice (blue) and *Bmal1* KO mice (red) were clustered separately according to Principal Coordinate Analysis of Unweighted UniFrac (*Left*) and Weighted UniFrac (*Right*) distances. The percentages of variation explained by principal coordinates PC1 and PC2 are indicated on the *x* and *y* axes. (*B*) The relative abundance of Proteobacteria was decreased and that of TM7 was increased in *Bmal1* KO mice, whereas those of Bacteroidetes and Firmicutes were not altered. (*C*) The inferred absolute abundance of Bacteroidetes, Firmicutes, and Proteobacteria were decreased in *Bmal1* KO mice, whereas that of TM7 was not altered. $N_{WT} = 5$, $N_{Bmal1\ KO} = 6$. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by Mann–Whitney test. All remained significant when adjusted to control for a false positive rate of 5%.

comparable for male and female mice. The microbiota composition of male *Bmal1* KO mice was more similar to female WT mice than male WT mice (Mann–Whitney $P < 0.001$ for unweighted and weighted UniFrac).

In male mice, *Bmal1* deletion was associated with a significant decrease of Proteobacteria and an increase of TM7 in their relative abundances (Fig. 4*B*). Consistently, the relative abundances of *Helicobacter* and *Sutterella*, two genera of Proteobacteria, were decreased, and that of *F16* spp., a TM7, was increased in male *Bmal1* KO mice (Fig. S5). In female *Bmal1* KO mice, however, Proteobacteria and TM7 were unchanged, but the relative abundances of Cyanobacteria and Tenericutes were increased (Fig. 4*B*). The increase in the relative abundance of *Ureaplasma* (Fig. S4) explained the changes in Tenericutes.

There were no significant differences between male and female *Bmal1* KO mice at the phylum level for Bacteroidetes and Firmicutes, but differences could be detected at the genus level (Fig. 4*B*). In male mice, *Bmal1* deletion was associated with significant increases of *Bacteroidales* spp., *Rikenellaceae* spp., *Rikenella*, *Clostridiales* spp., and *Clostridiaceae* spp. proportions, as well as significant decreases of *S24-7* spp., *Prevotella*, *Allobaculum*, and *Lactobacillaceae* spp. proportions (Fig. S5). In female mice, *Bmal1* deletion was associated with significant increases of *Bacteroidales* spp., *Clostridiaceae* spp., *Peptococcaceae* spp., and *Prevotella*, as well as significant decreases of *Lactobacillaceae* spp. (Fig. S5).

In male *Bmal1* KO mice, the absolute abundance of Proteobacteria was decreased and that of TM7 was increased (Fig. 4*C*), driven by the decrease of *Helicobacter* and *Sutterella* and the increase of *F16* spp., respectively. Bacteroidetes were decreased in male *Bmal1* KO mice, an effect of expansion of *Bacteroidales* spp., *Rikenellaceae* spp., and *Rikenella*, and reduction of *S24-7* spp. and *Prevotella* (Fig. S6). In female *Bmal1* KO mice, the absolute abundance of Tenericutes was increased (Fig. 4*C*), as driven by the increase of *Ureaplasma* abundance (Fig. S6). In addition, the inferred absolute abundances of Bacteroidetes, Firmicutes, and Proteobacteria were decreased in female *Bmal1* KO mice (Fig. 4*C*)–*Bacteroidales* spp., *Peptococcaceae* spp., *Prevotella*, and *Turicibacter* were expanded, whereas *Rikenellaceae* spp., *S24-7* spp., *Clostridiales* spp., *Lactobacillaceae* spp., and *Helicobacter* were reduced (Fig. S6).

Discussion

Here we provide evidence that the microbiota exhibit daily fluctuations, and begin to specify the factors that influence these oscillations. The fecal microbiota, including the microbial load and its composition, oscillate diurnally in mice. Two groups recently reported circadian variations of the relative taxonomic abundances in fecal microbiota (19, 20), but to our knowledge, this study is the first to show variation in the fecal bacterial load and the derived absolute abundances. We also find, unexpectedly, that this oscillation is more pronounced in female than in male mice. Deletion of *Bmal1*, the central component of the forward limb in host circadian clock, not only abolished the circadian behavior of fecal microbiota in both genders, but also shifted the microbiota configuration in a sex-dependent way.

Consistent with previous observations (19, 20), we observed diurnal oscillation in the relative abundance of Bacteroidetes and Firmicutes (Fig. 1). Bacteroidetes peaked several hours after the beginning of the dark phase and Firmicutes peaked around the beginning of the light phase. Cyclical fluctuations were detected in abundances at genus level, including *Lachnospiraceae* spp., *Oscillospira*, *Ruminococcaceae* spp., *Clostridiales* spp., *Clostridia* spp., and *Anaeroplasma*. Thaiss and colleagues found that the rhythmicity of microbiota composition is mostly lost after ablation of *Per1* and *Per2* genes, which are key components of the host molecular clock (19). A complication in the interpretation is that deletion of *Per1* and *Per2* has pleiotropic effects extending beyond just circadian behavior, such as involvement in cell growth and cell cycle regulation (26). Deleting *Bmal1*, which leads to arhythmicity without pleiotropic effects, also abolishes the diurnal oscillation in fecal microbiota. Collectively, both activator (*Bmal1*) and repressor (*Per1,2*) genes in the core clock of the host are fundamental to the oscillation of fecal microbiota composition, further strengthening the association of the host clock with microbiota rhythmicity in mice.

Measurement of the total microbial load provides an opportunity to assess changes in the absolute abundances of bacteria. We found that the absolute abundance of Bacteroidetes oscillates diurnally, whereas that of Firmicutes only showed slight fluctuation over time (Fig. 1). Additionally, the absolute abundance of Proteobacteria oscillates, although its relative abundance does not. This might be attributable to its low abundance. Therefore, variation of Bacteroidetes during the day is the main driving force of the circadian oscillation of total bacterial load, as well as the proportional changes of themselves and Firmicutes. Although microbiomic data are often presented as relative abundances, their discordance with absolute abundances as reflected in this study emphasizes the importance of also presenting the latter. Conclusions solely relying on the relative abundances may be misleading. In our work and in previous publications, analysis of relative proportions suggests oscillation

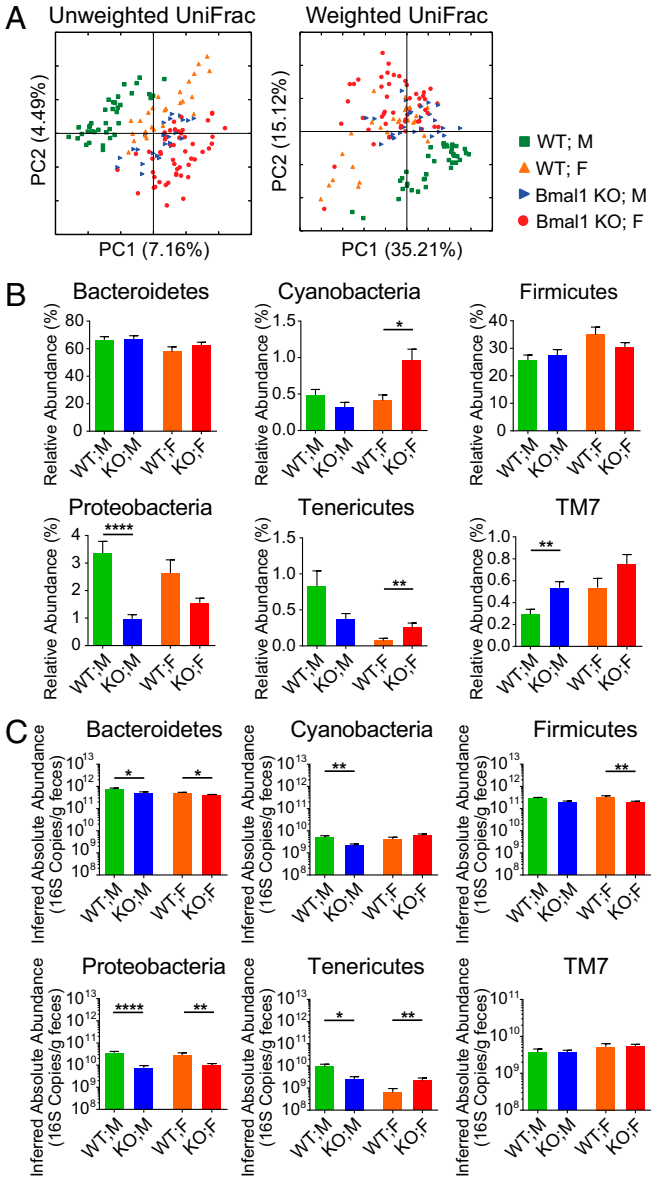

Fig. 4. Intestinal microbiota compositions of male and female mice respond differently to *Bmal1* deletion. Fecal microbiota profiles from individual mice at all time points across the day were compared based on sex and genotype. (*A*) Fecal microbiota composition of male WT mice (green), male *Bmal1* KO mice (blue), female WT mice (orange), and female *Bmal1* KO mice (red) were clustered according to Principal Coordinate Analysis of Unweighted UniFrac (left) and Weighted UniFrac (right) distances. Percentages of variation explained by principal coordinates PC1 and PC2 are indicated on the x and y axes. (*B*) The relative abundances at phylum level. (*C*) The inferred absolute abundances at phylum level. N_{WT} = 5, $N_{Bmal1\ KO}$ = 6. M, male; F, female. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ by Mann–Whitney test. Eleven out of 12 remained significant when adjusted to control for a false positive rate of 5%.

of Firmicutes during the light–dark cycle (19, 20). However, this is attributable to the significant variation of Bacteroidetes, which are highly abundant and oscillate, rather than the variation of Firmicutes, which are relatively stable throughout the day but change in proportion due to outgrowth of Bacteriodetes.

Consumed food influences the microbial communities in the intestine. Fasting is associated with Bacteroidetes dominance and ingestion of food with a proportional increase of Firmicutes (11, 12). Mice are active in the dark phase and consume the majority of their food during the first several hours of the dark phase (27). Therefore, during the daytime, when the mice are resting and consuming less food, Bacteroidetes are predominant, reaching the highest abundance toward the end of the light phase. The influence of food may reflect the varying nutrient availability to bacteria. Glycans and polysaccharides from the diet and the host mucus provide carbon sources to the intestinal bacteria (28). Unlike the host-derived glycans which are constantly available, dietary glycans fluctuate in both composition and abundance. As an adaption, several strains in Bacteroidetes evolved to use host mucosal glycans when dietary glycans are in short supply (29, 30). This may partially explain the blooming of Bacteroidetes in the resting phase.

Circadian feeding behavior has been suggested to influence microbial rhythmicity–diurnal oscillation was restored by restricting the feeding of *Per1/2* KO mice to the dark or light phase (19). Partial restoration of microbial oscillation could be accomplished by consolidating high-fat feeding to the dark phase (20). However, because circadian rhythms of host behavior and physiology can be entrained by daily cycles of restricted feeding (31), it is possible that feeding in the dark phase partially restored the circadian rhythm of the host. Hence, it remains to be determined whether the restoration of microbial oscillation is due to a direct influence of feeding, due to restoration of the host circadian clock, or due to the combination of the two.

In addition to regulating the circadian rhythmicity of microbiota, *Bmal1* also influenced both the abundance and composition of the microbiota. Given the evidence that *Bmal1* exerts an anti-inflammatory effect in mice (32), it is noteworthy that *Bmal1* deletion shifted the microbiota configuration toward a phenotype that may be proinflammatory. For example, expansion of *Rikenellaceae* has been reported due to high-fat diet feeding (33) and during the pathological progression of inflammatory bowel disease (34). *Clostridiaceae* had been found to be increased in patients following ileal pouch-anal anastomosis (35), in patients with ulcerative colitis and Crohn's disease (36), and in mice during the progression of lupus (37). Decrease of *Allobaculum* is associated with high-fat diet feeding in mice (38). Our findings do not presently identify the mechanism through which *Bmal1* affects the configuration of the microbiota and it is also unknown whether deletion of the Period genes influenced taxonomic composition (19).

Sex regulates the fecal microbial composition in line with the host circadian clock. Although microbiota in both genders exhibited circadian rhythmicity, females showed more significant oscillation than males (Fig. 1). However, the effect of host sex is secondary to the host circadian clock in shaping the rhythmicity, because *Bmal1* deletion abolished the rhythmicity irrespective of sex. This interpretation is further strengthened by the near-complete loss of diurnal oscillation of microbiota in *Per1/2* deficient mice, irrespective of sex (19). Sex also conditions the impact of *Bmal1* deletion on microbiota composition (Fig. 4 and Fig. S6), indicating an interaction between sex and genotype. Sex-dependent effects on the microbiome have been suggested in various animal models (39–41). In mice, male and female microbiota diverge after puberty, reflecting the sex bias in expression of autoimmune diseases, such as type 1 diabetes (40, 42). Although the mechanism remains unclear, a bidirectional interaction between microbiota and host hormone levels seems likely, because the divergence between male and female microbiota can be reversed by male castration (42). Moreover, differential effects of dietary manipulation are conditioned by sex in fish, mice and potentially humans (41).

Our findings specify pathways of host regulation of fecal microbiota composition and motivate exploration of the host circadian clock and microbial circadian rhythmicity. The present study and previous work (19, 20) highlight the need to consider sex, genotype, feeding pattern, and timing of sample collection in microbiome studies. Work presented here further highlights the importance of analyzing the absolute abundances of bacteria in addition to the proportions to understand the intestinal microbiome.

Materials and Methods

A detailed description of the methods is provided in *SI Materials and Methods*.

Animals. All C57BL/6 mice and *Bmal1* KO mice were raised in our animal facility. Mice 10–14 wk of age were used for all experiments. All animals were fed ad libitum with regular chow diet (5010, LabDiet) for the course of study. Mice were kept under 12-h light/12-h dark (LD) cycle, with lights on at 7:00 AM and off at 7:00 PM. Experimental protocols were reviewed and approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania.

Study Design.

Study 1: Microbial oscillation in WT mice. C57BL/6 mice were caged according to litter and sex. Seven male mice (housed in two cages with three in one cage and four in the other) and seven female mice (housed in two cages with three in one cage and four in the other) were studied. Fecal pellets from each mouse were sampled every 4 h for 48 h. A total of 182 samples were collected for microbiota composition analysis by pyrosequencing (*SI Materials and Methods*). Sequencing yielded 360,809 reads. After removing samples with less than 200 reads, 180 samples were available for analysis (*SI Materials and Methods*).

Study 2: Effect of Bmal1 deletion on microbial oscillation in mice. Homozygous *Bmal1* KO mice were established by mating heterozygous pairs. *Bmal1* KO mice were housed separately from the littermate wild-type (WT) controls immediately after the genotype was confirmed to minimize the influence of coprophagia (43). Four female (housed in two cages with two in each) and two male (housed in two cages with one in each) *Bmal1* KO mice as well as two female (housed in two cages with one in each) and three male (housed in two cages with two in one cage and one in the other) littermate control mice were studied. Fecal pellets from each mouse were sampled every 4 h for 48 h. A total of 143 samples were collected for microbiota composition analysis by pyrosequencing (*SI Materials and Methods*). Sequencing yielded 142,553 reads. After removing samples with less than 200 reads, 140 samples were available for analysis (*SI Materials and Methods*).

Statistical Analysis. Statistical tests for rhythmicity were performed using JTK_CYCLE algorithm as described (21), reporting Bonferroni-adjusted *P* values. Statistical analyses for bacterial abundance were performed with Mann–Whitney test, and *P* values were corrected for multiple comparisons using the procedure of Benjamini and Hochberg to control for a false discovery rate (FDR) of less than 5%. All data were expressed as means ± SEM. The PERMANOVA (44) procedure was applied based on pairwise UniFrac distance to assess the sources of variation, and *P* values were obtained using permutation tests.

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