Reviewed Preprint
v1 • September 2, 2024
Not revised

Reviewed Preprint
v2 • August 24, 2026
Revised by authors

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Competing interests: No competing interests declared

Funding: See page 26

Reviewing editor: Peter J Turnbaugh, University of California, San Francisco, United States

Targeted induction of gut-microbial metabolism acutely affects feeding patterns and clock gene expression in the host

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eLife Assessment

Feeding, the circadian rhythm, and the gut microbiota are all intimately linked, motivating new approaches to identify causal relationships while minimizing confounding factors. The authors employ an innovative combination of the osmotic laxative lactulose and a defined 3-member gut microbiota to acutely induce gut bacterial metabolism in mice during the daytime, resulting in changes in the ileal expression of clock genes and altered feeding behavior. Together, this study utilizes **convincing** methods to provide **important** new insights into the role of gut microbiota in the circadian rhythm, setting the stage for follow-on studies aimed at better understanding the mechanisms responsible.

https://doi.org/10.7554/eLife.100640.2.sa3

Abstract

The gut microbiota and host diurnal rhythm mutually influence each other, and microbiota metabolism has been shown to play a role in regulating host circadian function via secretion of fermentation products. Microbial metabolism is dependent on the availability of nutrients for the microbiota, typically through the host's food intake, making it challenging to disentangle the effect of host and microbiota metabolism. In this study, we acutely induced gut microbial metabolic activity without inducing host metabolism in mice. We found that increasing microbial metabolism in the gut altered clock gene expression locally. Actuating microbiota metabolism also reduced host food intake beyond the calories provided by the microbiota, suggesting a systemic signaling effect of microbial metabolism on the host.

Introduction

From regulating our daily sleep-wake cycles to influencing metabolic processes, the shift between day and night dominates the rhythm of most life on Earth (1, 2). The mammalian circadian clock is a fundamental biological process which functions through a tightly regulated network of transcriptional feedback loops (3–7) that trigger periodic fluctuations in physiology and behavior (8, 9). One of the key behavioral rhythms that is regulated by the circadian clock is food intake (10). In anticipation of the changing food availability with a change in light, the master pacemaker activates orexigenic pathways and hormone production to increase feeding and activity, which in turn triggers anorexigenic pathways through satiety signals to prepare for fasting and rest (11). Synchronization of these behaviors with the natural light-dark cycle increases fitness, while being asynchronous with the external environment is detrimental to health (12–14).

Recent research has revealed crucial links between the biological clock, feeding behavior, and the intricate ecosystem of the gut microbiota (15, 16). The circadian oscillations in food intake in the host result in oscillations in gut microbial metabolism and composition in a circadian pattern (17, 18). Oscillating immune function in the host also plays a role in shaping the gut microbial ecosystem. For example, immunoglobulin A, a key antibody that regulates commensal bacteria community composition, is secreted with a circadian rhythm, and host responses to the invasion of pathogenic bacteria are dependent on the time of day (19–21).

In addition, there is evidence that this regulation goes both ways: not only does the host clock regulate the microbiota, but also vice versa: the microbiota plays a significant role in the amplitude of host clock gene expression oscillations, and is associated with weight gain and reduced longevity in a dysregulated circadian rhythm (22–25). In line with this, germ-free (GF) mice, which do not have a microbiota, are less sensitive to the dysregulation of the circadian clock induced by high fat diets (26).

A major way of signaling between the gut microbiota and the host are microbial fermentation products (27). Fermentation products are metabolic byproducts of the microbiota which are used by the host for many metabolic and endocrine functions in the gut-brain axis, as well as for food intake regulation (28–30). Fermentation product excretion by the microbiota can trigger satiety signaling in the host and may play a role in regulating the host circadian rhythm due to the circadian nature of food intake (17, 31). However, metabolic oscillations in the gut microbiota arise from host food intake, making it difficult to disentangle host effects from microbial effects, and thus to evaluate mechanistic interactions between the microbiota and the host circadian clock (32).

In this study, we use an treatment with lactulose, a disaccharide that is not taken up in the small intestine of mice but can be metabolized by the gut microbiota, to increase microbial metabolism in the gut without directly providing calories to the host. This changes the normal microbial rhythm that depends on food intake patterns of the host The effect of this intervention is transient, and the system recovers rapidly from this disruption without lasting changes in the diurnal rhythm after an acute disruption. This experimental setup allowed us to evaluate how microbiota activity regulates host circadian clock gene expression and subsequent eating patterns. We show that microbial metabolism influences host clock gene expression and that this systemically regulates host feeding behavior.

Results

Microbial metabolism fluctuates with the host's diurnal feeding pattern

We first asked whether gut microbes exhibit diurnal variation in population size in a defined setting. To address this, we quantified bacterial load in fecal samples collected every 6 hours over two consecutive days. For these experiments, we used gnotobiotic mouse model colonized with a minimal microbiota of three species (3MM), consisting of *Bacteroides thetaiotaomicron* (*B. theta*), *Eubacterium rectale* (*E. rectale*), and *Escherichia coli* (*E. coli*), maintained across multiple generations. These species represent the phyla Bacteroidetes, Firmicutes, and Proteobacteria (recently renamed to Bacteroidota, Bacillota, and Pseudomonadota), respectively, which together dominate the gut microbiota (15, 33), and all three species reach high numbers in the gut of colonized mice (Figure 1A). Despite prior observations of phylum-level oscillations associated with feeding cycles (34), we detected no significant rhythmic changes in the population sizes of any of the three species over the course of two 24 hour cycles (Figure S1A).

Food intake was measured for each mouse using a TSE Phenomaster system that is fully enclosed in an experimental isolator. We have previously developed this system, which included extensive testing for sterility or gnotobiosis (35). As a readout for microbial metabolism, hydrogen gas excretion was also measured over time (Figure 1B and Figure S1B). Hydrogen is an abundant byproduct of butyrate production, and *E. rectale* is the only butyrate producer in the 3MM, and therefore the dominant hydrogen producer (Figure S1C) (36, 37). Hydrogen levels fluctuated in a

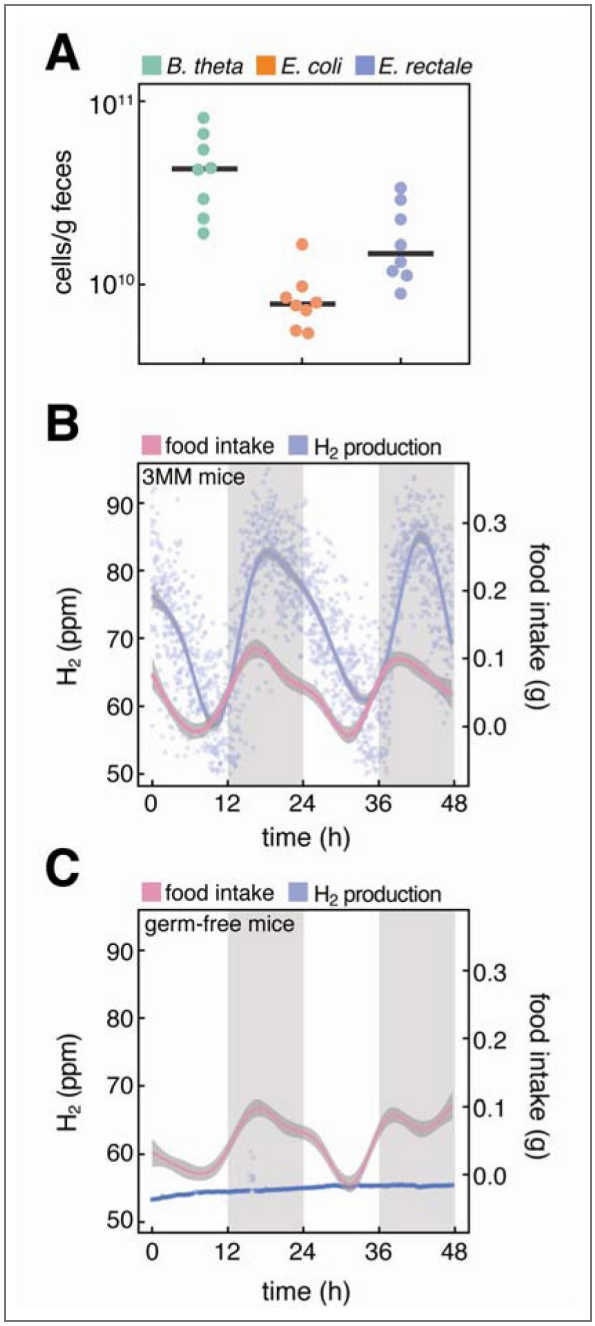


Figure 1. Timing of host food intake and microbial activity are linked.

(A) Cell counts per gram feces in the EAM mouse (n=8) at the end of the light cycle (zt=12h), measured by qPCR. **(B)** Food intake and hydrogen production of the EAM mouse over time. Two days of measurement are shown, measurements are taken every 24min (n=16). Curves were calculated using the geom_smooth function of the package ggplot2 in R with standard settings. SE is illustrated with grey shading surrounding the trend line. **(C)** Food intake and hydrogen production of the germ-free mouse over time (n=8).

24-hour period in the 3MM mouse, closely following mouse food intake, which provides the non-host-available fibers that the microbiota can metabolize in the gut (Figure 1B, Figure S1B). Germ-free mice did not produce measurable levels of hydrogen despite having the same food intake rhythm (Figure 1C).

Lactulose treatment acutely disrupts the diurnal pattern of microbial metabolism

To acutely disrupt the normal diurnal rhythm of microbial metabolism in the gut without directly providing nutrients to the host, we administered lactulose to 3MM mice. Lactulose is a disaccharide that cannot be metabolized by mammals, is known to be metabolized by the gut microbiota, and is associated with an increase in hydrogen production and reduced colon pH due to microbial metabolic activity (38–40). We administered the lactulose between Zeitgeber times 3 and 4, several hours after the mice had stopped eating and when hydrogen production was decreasing. To control for a potential effect of the oral gavage itself on the microbiota, PBS was administered as a negative control, and all experiments were replicated in germ-free mice to control for host effects independently of the microbiota.

Mice that were treated with lactulose showed increased hydrogen production two hours after treatment compared to PBS-treated mice, which continued in the normal rhythm of hydrogen production (Figure 2A). There was a significant difference in measured hydrogen levels between the mice in the two groups at the endpoint of the experiment at zt=8, 5h after lactulose treatment, indicating that the microbiota had metabolized the administered lactulose (Figure 2A, right). To evaluate the duration of the lactulose-dependent change in the diurnal pattern of microbiota metabolism, we repeated the experiment and monitored hydrogen production over the following dark cycle. Hydrogen levels in lactulose treated mice were equal to PBS treated mice by Zeitgeber 15, indicating that the microbiota had metabolized the lactulose and resumed its normal metabolic rhythm, and that our treatment therefore constitutes an acute and transient intervention (Figure 2B). To test whether these results were translatable to a complex microbial community, we performed this experiment in Specific Pathogen Free (SPF) mice. This effect was equally observed in SPF mice, indicating that a complex microbial community also metabolizes lactulose in an acute manner (Figure S2A).

Five hours after the initial lactulose treatment, after the microbiota had metabolized the lactulose, the mice were euthanized, and bacterial population sizes in cecum content were measured to evaluate the effect of lactulose on the 3MM microbiota. There was a significant increase in the cecal *E. rectale* population, but no significant change in the numbers of *B. theta* or *E. coli* (Figure 2C). Having observed increased hydrogen production in lactulose-treated mice, we further evaluated the effect of lactulose on microbial metabolism. Microbial metabolism results in an increase in excretion of acidic fermentation products, which should lead to a decrease in pH in the cecum. There was a significant drop in cecum content pH when 3MM mice were treated with lactulose, which was associated with an increase in fermentation product concentration in the cecum (Figure 2D and 2E). Lactulose-treated mice had significantly higher concentrations of acetate, butyrate, and succinate, indicating increased metabolic activity of all 3MM species, despite the lack of significant increase in *B. theta* and *E. coli* population densities (41, 42). *E. rectale* is the principal butyrate producer in the 3MM, while *B. theta* and *E. coli* both produce acetate, and *E. coli* produces succinate when grown on lactulose (Figure S2B). Germ-free mice that were treated with lactulose showed a small but significant drop in pH in cecum content, likely due to osmotic effects (Figure 2D). In GF mice, fermentation product concentrations did not increase after lactulose treatment (Figure 2F).

To ensure that the observed effects of lactulose treatment are due to the induction of microbial metabolism and not osmotic effects of lactulose treatment in our mouse models, we measured the water content of feces and cecum content after treatment in 3MM mice and germ-free mice in all experiments. In contrast to what would be expected for an osmotic laxative, water content in fresh feces of 3MM mice and the total fecal output in the 5h following treatment decreased with lactulose treatment, possibly due to the increased microbial biomass from lactulose metabolism,

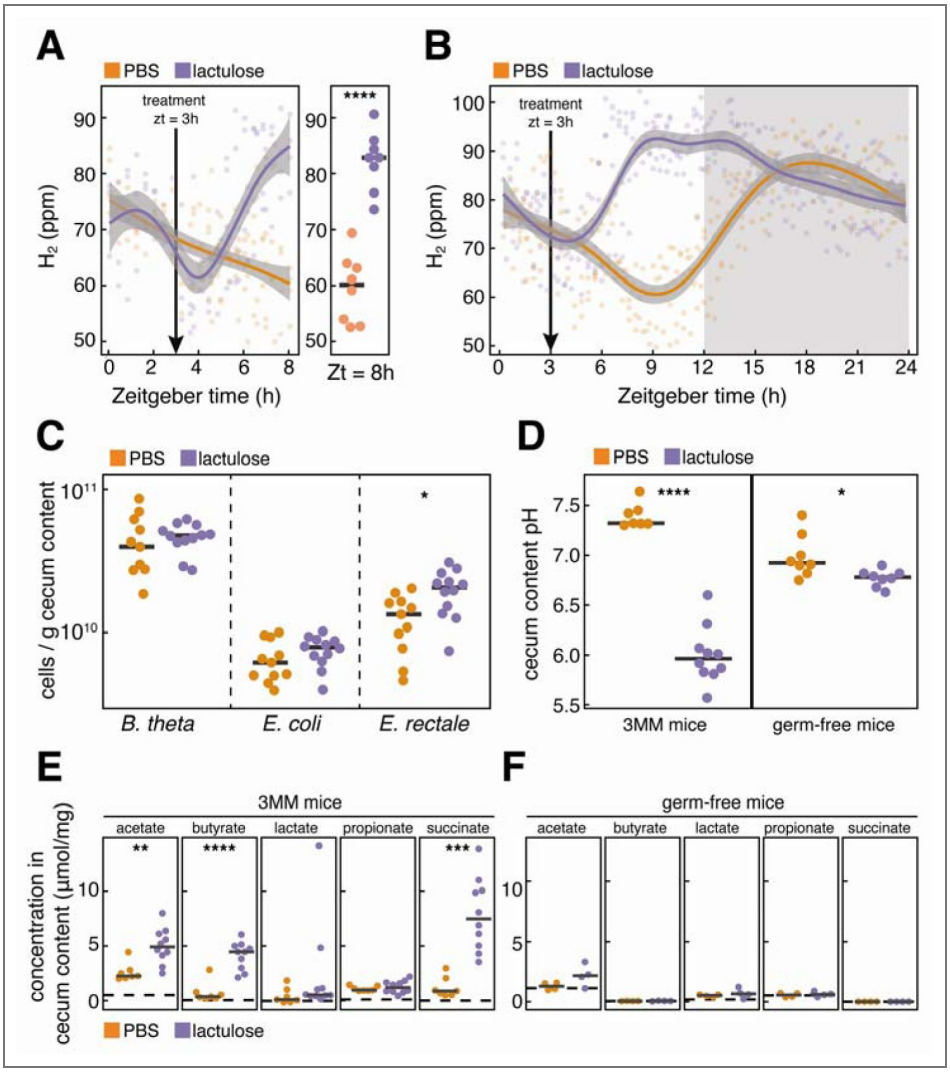


Figure 2. Lactulose treatment disturbs the diurnal rhythm of microbial metabolism.

(A) Hydrogen production increases after lactulose treatment (purple, n=8), but not after treatment with PBS (orange, n=8). Arrows at zt=3h indicate treatment. An unpaired t-test was performed on final hydrogen measurements (p>0.0001). **(B)** Hydrogen production after lactulose treatment (purple, n=6) is temporary and reverses back to normal levels after treatment, and is not observed in PBS treatment (orange, n=5). **(C)** Bacterial counts in cecum 5h after lactulose (purple, n=12) and PBS treatment (orange, n=10). *E. rectale* population increases in lactulose treated mice compared to PBS treated mice (p<0.05), *B. theta* and *E. coli* counts do not change significantly. **(D)** Cecum pH of 3MM (n=10 for lactulose, n=7 for PBS treatment) and germ-free (n=8 in each group) mice 5h after treatment. The pH decreases after lactulose treatment in 3MM (p<0.0001, unpaired t-test) and germ-free (p<0.05) mice. **(E)** Concentrations of microbial metabolites in cecum content of 3MM mice 5h after treatment with lactulose (purple, n=10) or PBS (orange, n=7). Acetate, butyrate, and succinate concentrations increase in lactulose treated as compared to PBS treated mice (**, ***, **** indicate significance levels smaller than 0.01, 0.001, and 0.0001, respectively); unpaired t-test, Benjamini-Hochberg corrected for multiple testing. **(F)** Concentrations of microbial metabolites in cecum content of germ-free mice 5h after treatment with lactulose (purple, n=8) or PBS (orange, n=8). There is no significant difference between groups (unpaired t-test, Benjamini-Hochberg corrected for multiple testing).

and indicating that the osmotic effect of lactulose treatment was small in these mice (Figure S2C,D). The water content of cecum content in 3MM and germ-free mice (Figure S2E) did not differ significantly between the different treatments. A factor that could potentially influence these parameters is the difference in cecum sizes between germ-free and colonized mice. While the cecum of 3MM mice is significantly smaller than that of germ-free mice (Figure S2F), both mouse models have large ceca when compared to SPF mice(35). Following up the surprising lack of an osmotic effect of lactulose treatment in 3MM mice, we treated 3MM and SPF mice with twice the amount of lactulose and measured fecal water content over time (Figure S3A), water content of cecum content (Figure S3B) and total fecal output during 5h after treatment (Figure S3C). Interestingly, lactulose only had a significant effect on the water content of fresh feces and cecum content in SPF mice, but not in 3MM mice. We concluded that the osmotic effect of lactulose is dependent on the colonization state of the animals, and not a major contributor to the observed effects in 3MM mice.

Inducing microbial metabolism acutely increases clock gene expression in small intestinal tissue

To test whether the induction of microbial metabolism affects gene expression driving the host clock, we studied the transcription of several core clock genes in the mouse gastrointestinal tract (Figure 3A). Clock regulation begins with the core clock genes (*clock* and *arntl*), which activate transcription of the *per* and *cry* repressor genes (43, 44). *Clock* and *arntl* also activate transcription of the *rev-erb* and *ror* genes in a second regulatory loop. Finally, *clock* and *arntl* regulate the transcription of *nfil3* and *dbp* in a third regulatory loop. These genes all function in a transcriptional autoregulatory feedback loop throughout the body.

As described above, 3MM mice were treated with lactulose or PBS between zeitgeber time 3-4 and euthanized five hours later. Tissue was harvested from the ileum, cecum, and colon for RNA extraction and RT-PCR. The gene expression of core clock genes was compared between the two treatment groups. When 3MM mice were treated with lactulose, there was a significant increase in several core clock genes in the ileum tissue, but no significant difference in gene expression in cecum and colon tissues, where most microbial metabolism occurs (Figure 3B and S3A). These differences in expression reflected the fluctuations seen in peripheral tissues in the normal mouse circadian rhythms (45). To control for the direct effect that lactulose may have on the host, this experiment was also conducted in germ-free mice. When germ-free mice were treated with lactulose, there was no significant increase in core clock genes in any of the studied tissues, indicating that microbial metabolism induced changes in the peripheral circadian clock (Figure 3B and S3B). Furthermore, to test whether this effect was translatable to a complex microbiota, this experiment was conducted in SPF mice. When SPF mice were treated with lactulose, there was a significant decrease in *cry1* expression in the ileum, indicating that the effect of microbial metabolism on SPF clock gene expression was diminished (Figure S3C).

To evaluate the extent of the effect of acute microbial metabolism on clock gene expression, we measured expression in ileum tissue at several time points over 24 hours following lactulose or PBS treatment in 3MM mice (Figure 3C). We found that gene expression of *cry1*, *cry2*, and *per2* was significantly different after treatment. This finding indicates that the effect of lactulose on microbial metabolism also resulted in an effect on host clock gene expression.

Inducing microbial metabolism decreases host food intake in following active cycle

We then tested whether increasing host clock gene expression through specific alteration of gut microbial metabolism has further effects on the behavioral rhythm of the host. We measured the rate and total amount of 3MM mouse food intake in the dark cycles before and after lactulose treatment. The dark cycle preceding treatment was used to ensure normal feeding behavior for each mouse and provided an undisturbed baseline measurement. Lactulose or PBS treatments were then administered as before at zeitgeber 3-4, and food intake was measured again in the

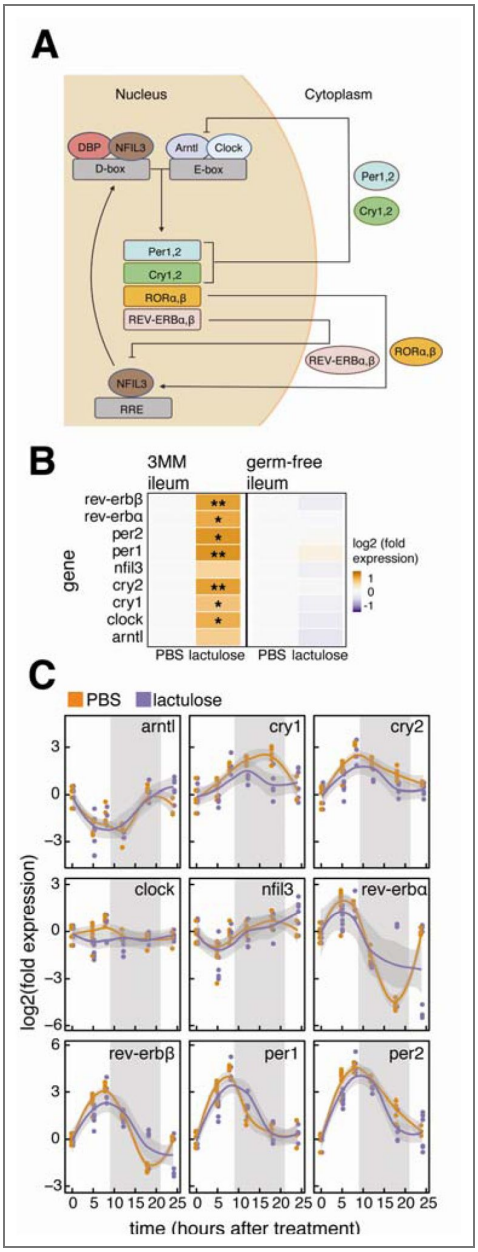


Figure 3. **Lactulose feeding acutely alters clock gene expression in small intestinal tissue.**

(A) Schematic of the clock gene network in mice. **(B)** Clock gene expression in ileum tissue in 3MM (N=10 for lactulose treatment, n=7 for PBS treatment) and germ-free (n=8 in each group) mice 5h after treatment, measured via qPCR. Fold change values are relative to the average of the respective PBS control groups. Statistical significance was tested using upaired t-tests and Benjamini-Hochberg corrected for multiple testing (* and ** indicate p<0.05 and p<0.01, respectively). **(C)** Clock gene expression in ileum tissue of 3MM mice during 24h after treatment (treatment starts at time 0h). All gene expression data was normalized to samples collected from untreated mice at zt3 (control). Expression differed significantly between treatment and control groups for cry1, cry2, and per2 (two-way ANOVA (time, treatment), p<0.05).

following dark cycle and compared to the baseline food intake in the previous dark cycle. 3MM mice treated with lactulose reduced both their food intake rate and total food intake in the following active cycle, while there was no significant reduction in food intake in 3MM mice that were treated with PBS (Figure 4A and S5A). We subsequently continued to measure food intake in 5 lactulose-treated mice and 5 PBS-treated mice for a second active cycle, and found that the decrease in food intake did not persist, indicating an acute effect of microbial metabolism on food intake (Figure S5B). When repeating this experiment with treatment during the active phase at zt=14-15, we did not see a significant change in food intake in the dark cycle after treatment (Figure S5C, D).

We repeated this experiment in GF mice and SPF mice and did not find any significant reduction in mice treated with lactulose (Figure 4B and S5E, F). The non-significant trend towards lower food intake in lactulose-treated GF mice can potentially be explained by the viscosity of the lactulose solution; viscous solutions given to mice via oral gavage have been shown to decrease food intake (46). However, the decrease in food intake is small in GF mice compared to 3MM mice, indicating that actuation of microbiota metabolism is the dominant cause for this of this effect.

To evaluate the mechanism by which microbial metabolism decreases food intake in 3MM mice, we allowed 3h for gene expression changes to take place in peripheral tissues after the peak of bacterial activity at 5h after lactulose administration. We thus measured circadian clock gene expression in the 3MM hypothalamus and liver 8h after lactulose or PBS treatment (Figure S6A). We did not find any significant change in clock gene expression in either of these organs, indicating that the mechanism does not lie in a systemic shift in circadian clock gene expression in hypothalamus or liver, at least at the timepoint we investigated.

Disruption of food intake patterns is due to systemic effects of microbial metabolic products

Microbial metabolism of lactulose in the mouse takes place largely in the cecum and colon (47). However, core clock gene expression was most significantly altered in the ileum when mice were administered lactulose. These mice then consumed less food following treatment, implying that the effect of microbial metabolism on the host is systemic and not localized to the point of metabolic processes (Figure 3B and 4A). To test the systemic effect of microbial metabolism on host food intake, 3MM mice were administered a fermentation product mix of succinate, acetate, and butyrate, as these were the fermentation products that were significantly produced by the 3MM in the cecum after lactulose treatment (Figure S2B). Orally administered fermentation products are taken up by the host in the small intestine, before reaching the cecum (48). 3MM mice reduced their rate of food intake and their total food intake following the treatment of fermentation products, indicating that the effect of the microbiota on host circadian behavior is systemic (Figure 4C). When we repeated this experiment in SPF mice, we did not find a significant decrease in food intake following fermentation product treatment (Figure S6B). This is possibly due to uptake efficiency differences in the SPF small intestine compared to the 3MM mouse, or due to differences in metabolic signaling processes between these mice.

Providing calories in the form of fermentation products is one potential mechanism by which the microbiota regulates host food intake systemically (49). To evaluate whether the reduction in host food intake was solely due to the supplemental energy provided by the fermentation products, we estimated the total number of kilocalories that were taken up by the host before and after the treatment with fermentation products, assuming that all orally administered fermentation products serve as an energy source (0.19 kcal). We found that mice reduced their food intake by more than these 0.19 kcal, going beyond compensation for the additional energy provided by the microbiota (Figure 4D).

To test if this effect of the fermentation products could be observed when they were not administered orally but produced by microbial metabolism, we estimated the calories in fermentation products produced by the microbiota after lactulose treatment (Figure 4B). To get an upper bound for this number, we assumed that the energy in fermentation products has to be

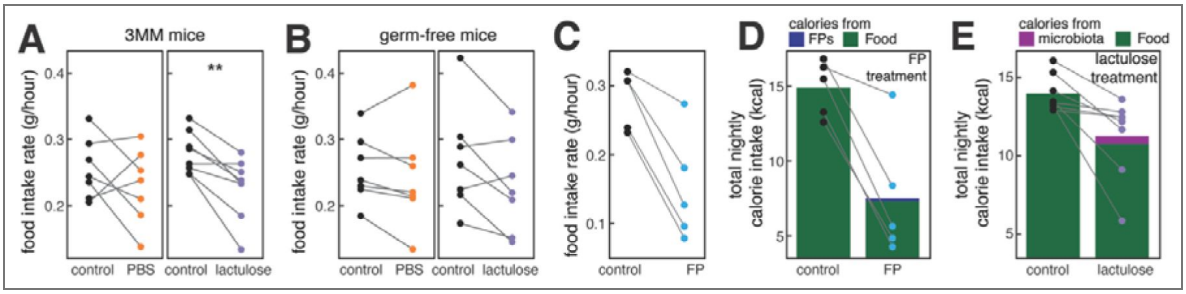


Figure 4. Lactulose treatment decreases host food intake in the following active cycle.

(A) Food intake rate in 3MM mice in the dark phase prior to treatment (control) and following treatment (treatment), after lactulose (n=8) and PBS (n=7) treatment. Food intake is significantly reduced after lactulose treatment (paired t-test, $p<0.01$). **(B)** Food intake rate in germ-free mice after lactulose (n=5) and PBS (n=4) treatment. Food intake is not significantly different between groups (paired t-test). **(C)** Food intake rate of 3MM mice during the dark phase prior to treatment with fermentation products (control) and the dark phase after treatment (treatment). Food intake is significantly reduced after treatment (paired t-test, $p<0.01$). Treatment was done by oral gavage of 66mg of fermentation product in 100µL of PBS, with a 4:3:3 ratio of sodium succinate, sodium acetate, and sodium butyrate, respectively. **(D)** 3MM mouse total calorie intake after accounting for the calories directly provided by fermentation products in treatment. Calory intake remains significantly reduced after treatment when calories provided by the treatment are considered (paired t-test, $p<0.05$). **(E)** 3MM mouse total calorie intake after accounting for calories provided by the microbiota after lactulose metabolism. Calory intake remains significantly reduced after lactulose treatment when calories provided by microbial metabolism are taken into account (paired t-test, $p<0.05$).

lower than the combustion enthalpy of the fed lactulose (0.52 kcal). We found that 3MM mice reduced their food intake following lactulose treatment by even more than these 0.52 kcal, showing that microbiota metabolism affects host behavior through a mechanism other than energy homeostasis of the host (Figure 4E).

To understand the mechanism by which microbial metabolism regulates host food intake, we measured various satiety-associated hormones in 3MM, SPF, and GF mice. We measured concentrations of the hormones PYY and GLP-1, known to be associated with gut microbiota changes (50, 51), in portal vein plasma 5h after treatment with PBS or lactulose. We found no significant difference in PYY or GLP-1 concentrations between control and treatment groups in 3MM, GF, or SPF mice 5h after treatment (Figure S7A, B). We also measured ghrelin and leptin concentration in cardiac serum of 3MM and GF mice 5h after PBS or lactulose treatment and found no significant effect of lactulose on their concentrations in 3MM mice 5h after treatment (Figure S7C, D). There was a small but significant increase of ghrelin concentration in lactulose treated GF mice, potentially due to the osmotic effect of lactulose increasing hunger signaling (Figure S7C).

Discussion

The gut microbiome and host circadian clock are intricately linked, with evidence suggesting that the host circadian clock can influence the gut microbiota and vice versa (34, 52). In this study, we show that the microbiota regulates host circadian clock gene expression and feeding behavior through metabolic rhythmicity. We first demonstrated that the EAM gut microbiota oscillates metabolically in a periodic manner which is closely associated with the periodic food intake of the host. Similar rhythmic oscillations in bacterial metabolism have been found independently of microbiota complexities (18, 35, 53). We disrupted the metabolic rhythmicity of the 3MM by providing a nutrient that is available only to the microbiota during the host's inactive period, during which food intake is typically low (Figure 1C). We observed an acute increase of microbial metabolism following one treatment of lactulose that was comparable to normal production during the host's active state, simulating host food intake for the microbiota (17). Consequently, we observed an increase of expression of several clock genes in the ileum tissue, some of which persisted for several hours (45).

Most microbial metabolism takes place in the cecum, and interestingly, we found no change in cecum tissue gene expression after treating 3MM mice with lactulose. This may indicate a systemic effect of microbial metabolism on the host, rather than a localized one. In fact, a systemic relationship between the gut microbiota and small intestinal epithelial rhythmicity has previously been described as it was found that altering the microbiota through diet causes changes in small intestinal immune function (54, 55). The systemic effect of the microbiota on the host clock gene expression likely influenced host food intake following the lactulose treatment. We observed an increase in fermentation product concentration in the mouse cecum after administering lactulose to 3MM mice, and a subsequent decrease in food intake in the following active cycle. We observed a similar decrease in food intake when we administered a mix of fermentation product directly to the host, indicating that microbial metabolism is in part regulating host feeding behavior. One possible mechanism by which this may occur is through microbial feeding of the host (49). The microbiota regulates host appetite through energy harvesting of inaccessible fibers: GF mice eat more food in a day than colonized mice to compensate for the reduced energy extraction in the large intestine that is otherwise provided by the microbiota (35). By treating 3MM mice with lactulose, we assumed that the host would receive energy indirectly from microbial fermentation, and that the host would reduce its food intake in the following dark cycle by the same number of kilocalories. However, after accounting for the energy that the microbiota was providing the host through lactulose metabolism, we still observed a reduction in total energy intake in mice. We observed similar results when we provided the host directly with fermentation products. This finding indicates that providing energy is not the only mechanism by which microbial metabolism reduces host feeding, and that there is a further mechanism that regulates the host's decrease in total energy harvest.

Surprisingly, when we repeated our experiments in SPF mice we did not observe the same results as in 3MM mice. First, we did not measure the same changes in clock gene expression in mice that were treated with lactulose. Furthermore, mice treated with lactulose did not decrease their food intake in the following active cycle. We hypothesized that the microbial composition of the SPF mice resulted in the utilization of fermentation product that were generated after lactulose treatment. However, direct treatment of SPF mice with fermentation product also did not decrease their food intake, indicating that this was not the case. One possible explanation for this difference might be that SPF mice have decreased sensitivity to changes in fermentation products, and that further lactulose doses would induce similar changes in clock gene expression and food intake in SPF mice. Alternatively, there might be a signaling factor in the SPF microbiota that is absent in the 3MM, which can block the systemic effect of fermentation products downstream of uptake into the systemic circulation.

The experimental approach of using lactulose feeding to acutely induce microbial metabolism also has its limitations. Lactulose can have an osmotic effect, which we found to be dependent on microbiota complexity (Figure S3), and could possibly also have microbiota independent effects on the host. While we tried to control for these effects by ensuring that the osmotic effect in our sytem was minimal (Figure S2C-E), it would be desirable to use different methods of specifically actuating microbiota metabolism in future studies. One possibility could be the use of genetic systems that can be activated by experimentally tunable chemical inducers (56), which may avoid the more complex physiology of lactulose metabolism.

Administration of fermentation products may have a systemic effect on the host through various mechanisms, and there is an association between fermentation product intake and a reduction in food intake (51, 57–59). Butyrate dietary supplementation in mice decreases food intake and promotes fat oxidation, and acetate supplementation can decrease appetite directly (60, 61). It has also been found that dietary supplementation of fermentable carbohydrates which can be broken down by the microbiota result in changes in regulation of satiety hormones which are produced in a circadian manner (50, 62–64). However, we did not find any of these hormones to be acutely altered 5h after treatment of mice with lactulose, indicating that the systemic effect of microbial metabolism on food intake might involve a different mechanism. However, it remains possible that changes in hormone levels happen at a different time point, and a systematic analysis of microbial effects on the temporal development of hormone levels would be of intertest. It has previously been found that mice with gene deletions in the circadian clock lose rhythmicity in food intake and fermentation product concentration in the cecum, indicating the intricate association between the host circadian rhythms and the microbiota (65–67). *Cry1* and *cry2* were upregulated in the ileum for several hours after lactulose treatment, and are known to decrease insulin resistance through the reduction of hepatic gluconeogenesis (68–71). Insulin is a hormone that is also associated with satiety, and decreased insulin resistance may have increased satiety in lactulose treated mice (72). However, there was no significant change in *cry1/2* in the 3MM liver. It is therefore possible that the changes in expression in the ileum resulted in a signaling mechanism to the liver which resulted in acute changes in glucose metabolism and insulin production.

In conclusion, our work suggests that gut microbiota affects host food intake through the modulation of the circadian clock. We propose a mechanism in which the microbiota breaks down dietary fibers ingested by the host and the metabolic products of microbial metabolism regulate host circadian clock gene expression, which in turn regulates eating behavior. While our results demonstrated an acute effect of diet on the host due to the rapid decoupling of diurnal rhythm and diet, we speculate that the chronic effect of the microbiota on the host through the usual diet of the mouse extends to factors associated with host circadian rhythms such as endocrine signaling, and that the microbiota plays a significant role in maintaining regular satiety-associated behavior through its metabolic byproducts.

Methods

Mice

All strains and resources are listed in the key resources table.

Wild type C57BL/6 mice were maintained on a standard diet at the ETH Phenomics center. Mice were re-derived Germ-free or bred with the 3MM microbiota and kept under strict hygienic conditions in breeding isolators. The 3MM microbiota was derived using strains *Bacteroides thetaiotaomicron* (73), *Eubacterium rectale* ATCC, and *Escherichia coli* HS (74). Specific pathogen free (SPF) mice were bred and housed in individually ventilated cages. All experiments began with 9-15 weeks of age with mixed-sex groups. Mice were fed autoclaved mouse chow (Kliba Nafag 3807) *ad libitum* for the duration of all experiments and were kept with a dark and light period of 12 hours each. All animal experiments were approved by the Swiss Kantonal authorities (License ZH120/19 and ZH016/21) according to the legal and ethical requirements.

Metabolic cages

At the start of the experiment, mice were transferred from the breeding isolators to the isolator-housed TSE PhenoMaster (TSE Systems) system and were single housed for the duration of the experiment. Mice were acclimated for 4 days after transfer to the metabolic cages. The TSE PhenoMaster system allows measurements of oxygen, carbon dioxide, and food and water consumption for 160 seconds in 24-minute intervals. Eight metabolic cages were housed in two sterile isolators, and each cage was connected to the system through a HEPA filter. Hydrogen in each cage was measured through a hydrogen sensor (SGP30, Sensirion) which was attached to the TSE Phenomaster system. Air flow in each cage was set to 0.4 L/min. A two-point calibration of all gases using reference gases was performed within 24 hours of each experiment. The TSE Phenomaster data and hydrogen data were combined in the data analysis post-experiment.

Mouse experiments

On the fifth day after mouse transfer to the TSE Phenomaster, mice were treated by oral gavage with either 132mg Lactulose in 100µL PBS, or 100µL PBS. In cases where 2 lactulose doses were given, the second dose of 132mg was administered 1h after the first. The oral gavage was administered between Zeitgeber 3-4, except when otherwise noted. To measure circadian rhythm changes in the host and changes in the gut, mice were euthanized 5 hours after the gavage. To measure the changes in clock gene expression over 24 hours, mice were euthanized 8 hours, 12 hours, 18 hours, and 24 hours after the gavage. To measure the effect of lactulose on host food intake, mice were euthanized the day following the treatment or two days following treatment. To measure the effect of fermentation product on host food intake, mice were treated between Zeitgeber 3-4 with 66mg of fermentation product in 100µL of PBS, with a 4:3:3 ratio of sodium succinate, sodium acetate, and sodium butyrate, respectively. Mice which did not eat anything in the 24 hours following oral gavage were excluded from the study.

Bacterial quantification

Mice were euthanized when a peak in hydrogen production was observed following gavage. Wet feces or cecum content were collected from mice and weighed for bacterial quantification. Bacterial DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen). qPCR was performed using the FastStart Universal SYBR Green Master Mix (Roche). Primers for each tested strain were diluted to a final concentration of 1µM. DNA was amplified using a QuantStudio 7 Flex instrument (Applied Biosystems) or a StepOnePlus Real-Time PCR System instrument (Applied Biosystems), with an initial denaturation step of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Bacteria were quantified in each run by comparing the obtained Ct values to a standard curve of Ct values for bacterial DNA from a known bacterial cell count in the respective strain, which was extracted using the same protocol. Germ-free cecum content and water samples were used to generate a detection limit. For each experiment, we included a quality

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial Strains		
B. theta Δtdk	Martens et al. 2009	N/A
E. coli HS	Desai et al. 2016	N/A
E. rectale	ATCC	33656
Chemicals, Peptides, and consumable materials		
BHI	Oxoid	Cat. CM1135
L-Cysteine	Merck	Cat. 168149-25G
Lactulose	Sigma	Cat. 61360-25G
PBS pH 7.4	Gibco	Cat. 10010-015
Sodium acetate	Merck	Cat. 1.06268.0250
Sodium butyrate	Sigma	Cat. 303410-100G
Sodium succinate dibasic Hexahydrate	Merck	Cat. S2378-100G
Isopropanol	Fisher chemical	Cat. A461-212
Formic acid	Sigma	Cat. 1.00264
Acetonitrile	Fisher chemical	Cat. 10001334
Trizol	Life technologies	Cat. 15596018
Chloroform	BDH chemicals	Cat. 22711.290
Ethanol	Emsure	Cat. 1.00983.1000
RNAse inhibitor	Life technolog	Cat. AM2694

Key resources table.

	ies	
0.1 mm beads	OPS diagnostics	Cat. PFMB 100-100-12
1.0mm silicon carbide beads	Biospec	Cat. N11079110sc
DPP IV Inhibitor	Sigma	Cat. DPP4
Critical Commercial Assays		
DNeasy PowerSoil Pro Kit	Qiagen	Cat. 47014
Direct-zol RNA kit	Zymo	Cat. R2050
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat. 4368814
Mouse PYY ELISA kit	Crystal chem	Cat. 81501
Mouse GLP-1 ELISA kit	Crystal chem	Cat. 81508
Mouse Ghrelin ELISA kit	Sigma	Cat. EZRGRT-91K
Mouse Leptin ELISA kit	Sigma	Cat. EZML-82K
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Jackson Laboratories; bred at EPIC mouse facility of ETH Zurich, Switzerland	Jax Strain#: 000664, RRID: IMSR_JAX:000664
Oligonucleotides		
B._theta_fwd : TACTCGCCTCTTTGCAACCCTACC	Steimle et al. 2021	
B._theta_rev : GGCCCCAGATCCGAACAACAC	Steimle et al. 2021	

Key resources table. (continued)

E._coli_fwd : GGTACAACAGGCGTTATTGTATC	This study	
E._coli_rev : CGAAAGCACC-GATCTTCTT	This study	
E._rectale_fwd : GGTGGCTGGGTGATGTAAAACTGA	This study	
E._rectale_rev : ACCGCCGAGCAAAATGAAGC	This study	
B._coccoides_fwd : GAAGAGCAAGTCTGATGTGAAAGG	Brugiroux et al. 2016	
B._coccoides_rev : CGGCACTCTAGAAAAACAGTTTCC	Brugiroux et al. 2016	
RPL19_fwd: GAAGGTCAAAGGGAATGTGTTCA	Cuddihey et al. 2022	
RPL19_rev: CCTTGTCTGCCTTCAGCTTGT	Cuddihey et al. 2022	
Arntl_fwd: TGCAGGAAGCTCATTGG	This study	
Arntl_rev: TGTCGAGCGAAAACCAG	This study	
CLOCK_fwd: GTGGAAGTCCGAGGTAG	This study	
CLOCK_rev: GCAATAGAAAACACCAGAGTC	This study	
REVERBα_fwd: CCCCTTTATTCTAATCATGCC	This study	
REVERBα_rev: CGACACAAAGTAGTACGTTG	This study	
REVERBβ_fwd: GCAGTGATGACCCTTATACC	This study	

Key resources table. (continued)

REVERBβ_rev: CGGCAATGACTAAGAAGGAC	This study	
Nfil3_fwd: CTTTCAGGACTACCAGACATCCAA	Wang et al. 2017	
Nfil3_rev: GATGCAACTTCCGGCTACCA	Wang et al. 2017	
Cry1_fwd: GGCCACATTCGTCATAATTG	This study	
Cry1_rev: CCCCAGTGTGCAAAG	This study	
Cry2_fwd: GGATCCCTGTGTAGTTATAACG	This study	
Cry2_rev: CCTGGGAGTTATCACCG	This study	
Per1_fwd: ATGAAGCAGACAATTAAGGC	This study	
Per1_rev: CCCCAACTCTATCGCTATTC	This study	
Per2_fwd: TGCTCAGGACTTGTGTAGG	This study	
Per2_rev: CTGGTCACTGAACATTAGC	This study	
Analysis Software		
R studio V4.2.0		https://cran.rstudio.com/
Skyline V21.1		https://skyline.ms/project/home/software/Skyline/begin.view

Key resources table. (continued)

control for gnotobiosis, using *Blautia pseudococcoides* as an indicator strain for contaminations. This strain is present in our mouse colony, forms spores, and has been the most likely contaminant in our gnotobiotic sytem. Consequently, probes for *B. pseudococcoides* were included in the qPCR assays, and mice with a C_t lower than 26 in these runs were excluded from the study.

Cecal pH measurement

Cecum content was collected in a 2mL Eppendorf tube (Sarstedt) and snap frozen in liquid nitrogen 5 hours after treatment. At the time of measurement, cecum content was thawed and spun down at 1000rpm for 30 seconds to accumulate all content at the bottom of the tube. A pH sensor (Sevencompact S220, Mettler Toledo) was completely inserted into the cecum content and pH was measured.

Short chain fatty acid quantification in cecum content

Cecum content was collected in a 2mL Eppendorf tube 5 hours after treatment, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. For UPLC/MS, cecum content was thawed on ice, then homogenized in 70% isopropanol and centrifuged. The supernatant was used for fermentation product quantification using a protocol previously described (75). A 7-point calibration curve was generated with fermentation product in known concentrations. Calibrators and samples were spiked with a mixture of isotope-labeled internal standards, derivatized to 3-nitrophenylhydrazones, and then quenched with 0.1% formic acid.

An ACQUITY UPLC system (I-Class, Waters, MA, USA) coupled with an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Scientific) were used for UPLC/MS analysis. To quantify fermentation products, a Kinetex 2.6 µm XB-C18, 50 × 2.1 mm column (Phenomenex) and a flow rate of 250 µL/min was used with a binary mixture of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient starts from 90% of A, then gradually decreases to 75% of A within 6 min and then to 0% of A within 1 min. Then a 0% of A is kept for 1 min and a 90% of A is kept for 1 min to restore the initial solvent ratio. The column was kept at 40°C and the autosampler at 5°C. Acquired raw data were imported to Skyline (V21.1) and MS2 peaks were integrated. A calibration curve for each compound was built based on the ratio between peak area of unlabeled and labeled form using custom R code. Limit of blank was calculated as mean of the concentration detected in technical blank samples ±1.645 standard deviation.

Short chain fatty acid quantification in medium

All EAM strains were grown to stationary phase in BHI medium supplemented with 1g/L L-cysteine and 2.5g/L hemin. After growth, strains were subcultured into 10mL of Epsilon medium (50mM NaCl, 0.02M NH_4Cl, 0.028M K_2HPO_4, 0.072M KH_2PO_4, 50µM $MnCl_2x4H_2O$, 50µM $CoCl_2$, 0.4mM $MgCl_2x6H_2O$, 0.5mM $CaCl_2x2H_2O$, 4µM $FeSO_4x7H_2O$, 20mM $NaHCO_3$, 5mM L-cysteine, 1.2mg/l hemin, 1mg/l menadione, 2mg/l folinic acid, 2mg/l vitamin B12) with 20mM lactulose as a carbon source and grown overnight. Medium for growing *B. theta* and *E. rectale* was supplemented with 1% tryptone, and medium for growing *E. coli* was not supplemented with tryptone. 1ml of culture was extracted and filtered in a 0.22µm filter, and fermentation product quantification was performed as previously described (76). In short, isocratic HPLC was performed in on a Thermo Scientific Ultimate 3000 (Thermo) using 2.5mM H_2SO_4 as mobile phase at a 0.4ml/min flow rate. 20µl of sample was injected, separated over a Phenomenex Rezex RoA organic acid H+ (8%) column kept at 40°C, and compounds were detected using a refractive index detector. Data was recorded for 40min after injection for each sample. Data was analyzed using Python and the package hplc-py (77).

Determining water content of feces and cecum content and total fecal output

Fresh fecal pellets and cecum content were collected 5 hours after treatment and the wet weight was measured. Samples were then lyophilized (Alpha 2-4 LD plus, Martin Christ GmbH) for 48 hours and dry weight was measured. The ratio of dry weight to wet weight was calculated to

estimate the water content in the feces and cecum content after the gavage.

For measuring total fecal output after gavage, the bedding in each cage was replaced at the time of the oral gavage. At the end of the experiment, all fecal pellets were collected from the bedding in each cage, and lyophilized to determine the total fecal dry weight that was produced since lactulose gavage. This dry weight of the feces was measured and was then normalized by the water content in fresh feces collected from each mouse to estimate total fecal wet weight produced per animal in the respective time window. For this measurement, we disregard coprophagia, which might lead to a slight underestimation of total fecal output.

RNA extraction

Mouse ileum, cecum, and colon tissues were collected in RNAlater (Thermo Scientific) and snap frozen in liquid nitrogen. Samples were stored at -80°C until further analysis. For RNA extractions, samples were thawed on ice and weighed. RNA was extracted using a modified protocol of the Direct-zol RNA kit (Zymo). Samples were mixed with Trizol (Thermo Scientific) and incubated for 5min at room temperature. Samples were then homogenized twice with 0.1mm beads (BioSpec Products) and 1.0mm silicon carbide beads (BioSpec Products), for 90s at 30Hz. Further Trizol was mixed with the samples, and centrifuged. Supernatant was mixed with chloroform (Sigma Aldrich) and centrifuged. Supernatant was then washed in 100% ethanol and centrifuged in collection tubes. RNA was washed in Pre-wash buffer and incubated for 15min at room temperature with 60 units of DNAse. Finally, RNA was washed in Wash buffer and eluted in Nuclease-free water (Invitrogen).

RNA concentration and quality were measured using a nanodrop machine (ND-1000 Spectrophotometer, Thermo Scientific) and RNA was diluted to a final concentration of 20ng/µL in nuclease-free water. Reverse transcription was done using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was diluted 1:25 in nuclease-free water for qPCR.

Circadian clock gene expression

Host circadian clock gene primers were used to estimate expression using RT-PCR. A housekeeping gene (rpl19) was used for normalization. The RT-PCR was carried out as previously described CITE, and sample extracted RNA was included in a reaction with the housekeeping gene to control for DNA contamination.

GLP-1 and PYY measurements

Mice were anaesthetized with isofluorane before exsanguination and tissue sampling. Portal vein blood was collected. Plasma was isolated and immediately transferred to tubes with 20µL of DPP IV inhibitor (Sigma Aldrich) and immediately flash frozen. GLP-1 and PYY measurements were done using an ELISA kit (Crystal Chem), according to the manufacturer's instructions.

Leptin and Ghrelin measurements

Mice were euthanized using CO_2. Blood was collected by cardiac puncture, and serum was collected and flash frozen. Leptin and total Ghrelin measurements were done using an ELISA kit (Sigma Aldrich), according to the manufacturer's instructions

Data analysis

Phenomaster quality control

For all measurements, a variable of zeitgeber time (h) was created where 0-12 represents the light phase and 12-24 represents the dark phase animals experience in the animal facility. The day number of the experiment was calculated based on the zeitgeber time of the first measurement. Each new day began at zeitgeber time 0, at the start of the light cycle. To account for faulty measurements due to disruptive events and measurement noise, some datapoints were excluded from the raw dataset: food intake above 1g and water intake above 1ml in one 24-minute interval for one mouse was considered to have been caused by human disruption and was excluded, food

intake of 0.01g as well as negative food and drink values were considered measurement noise and were excluded; for each mouse, the interquartile range of food and water intake measurements was calculated, and values greater than the 75th percentile + 1.5 times the interquartile range were considered outliers and excluded from the dataset. Potential sources of outlier measurements include food and water loss during handling of the experimental setup, as well as leaky water bottles. We analyzed whether the probabilities for excluding values was the same between the experiments show in in Figures 4A/S5A, 4B/S5B, and S5F, and found that they were significantly different (Chi-square test, p=3.9 x 10^{-18}), which could be due to differences between the experiments in the number of times cages were handled. Exclusion probabilities between treatment and control were significantly different in Figure 4A/S5A (Fisher's exact test, p= 1.7 x 10^{-6}), but not significantly different in the experiments shown in Figures 4B/S5E and S5F (Fisher's exact test, p=0.71 and p=0.10, respectively). While we cannot strictly rule out any influence of our data exclusion strategy, these results speak against a systematic effect of treatment on behavior that would lead to changes the amount of excluded data points. After data cleanup, mice which had a total daily food intake difference of more than 1g in the two days prior to treatment were considered not acclimated to the metabolic cages and were excluded from further analysis.

Hydrogen sensor data

The hydrogen sensors attached to the TSE Phenomaster system (Sensirion SGP30) measured hydrogen every second, and hydrogen measurements were recorded in a separate file. The median and maximum hydrogen values for each experimental box and reference box measurement were calculated. Median hydrogen values were used for further analysis. If the reference box measurements remained visually constant for the duration of the experiment, the hydrogen data was accepted and merged with the Phenomaster data for further analysis.

Metabolic cage statistical analysis

For estimating daily mouse food intake and hydrogen production, data was extracted from the raw dataset in the two days prior to mouse treatment. For each mouse, the average hydrogen value and food intake measurement per timepoint was calculated. A general additive model was used to fit a curve (using R function mgcv::gam) to these points using the formula y~s(x; bs="cs).

For estimating food intake per day, data was extracted from the beginning of the day prior to mouse treatment to the end of the first dark cycle following treatment, resulting in data from two dark cycles. Cumulative food intake and total food intake were calculated per mouse for each dark cycle, and food intake rate was calculated as the slope of the linear regression of cumulative food intake. A paired T-test was used to compare food intake rate and total food intake for each mouse in the dark cycle prior to treatment and in the dark cycle following treatment.

Energy harvesting estimations

For estimating the indirect caloric intake of the EAM mouse in the lactulose treatment, we made a deliberate overestimation, assuming that the entire energy contained in the administered lactulose would reach the mice. We estimated the combustion enthalpy of lactulose to be the same as that of lactose (1346 kcal/mol), and the combustion enthalpy of 132mg (0.39mmol) of lactulose is therefore 0.52kcal (78). For estimating the caloric intake of the EAM mouse in the fermentation product treatment, it was assumed that 100% of fermentation product given to the host were used for energy harvesting. The combustion enthalpies and masses provided to the host of acetate (0.21kcal/mmol, 19.8mg), butyrate (0.52kcal/mmol, 19.8mg), and succinate (0.36kcal/mmol, 26.4mg) were used to calculate the maximum possible calories provided to the host (0.19 kcal).

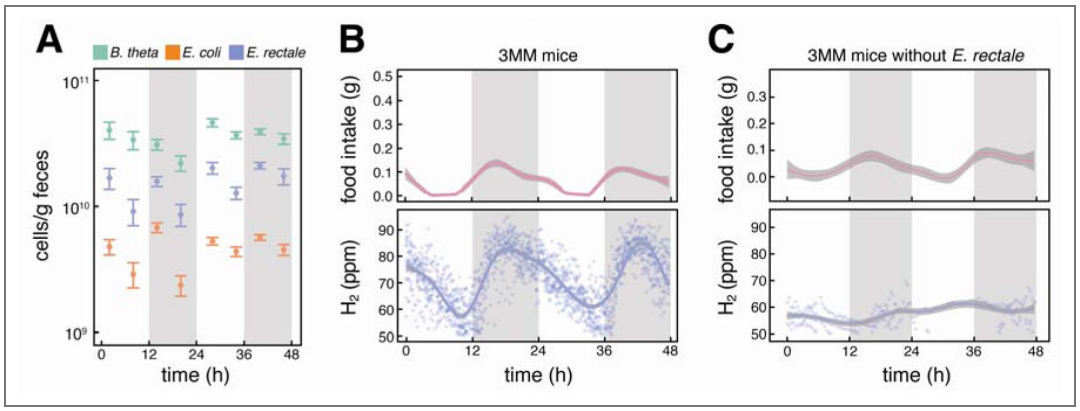
Energy density of mouse chow was previously measured to be 3.94kcal/g [35]. We calculated the total caloric intake per mouse for the dark cycle preceding and following lactulose treatment based on the energy from food intake and indirect feeding from the microbiota and fermentation product treatment. A paired t-test was used to compare caloric intake for each mouse in the dark cycle prior to treatment and in the dark cycle following treatment.

Gene expression

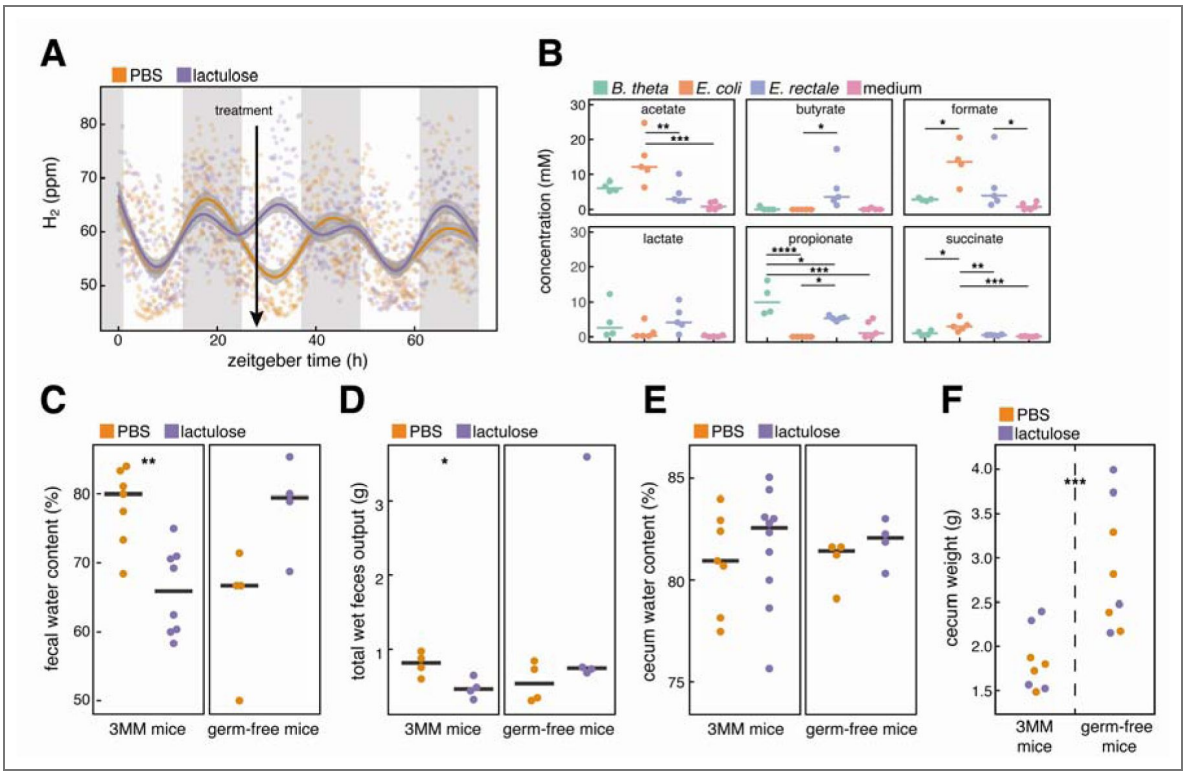
A fluorescence threshold of 0.3 was set for all primers. For data analysis, the $\Delta\Delta C_t$ method was used to estimate relative gene expression for each experiment (79). In all samples, the C_t of the gene of interest was first normalized by that for the housekeeping gene rpl19. For samples collected over 24 hours (data shown in Figure 3C), gene expression values were then normalized to those of untreated mice euthanized at 9AM. For all other samples, gene expression was normalized to the PBS-treated control mice.

For estimating clock gene expression differences in gastrointestinal tissue samples, and for calculating fermentation product concentration in cecum content, unpaired t-tests were used to compare both groups. Benjamini Hochberg multiple testing correction was used on both these datasets. For all further comparisons between treatment groups, unpaired t-tests were used. All data analysis was carried out using R V4.2.0.

Supporting information

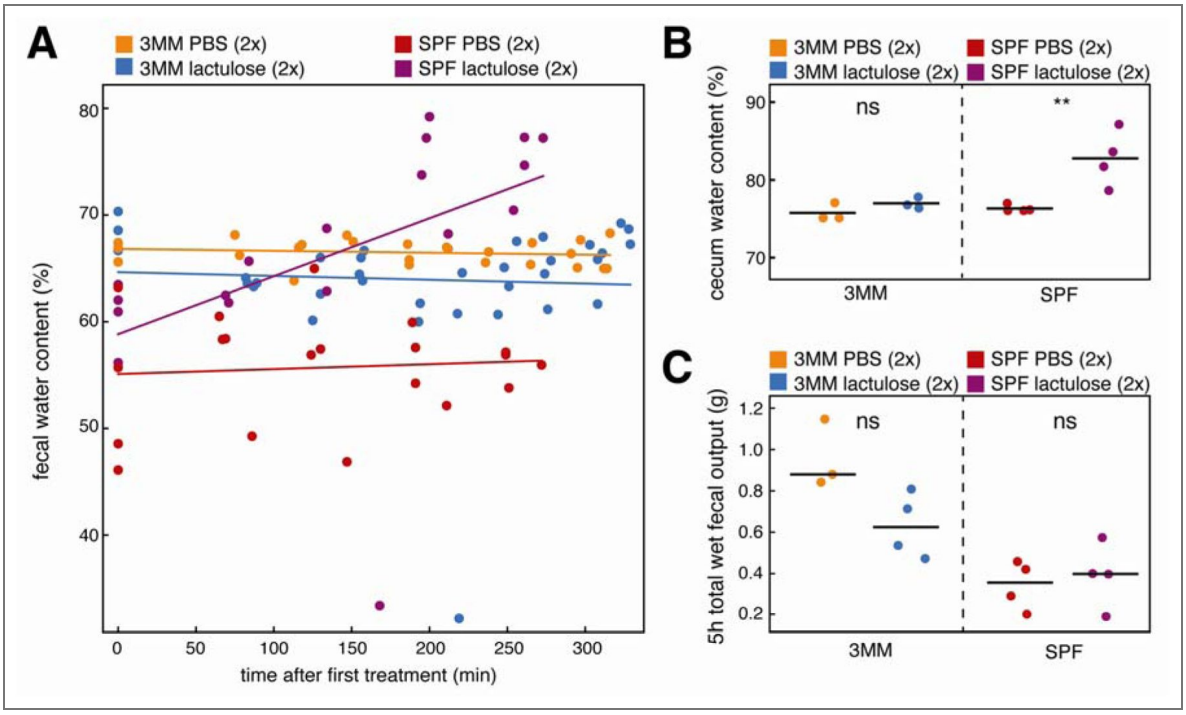


Supplementary Figure 1. ***E. rectale*** **is the principal hydrogen producer in the 3MM mirobiota. (A)** Microbial cell counts based on qPCR in 3MM mouse feces. Fecal pellets were sampled over 2 days in 8 3MM mice. **(B)** Food intake and hydrogen production in 3MM mice (n=16) over two days, with measurements taken every 24min. Trend lines were plotted using the geom_smooth function in the ggplot2 package in R with standard settings, shaded areas around the trend lines represent standard error of the mean. **(C)** Food intake and hydrogen production of gnotobiotc mice colonized with *B. theta* and *E. coli* only (n=4). In all panels, shaded background indicates dark periods in the animal facility, from 6pm (zt=12) to 6am (zt=24).



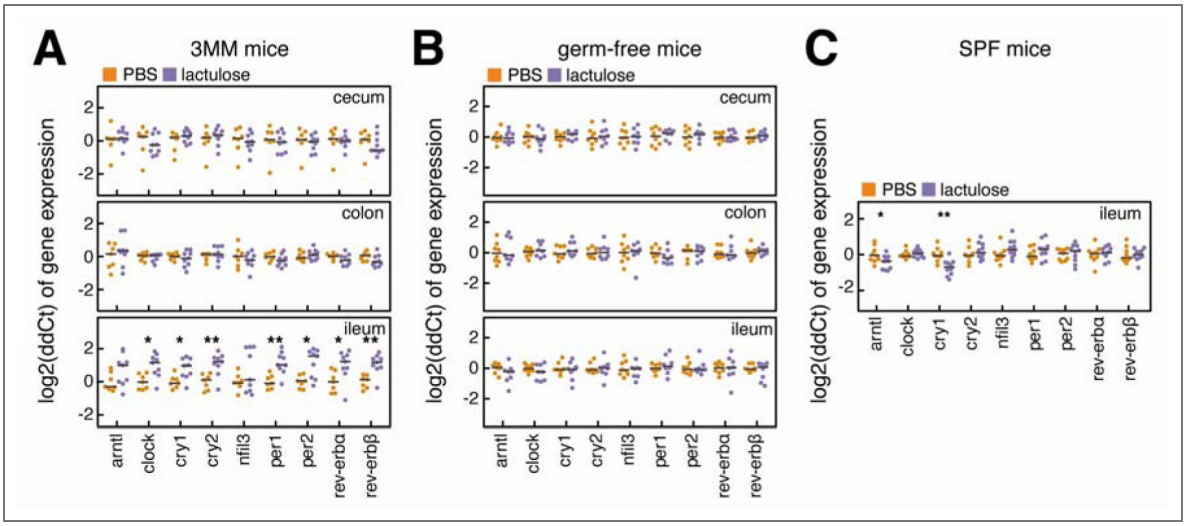
Supplementary Figure 2. Lactulose changes the diurnal pattern of microbial metabolism.

(A) Hydrogen production temporarily increases after treatment with lactulose (n=5), but not after treatment with PBS (n=6), in SPF mice. Treatment is indicated by a black arrow. **(B)** Production of fermentation products by the bacterial strains constituting the 3MM microbiota grown in axenic culture in media with lactulose as carbohydrate source. Measurements were taken in stationary phase (*, **, ***, ****, indicate p<0.05, 0.01, 0.001, and 0.0001, respectively; ANOVA with Tukey's post-hoc test). **(C)** Fecal water content in 3MM mice treated with lactulose (n=8) and PBS (n=7), and germ-free mice treated with lactulose (n=4) and PBS (n=4). The water content decreases significantly upon lactulose treatment in 3MM mice (p<0.01, unpaired t-test). **(D)** Estimated production of total fecal wet weight during 5h in 3MM mice after treatment with lactulose (n=4) and PBS (n=4), and in germ-free mice treated with lactulose (n=4) and PBS (n=4). This data was calculated by experimentally measuring total fecal dry weight and correcting it with the fecal water content determined for the respective microbiota and treatment in **(C)**. Output of fecal wet weight increases significantly in 3MM mice after lactulose treatment (unpaired t-test, p<0.05). **(E)** Water content of cecum content in 3MM mice treated with lactulose (n=10) and PBS (n=7). **(F)** Cecum weight of 3MM and germ-free mice treated with PBS or lactulose. Germ-free mice have significantly higher cecum weights than 3MM mice (p<0.01, unpaired t-test), which already have higher cecum weights than conventionally colonized mice (around 0.5g)(35).



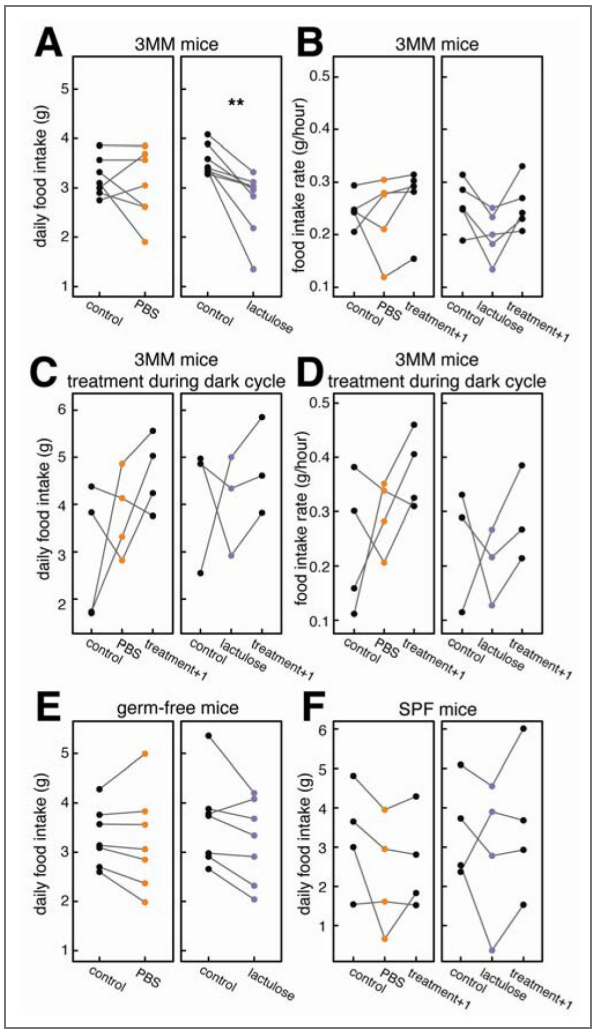
Supplementary Figure 3. Even higher dose lactulose treatment does not lead to an osmotic effect in 3MM mice, but does so in SPF mice.

(A) Water content in fresh fecal pellets collected in regular intervals after PBS or lactulose treatment, in 3MM and SPF mice. Lines indicate linear regression on the data. Fecal water content does not increase after PBS treatment in 3MM or SPF mice. Lactulose treatment does also not lead to an increase in water content in 3MM mice, but did lead to an increase in SPF mice, indicating that the osmotic effect of lactulose depends on the colonization state of the animals. **(B)** Cecum water content 5h after treatment. Only lactulose treated SPF mice showed a significant increase in water content in cecum content 5h after treatment ($p<0.01$, ANOVA with Tukey's post-hoc test). **(C)** Total fecal output during 5h after treatment was not significantly different between groups (ANOVA). This data was collected the same way as in Figure S2D, by collecting all fecal pellets from cage bedding, measuring their dry weight, and correcting it by the mean water content of the fresh feces in the respective group.



Supplementary Figure 4. Targeted induction of microbial metabolism by lactulose treatment acutely alters clock gene expression in small intestinal tissue.

(A) Clock gene expression measured by qPCR in tissue samples of ileum, cecum, and colon of 3MM mice after treatment with lactulose (n=10) and PBS (n=7). Significant differences in gene expression between groups were detected in samples from the ileum (unpaired t-test, multiple testing correction). **(B)** Clock gene expression in tissue samples of ileum, cecum, and colon of germ-free mice after lactulose (n=8) and PBS (n=8) treatment. No significant differences were found in gene expression levels between treatment groups (unpaired t-test, multiple testing correction). **(C)** Clock gene expression in ileum tissue of SPF mice after treatment with lactulose (n=5) or PBS (n=5). Cry1 expression was significantly different between treatment groups (unpaired t-test, Benjamini-Hochberg correction). In all panels, * and ** indicate $p<0.05$ and $p<0.01$, respectively.

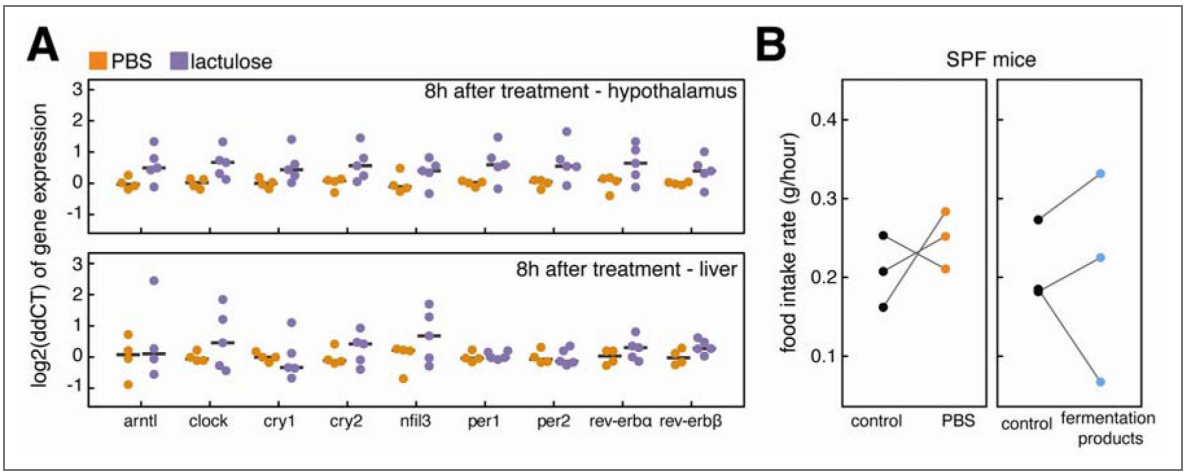


Supplementary Figure 5. Induction of microbial metabolism transiently decreases host food intake in the following active cycle.

(A) Total food intake in 3MM mice before treatment (control) and after lactulose (n=8) and PBS (n=7) treatments. Food intake was significantly lower after lactulose treatment (paired t-test, $p<0.01$). **(B)** Food intake rate in 3MM mice in the dark phase before treatment (control) and the two dark phases following treatment (treatment, treatment+1). The food intake rate is not significantly different between the days (ANOVA). **(C)** Total food intake in 3MM mice before treatment (control) and in the two dark cycles after treatment with lactulose (n=3) and PBS (n=4), when treated at 8:30pm (zt=14.5, 2.5h into the dark cycle). No significant differences were found between groups (ANOVA). **(D)** Food intake rate for the experiment shown in in **(C)**. **(E)** Total food intake in germ-free mice in the dark cycle before treatment (control) and the dark cycle after treatment with lactulose (n=7) and PBS (n=7). No significant differences were found between the days (paired t-test). **(F)** Total food intake of SPF mice before treatment (control) and in the two dark cycles after treatment with lactulose (n=4) and PBS (n=4). No significant differences were found between the days (ANOVA).

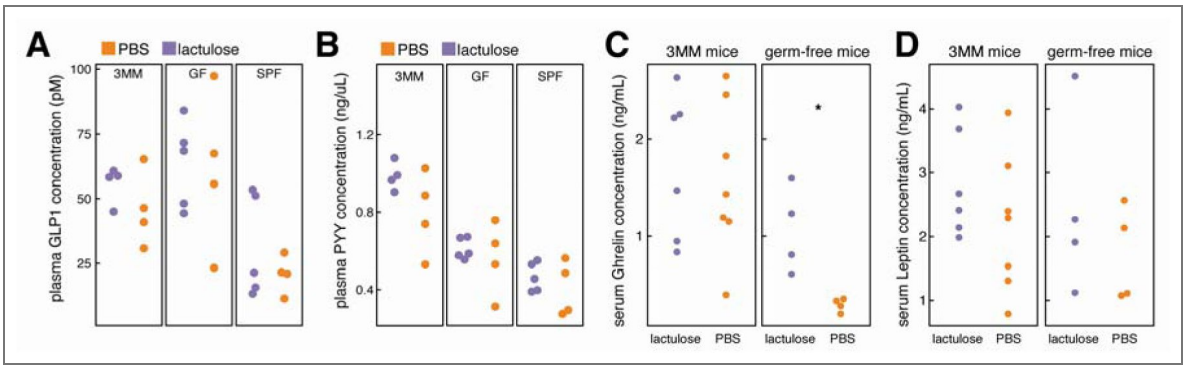
Supplementary Figure 6. Clock gene expressing in perpheral tissues and food intake rates in SPF mice after PBS or lactulose treatment.

(A) Clock gene expression in hypothalamus and liver of EAM mice after lactulose (n=5) and PBS (n=4) treatment, measured via qPCR. Gene expression levels are not significantly different between groups (unpaired t-test). **(B)** Food intake rates of SPF mice in the dark phase before treatment (control) and the dark phase following treatment with fermentation products. Food intake rates did not differ significantly between the days (paired t-test).



Supplementary Figure 7. Concentration measurements of metabolic hormones after treatment with PBS or lactulose.

(A) Total GLP1 concentration in portal vein plasma 5h after lactulose or PBS treatment in 3MM, germ-free, and SPF mice. We found no significant differences between groups (unpaired t-test). **(B)** PYY concentration in portal vein plasma after lactulose or PBS treatment in 3MM, germ-free, and SPF mice. Groups were not significantly different (unpaired t-test). **(C)** Total Ghrelin concentration in serum from cardiac puncture after lactulose or PBS treatment in 3MM and germ-free mice. No significant differences were found between groups in 3MM mice. Ghrelin was significantly increased ($p<0.05$) in germ-free mice after lactulose treatment (unpaired t-test). **(D)** Total Leptin concentration in serum from cardiac puncture after lactulose or PBS treatment in 3MM and germ-free mice. Concentrations were not significantly different between groups (unpaired t-test).



Data availability

All data relevant for generating figures is available in a permanent data repository (doi: 10.5281/zenodo.20624461).

Acknowledgements

We would like to thank Daniel Hoces for providing the base R script which was adapted for analyzing the Phenomaster data. We would further like to thank Sven Nowok and Dominik Bacovcin and the animal caretakers at the EPIC facility at ETHZ for maintaining the mouse lines. We would like to thank Sandra Kaiser, Bidong Nguyen, Sanne Kroon, and Wolf Dietrich-Hardt for providing the Lactulose dosage for this study.

Additional information

Funding source

This work was supported by the Swiss National Science Foundation (No. 1851228, to ES) and was supported as a part of NCCR Microbiomes, a National Centre of Competence in Research, funded by the Swiss National Science Foundation (No. 180575, to ES). It was also supported by a project grant from Innosuisse (No. 120.452 IP-LS, to MA).

Author contributions

GG, ES, and MA designed the research. GG, MA, CM, SO, AWO, ECB, JL, and DK performed experiments. GG and MA evaluated and analyzed the data and wrote the manuscript in cooperation with all authors.

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Markus Arnoldini (*markus.arnoldini@hest.ethz.ch*) or Giorgia Greter (*giorgia.greter@hest.ethz.ch*).

Materials availability

All materials are available upon request to the corresponding author.

Funding

Funder	Grant reference number	Author
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (SNF)	1851228	Emma Slack
SNF | Swiss National Centre of Competence in Research Kidney Control of Homeostasis (NCCR Kidney.CH)	180575	Emma Slack
Innosuisse - Schweizerische Agentur für Innovationsförderung (Innosuisse)	120.452 IP-LS	Markus Arnoldini

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Peer reviews

Reviewer #2 (Public review):

Summary

The authors aimed to investigate how microbial metabolites, including hydrogen and short-chain fatty acids, influence feeding behavior and clock-gene expression in mice. Specifically, they examined these effects across different microbial environments, including a reduced-community model, germ-free mice, and specific-pathogen-free mice. The study addresses an important and poorly understood question concerning how microbial metabolism may influence host daily rhythms and feeding patterns.

Strengths

The manuscript presents a thoughtful and innovative investigation into the relationship between microbial metabolism, feeding behavior, and host clock-gene expression. A major strength is the use of a reduced microbial community together with real-time measurements of hydrogen production, which provides a useful experimental framework for separating microbial metabolic activity from direct host nutrient intake. The inclusion of germ-free and specific-pathogen-free mice also allows the authors to examine how these effects depend on microbial complexity.

The revised manuscript has addressed several concerns raised in the original review. The authors have clarified aspects of stool collection, provided additional methodological information, refined their use of the terms "circadian" and "diurnal," and added experiments examining the osmotic effects of lactulose across different microbial environments. These revisions improve the clarity and interpretation of the work. The finding that lactulose-induced microbial activity is associated with altered feeding behavior and clock-gene expression in the reduced-community model, but not consistently in germ-free or specific-pathogen-free mice, remains intriguing and highlights the complexity of microbial-host interactions.

Weaknesses

Despite these improvements, several important concerns remain unresolved. Most notably, the response regarding excluded food-intake measurements is insufficient. The authors report that the probability of excluding measurements differed significantly between treatment and control groups in the key feeding experiment shown in Figure 4A/S5A. However, they do not provide the underlying number or percentage of excluded observations, the direction of the imbalance, how many animals were affected, whether the exclusions occurred before or after treatment, or which exclusion criteria accounted for the removed values. Because food-intake rate was calculated from repeated measurements of cumulative intake, differential exclusion of observations could influence both the estimated slope and the reported treatment effect. The authors should provide these details and demonstrate that the principal feeding result is robust to the exclusion procedure.

There is also ambiguity surrounding the analysis and presentation of the quantitative polymerase chain reaction data. Figure 3B-C and Supplementary Figure 4A-C use conflicting mathematical labels, the raw cycle-threshold and replicate-level delta cycle-threshold values are not provided, and the rebuttal appears to conflate logarithmic transformation with exponentiation. I suspect that this may primarily reflect terminology or figure-labeling errors rather than an incorrect underlying analysis. The negative values shown in Figure 3 suggest that the authors may have plotted negative delta-delta cycle-threshold values, corresponding to log2 fold change. Nevertheless, the current description makes the workflow difficult to verify.

Several mechanistic and interpretive issues were acknowledged but only partially addressed. The authors appropriately softened their interpretation of the clock-gene findings and clarified that hormone concentrations were measured at only one time point. However, they did not provide baseline evidence that the positive and negative limbs of the clock-gene network are normally in counterphase, did not discuss the possible involvement of AMP-activated protein kinase, and did not address the limitation of measuring total rather than active glucagon-like peptide 1. These issues should be explicitly discussed as limitations of the current study and as priorities for future work.

Overall assessment

The authors have mostly achieved their aims by providing novel evidence that acute changes in microbial metabolism may influence feeding behavior and clock-gene expression in a simplified microbial environment. The experimental approach is creative, and the study has the potential to contribute meaningfully to the fields of microbiome research and circadian biology. However, unresolved concerns regarding differential data exclusion and the transparency of the gene-expression analysis currently limit confidence in the strength of the evidence supporting the principal conclusions.

Addressing these reporting and analytical issues would substantially strengthen the manuscript and improve its value to researchers studying microbial metabolism, feeding behavior, and host biological rhythms.

https://doi.org/10.7554/eLife.100640.2.sa2

Reviewer #3 (Public review):

Summary:

In the manuscript by Greter, et al., entitled "Targeted induction of gut-microbial metabolism acutely affects feeding patterns and clock gene expression in the host" the authors investigate whether acute exposure to a non-nutritive disaccharide (lactulose) promotes microbial metabolism that feeds back onto the host to impact circadian networks. The premise of the study is interesting, and the experiments are thoughtfully designed to dissect these relationships. The evidence presented generally supports the authors' conclusions regarding the impact of lactulose administration during the fasting period, which is intended to mimic a feeding-associated perturbation of the gut microbiota, and its comparison with lactulose administration during the fed state. The studies employ complementary model systems, including germ-free mice, mice colonized with a simplified three-member microbial community, and conventionally colonized animals. These approaches support the authors' conclusions regarding the relationship between diurnal rhythms of microbial fermentation and host circadian clock gene networks. Overall, the work provides a useful experimental framework for developing a deeper mechanistic understanding of how microbial fermentation products contribute to diurnal host-microbe interactions.

Strengths:

Attempting to disentangle nutrient acquisition from microbial fermentation and its impact on diurnal dynamics of gut microbes on host circadian rhythms is an important step for providing insights into these host-microbe interactions.

The authors utilize a novel approach in leveraging lactulose coupled with germ-free animals and metabolic cages fitted with detectors that can measure microbial byproducts of fermentation, particularly hydrogen, in real time.

The authors consider several interesting aspects of lactulose delivery, including how it shifts osmotic balance as well as providing calculations that attempt to explain the caloric contribution of fermentation to the animal in the context of reduced food intake. This provides interesting fundamental insights into the role of microbial outputs on host metabolism.

The authors employ complementary systems, including a simplified three-member microbial community, providing insight into the minimal set of functionally distinct community members necessary to promote the rhythmic production of fermentation products that can affect host physiology.

Residual limitations:

Hypothesis and study framing: The manuscript still does not clearly articulate a specific, testable hypothesis. While the Introduction provides motivation and objectives (e.g., line 53 onward), it remains unclear what precise hypothesis was being evaluated. A more explicit statement would strengthen the conceptual framework of the study.

Interpretation of circadian gene expression changes: The authors have not fully reconciled the differing effects of lactulose treatment on circadian gene expression in the 3MM and SPF settings. In particular, it remains unclear how the increased expression of certain circadian genes observed in lactulose-treated 3MM mice, particularly Cry1, relates to the decreased expression seen in SPF mice, and how the reduction in Arntl expression observed in lactulose-treated SPF mice fits within the proposed model. The authors acknowledge that resolving these mechanistic differences is beyond the scope of the current study, but the limitation should be discussed more explicitly.

https://doi.org/10.7554/eLife.100640.2.sa1

Author response:

The following is the authors' response to the original reviews.

We thank the three reviewers for their encouraging and constructive comments. We have addressed them by increasing clarity of the writing and adding details to the methods section that were previously missing or not stated clearly enough. We have included additional experimental data. Specifically, we tested how the osmotic effect of lactulose depends on lactulose dosage and colonization state, added data on cecum sizes of mice with different microbiomes, and tested how lactulose treatment in the active phase affects feeding behavior.

> ***Public Reviews:***
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> ***Reviewer #1 (Public Review):***
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> *Greter et al. provide an interesting and creative use of lactulose as a "microbial metabolism" inducer, combined with tracking of H2 and other fermentation end products. The topic is timely and will likely be of broad interest to researchers studying*

> *nutrition, circadian rhythm, and gut microbiota. However, a couple of moderate to major concerns were noted that may impact the interpretation of the current data:*
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> *(1) Much of the data relies on housing gnotobiotic mice in metabolic cages, but I couldn't find any details of methods to assess contamination during multiple days of housing outside of gnotobiotic isolators/cages. Given the complexity of the metabolic cage system used, sterility would likely be incredibly challenging to achieve. More details needed to be included about how potential contamination of the mice was assessed, ideally with 16S rRNA gene sequencing data of the endpoint samples and/or qPCR for total colonization levels relative to the more targeted data shown.*

We thank the reviewer for pointing out that we have not made the experimental setup clear in the text. One of the unique features of our metabolic cage setup is that the mice do not need to be housed outside gnotobiotic isolators, but that the whole system is placed inside an isolator. We have developed and published this system recently (Hoces et al, PLOS Biol 2022), including extensive testing for sterility/gnotobiosis. We have now adapted the main text to increase clarity on this issue (lines 76ff).

Given that 16S sequencing of germ-free mice will typically produce false-positive reads, we used *Blautia pseudococcoides* as an indicator strain for contamination. This strain is present in our SPF mouse colony, forms spores that are highly resilient to decontamination measures, and has been the most likely contaminant in our gnotobiotic system. We have checked for presence of this strain in the cecum content of all our animals at the end of each experiment, and only included experiments which had a *B. pseudococcoides* signal below threshold level. We have now added this information to the methods section (lines 389ff).

> *(2) The language could be softened to provide a more nuanced discussion of the results. While lactulose does seem to induce microbial metabolism it also could have direct effects on the host due to its osmotic activity or other off-target effects. Thus, it seems more precise to just refer to lactulose specifically in the figure titles and relevant text.*

We have adapted all figure legends to not contain interpretations, but rather state what was done in the experiments shown in the figures. We have also adapted the text in multiple places to soften the language and avoid overinterpretation of our experimental results.

> *Additionally, the degree to which lactulose "disrupts the diurnal rhythm" isn't clear from the data shown, especially given that the markers of circadian rhythm rapidly recover from the perturbation. It is probably more precise to instead state that lactulose transiently induces fermentation during the light phase or something to that effect.*

We tried to make the argument that what we call disruption of the diurnal rhythm is acute, meaning that it is not disrupting the rhythm "chronically" (i.e., for longer), but that it recovers rapidly from this transient disruption. Given the confusion this wording is causing we are introducing this conceptually in the new version of the manuscript (lines 56ff).

> *The discussion could also be expanded to address what methods are available or could be developed to build upon the concepts here; for example, the use of genetic inducers of metabolism which may avoid the more complex responses to lactulose.*

We also appreciate the mention of concepts from our study that can be built on in future studies, and we added a paragraph on potential further research. (lines 301ff).

> *Despite these concerns, this was still an intriguing and valuable addition to the growing literature on the interface of the microbiome and circadian fields.*

We thank the reviewer for all their encouraging and constructive remarks!

> ***Reviewer #2 (Public Review):***
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> *Summary:*
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> *The authors aimed to investigate how microbial metabolites, such as hydrogen and short-chain fatty acids (SCFAs), influence feeding behavior and circadian gene expression in mice. Specifically, they sought to understand these effects in different microbial environments, including a reduced community model (EAM), germ-free mice, and SPF mice. The study was designed to explore the broader relationship between the gut microbiome and host circadian rhythms, an area that is not well understood. Through their experiments, the authors hoped to elucidate how microbial metabolism could impact circadian clock genes and feeding patterns, potentially revealing new mechanisms of gut microbiome-host interactions.*
>
> *Strengths:*
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> *The manuscript presents a well-executed investigation into the complex relationship between microbial metabolites and circadian rhythms, with a particular focus on feeding behavior and gene expression in different mouse models. One of the major strengths of the work lies in its innovative use of a reduced community model (EAM) to isolate and examine the effects of specific microbial metabolites, which provides valuable insights into how these metabolites might influence host behavior and circadian regulation. The study also contributes to the broader understanding of the gut microbiome's role in circadian biology, an area that remains poorly understood. The experiments are thoughtfully designed, with a clear rationale that ties together the gut microbiome, metabolic products, and host physiological responses. The authors successfully highlight an intriguing paradox: the significant influence of microbial metabolites in the EAM model versus the lack of effect in germ-free and SPF mice, which adds depth to the ongoing exploration of microbial-host interactions. Despite some methodological concerns, the manuscript offers compelling data and opens up new avenues for research in the field of microbiome and circadian biology.*

We thank the reviewer for their encouraging remarks, specifically on the surprising findings that microbial metabolism seems to affect circadian clock gene expression and behavior differently in EAM and SPF mice.

> *Weaknesses:*
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> *The manuscript, while providing valuable insights, has several methodological weaknesses that impact the overall strength of the findings. First, the process for stool collection lacks clarity, raising concerns about potential biases, such as the risk of coprophagia, which could affect the dry-to-wet weight ratio analysis and compromise the validity of these measurements.*

We thank the reviewer for pointing out that our description of the specific methods used for collecting feces were presented in a somewhat confusing manner. In short, dry and wet faecal weights were determined based on fecal pellets that were freshly produced and directly collected from restrained mice. To determine total fecal output over time, we collected all fecal pellets produced in a 5-hour window in a cage, determined their dry weight, and then used the water content determined for fresh faeces to calculate wet weight. Using this method, we cannot account for potential differences in coprophagia between the groups. However, this is not likely to affect the dry-to-wet ratio of faecal output in our results. We have now adapted the section in the methods to increase clarity (lines 440ff), and changed the quantity shown in Figure S2C and E to "water content", which is a more intuitive measure for the same thing.

> *Additionally, the use of the term "circadian" in some contexts appears inaccurate, as "diurnal" might be more appropriate, especially given the uncertainty regarding whether the observed microbiome fluctuations are truly circadian.*

Similarly to our answer to reviewer 1 above, we appreciate this remark about imprecise language and have addressed this issue in the text and the figure legends. Indeed, we do not think the fluctuations in microbiota activity are truly circadian, but likely a result of the entrainment through the host's food intake.

> *Another significant issue is the unexpected absence of an osmotic effect of lactulose in EAM mice, which contradicts the known properties of lactulose as an osmotic laxative. This finding requires further verification, including the use of a positive control, to ensure it is not artifactual.*

This is a good point. We have used this lactulose dosage specifically to induce microbial metabolism without causing osmotic diarrhoea and went to some lengths do demonstrate this (FigureS2C-E). In response to this comment (and one by reviewer 3 below about transit time), we have now performed additional experiments using higher lactulose dosage (new FigureS3). Our results indicate that the effect of lactulose on water content and transit time depends on microbiota complexity, with no change in fecal water content in 3MM mice even when treated with higher lactulose doses, and a stronger change in SPF mice. We now address this in the main text (lines 127ff).

> *The presentation of qRT-PCR data as log2-fold changes, with a mean denominator, could introduce bias by artificially reducing variability, potentially leading to spurious findings or increased risk of Type I error. This approach may explain the unexpected activation of both the positive and negative limbs of the circadian clock.*

While we agree that our description of the qPCR method used for measuring circadian clock gene expression was lacking detail, we do not see how our analysis would lead to an increased risk of Type 1 error.

Briefly, we use the standard ΔΔCt method to analyze our RT-PCR results. We first normalize gene expression values for each gene of interest to an internal housekeeping gene. Then, we use these normalized values to compare gene expression values in treatment vs control groups (or to the control group at time point 0 in the case of Figure 3C). We then use a log2 transformation to convert the logarithmic RT-PCR values to fold changes. We apologize for the confusing labeling in the figures, where we called the values "log2(fold changes)", which we have now changed to "log2(ΔΔCt) of expression" in Figures 3B, C and S4.

The simultaneous activation of both limbs of the circadian clock is indeed a surprising result and somewhat complicates interpreting the effect of lactulose treatment on clock gene expression. We take it as evidence that it generally interferes with clock gene expression, while a clearer understanding of the effects would require further research.

> *Moreover, the lack of detailed information on the primers and housekeeping genes used in the experiments is concerning, particularly given the importance of using non-circadian housekeeping genes for accurate normalization.*

It seems like the resource table describing these important experimental details was omitted in the original submission. We have now included it in the revised version (Table S1).

> *The methods for measuring metabolic hormones, such as GLP-1 and GIP, are also not adequately described. If DPP-IV/protease inhibitor tubes were not used, the data could be unreliable due to the rapid degradation of these hormones by circulating proteases.*

We thank the reviewer for pointing out this omission. We have now added details of how we measured the metabolic hormones to the methods section, including the fact that we have added a DPP-IV inhibitor to the tubes at sampling (lines 469ff).

> *Finally, the manuscript does not address the collection of hormone levels during both fasting and fed phases, a critical aspect for interpreting the metabolic impact of microbial metabolites.*

While we agree that it would be interesting to measure hormone levels also in the fed phase, a more thorough examination of hormone levels over the diurnal cycle, as suggested by reviewer 3, would be relevant for a full-scale follow-up. Given our data, we of course cannot exclude that there may be time-point-specific differences and therefore have softened the language around this conclusion to state that hormone levels are not acutely changed after a lactulose intervention at the time-points examined. (lines 318ff).

> *These methodological concerns collectively weaken the robustness of the study's results and warrant careful reconsideration and clarification by the authors.*
>
> *Because of these weaknesses, the authors have partially achieved their aims by providing novel insights into the relationship between microbial metabolites and host circadian rhythms. The data do suggest that microbial metabolites can significantly influence feeding behavior and circadian gene expression in specific contexts. However, the unexpected absence of an osmotic effect of lactulose, the potential biases introduced by the log2-fold change normalization in qRT-PCR data, and the lack of clarity in critical methodological details weaken the overall conclusions. While the study provides valuable contributions to understanding the gut microbiome's role in circadian biology, the methodological weaknesses prevent a full endorsement of the authors' conclusions. Addressing these issues would be necessary to strengthen the support for their findings and fully achieve the study's aims.*

We thank the reviewer again for their careful and critical reading of our work, and for their constructive input. In the revised version of our manuscript, we address the reviewer's concerns by providing more methodological detail and additional experimental data.

> *Despite the methodological concerns raised, this work has the potential to make a significant impact on the field of circadian biology and microbiome research. The study's exploration of the interaction between microbial metabolites and host circadian rhythms in different microbial environments opens new avenues for understanding the complex interplay between the gut microbiome and host physiology. This research contributes to the growing body of evidence that microbial metabolites play a crucial role in regulating host behaviors and physiological processes, including feeding and circadian gene expression.*

We thank the reviewer for their encouraging remarks!

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
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> *In the manuscript by Greter, et al., entitled "Acute targeted induction of gut-microbial metabolism affects host clock genes and nocturnal feeding" the authors are attempting to demonstrate that an acute exposure to a non-nutritive disaccharide (lactulose) promotes microbial metabolism that feeds back onto the host to impact circadian networks. The premise of the study is interesting and the authors have performed several thoughtful experiments to dissect these relationships, providing valuable insights for the field. However, the work presented does not necessarily support some of the conclusions*

> *that are drawn. For instance, lactulose is administered during the fasting period to mimic the impact of a feeding bout on the gut microbiota, but it would be important to perform this treatment during the fed state as well to show that the effects on food intake, etc. do not occur.*

We thank the reviewer for this important point. In the revised version, we include an experiment where we administer lactulose during the fed state and do not observe a significant change in food intake. We describe this in the text (lines 189ff) and in the new Figure S5C and D.

> *To truly draw the conclusion that the current outcomes are directly connected to and mediated via an impact on the host circadian clock, it would be ideal to perform these studies in a circadian gene knock-out animal (i.e., Cry1 or Cry2 KO mice, or perhaps Bmal-VilCre tissue-specific KO mice). If the effects are lost in these animals, this would more concretely connect the current findings to the circadian clock gene network.*

We agree that these would be interesting experiments to follow up on the question how the observed effects are actuated by host functions. However, they would require a large amount of preparatory work (including rederiving the KO mice to get them germ-free in our gnotobiotic facility), we argue that they are beyond the scope of this study.

> *Despite these reservations, the work is promising.*

We thank the reviewer for their encouraging assessment.

> *Strengths:*
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> *Attempting to disentangle nutrient acquisition from microbial fermentation and its impact on diurnal dynamics of gut microbes on host circadian rhythms is an important step for providing insights into these host-microbe interactions.*
>
> *The authors utilize a novel approach in leveraging lactulose coupled with germ-free animals and metabolic cages fitted with detectors that can measure microbial byproducts of fermentation, particularly hydrogen, in real-time.*
>
> *The authors consider several interesting aspects of lactulose delivery, including how it shifts osmotic balance as well as provides calculations that attempt to explain the caloric contribution of fermentation to the animal in the context of reduced food intake. This provides interesting fundamental insights into the role of microbial outputs on host metabolism.*

Thank you!

> *Weaknesses:*
>
> *While the authors have done a large amount of work to examine the osmotic vs. metabolic influence of lactulose delivery, the authors have not accounted for the enlarged cecum and increased cecal surface area in germ-free mice. The authors could consider an additional control of cecectomy in germ-free mice.*

We thank the reviewer for pointing out the potential effect of the anatomical differences of germ-free and conventionally colonized mice. We agree that when comparing germ-free mice to SPF mice, the enlarged cecum area in germ-free animals could lead to differences in water release or uptake. However, this difference is smaller in gnotobiotic mice colonized with our minimal microbiota, even though their ceca are still slightly smaller than those of germ-free mice (new Figure S2F). While we agree that including control of cecectomy in germ-free mice could be interesting, we do not have the option of doing surgery on germ-free mice given our

current experimental setup. We have now added information on cecum weight, a good proxy for cecum size, in the new Figure S2F done.

> *The authors have examined GI hormones as one possible mechanism for how food intake is altered by microbial fermentation of lactulose. However, the authors measure PYY and GLP-1 only at a single time point, stating that there are no differences between groups. Given the goal of the studies is to tie these findings back into circadian rhythms, it would be important to show if the diurnal patterns of these GI hormones are altered.*

We fully agree that a deeper investigation of the diurnal fluctuations of hormone levels would be an interesting next step in studying whether perturbations in food intake can disturb these rhythms. Doing this for the whole rhythm would really require a full second study.

In response to the reviewer's comments, we have changed the statements made around these data to point out just that hormone level fluctuations could not be detected during specific time points after lactulose treatment and therefore do not seem to explain the imminent behavioral changes (lines 318ff).

> *Considerations of other factors, such as conjugated vs. deconjugated bile acids, microbial bile salt hydrolase activity, and bile acid resorption, might be an important consideration for how lactulose elicits more influence on ileal circadian clock genes relative to cecum and colon.*

We absolutely agree that investigation of microbial bile acid modification and their metabolism by the host would be an interesting topic for a follow-up study.

> *Measurements of GI transit time (both whole gut and regional) would be an important for consideration for how lactulose might be impacting the ileum vs. cecum vs. colon.*

This is also an interesting point. While we did not add an experiment in which we specifically measure transit time to the revised version, we now measure total faecal output in a 5 h time period after PBS or lactulose treatment (Figure S3C). Faecal output is known to be a good proxy for transit time, and we see no significant difference between lactulose treatment (even with a two-fold higher dose than used before, new Figure S3) and PBS treatment.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *(1) Line 126 - see the point in the public review, this data argues against disrupting the rhythm.*

See our response above

> *(2) Line 156 - the metric used for water content is confusing. Why not just subtract dry weight from wet weight to get water content? The ratio is much harder to think about for me. Perhaps more importantly, this data is very confusing given that colonization seems to impact the activity of lactulose, which complicates the interpretation. Could be an interesting area for future study that you might highlight more in the discussion.*

We thank the reviewer for pointing out that our presentation of water content could be clearer. We have changed the dry/wet ratio we have used in the previous version to the "water fraction" (new Figure S2C, E; new Figure S3A,B), i.e., the per cent of weight of the wet sample that is made up by water. We would argue that this is a measurement that is easier to interpret than the difference suggested by the reviewer, because it is independent of the absolute sample weight.

We also agree that it is surprising that colonization state changes the osmotic state of the gut environment and have now added text discussing that (lines 127ff).

> *(3) Line 188 - the lack of expression changes in the distal gut (cecum/colon) potentially conflicts with the model, warranting additional discussion/qualifications. Is lactulose getting metabolized in the small intestine? Alternatively, does lactulose have a direct effect on the host? The current text seems to imply that lactulose is fermented in the colon, the fermentation products are absorbed, and then they only impact the ileum through circulation, which doesn't seem physiologically possible.*

It is possible that lactulose affects small intestinal tissue directly, but we show that the effect depends on microbial activity. Microbial activity is much larger in the large intestine than in the small intestine, which is why we hypothesize that it acts through systemic signals rather than locally. These systemic signals might be fermentation products impacting the ileum through circulation. We would argue that this is not implausible, given that there are well-documented systemic effects of fermentation products in circulation (e.g., den Besten et al, 2013). It is, however, also possible that, e.g., metabolism of fermentation products in the liver triggers a secondary signal that acts systemically.

> *(4) Line 241 - need to weaken this sub-heading. The experimental design shows that fermentation products impact feeding behavior, but this does not necessarily imply that fermentation products are responsible for the lactulose effect.*

Done.

> *(5) Line 256 - The lack of an effect in SPF mice is surprising and potentially conflicts with the model proposed. Given this and other confusing results (gene expression site specificity, osmolarity effects, etc) it seems prudent to be more cautious as to the potential mechanisms through which lactulose supplementation impacts host gene expression and feeding behavior, which would likely require a lot more experiments to provide a definitive answer.*

While the lack of an effect in SPF mice is surprising, we would argue that this is rather points towards the need for a better understanding of host-microbiota-diet interactions than a conflict with our interpretation. We do agree with the reviewer that more work is necessary for providing definitive proof of the underlying mechanisms of the observed effects and have adapted the language throughout the text.

> *(6) Line 260 - A lot of text is devoted to the potential caloric effects of the fermentation products and the lactulose itself. Was this in response to a prior reviewer? Either way, it seems too speculative to me and I would recommend trimming it down and moving it to the discussion.*

We have rewritten this section to make it more concise and increase readability (lines 224ff).

> *(7) Line 305 - Not fasting, just lower caloric intake, as shown by Figure 1c.*

We have changed the text to reflect that.

> *(8) Line 371 - Too definitive given the current data. Need to qualify the interpretation here.*

We have adapted the text and qualified the interpretation.

> *(9) Figure 2 - Need to revise the title, no data showing that the rhythm is disrupted.*

Done.

> *(10) Figure 3b - Clarify what timepoint is shown in the legend.*

Done.

> *(11) Figure 3c - label when the treatment started. Consider changing to 2-way ANOVA which is probably more appropriate than t-tests.*

We have added information on treatment start to the figure legend and have changed the statistical analysis to a two-way ANOVA (time, treatment).

> *(12) Figure 4c - move to supplement as this negative data isn't sufficient to rule out an impact on the hypothalamus or liver. Even when only considering transcript levels it's possible that the timepoint is just not ideal.*

We agree that this data only represents a snapshot of gene expression at one timepoint after treatment and does not rule out involvement of hypothalamus or liver in this process. We have now moved the previous Figure 4C to the supplementary information (new Figure S6A).

> *(13) Figure 5 - defined the "fermentation products" and their concentrations in the legend. Consider moving panels d, and e to the supplement - negative results with unclear interpretation. Clarify in the legend how many calories/g were assumed for the fermentation products and provide a scientific rationale for this decision. Modify the title to be more cautious - as discussed above.*

We thank the reviewer for pointing out that this was not clear. We have now added the formulation of the fermentation products to the figure legend and moved panels D and E to the supplement. We have also combined the former Figures 4AB and 5ABC into the new Figure 4.

> *(14) Figure S1 - The patterns in panel a are really intriguing and could be discussed more. For example, what do you think is driving the rapid oscillations in E. rectale?*

We agree that the patterns are potentially interesting, but the fact that the patterns we observe do not replicate well between the two light-dark cycles we monitor suggest a large contribution of experimental noise. We therefore refrain from interpreting too much into this dataset.

> *(15) Figure S2 - Could changing the metric for water content be more easily interpretable? Modify the title to better match the data shown.*

We thank the reviewer for pointing that out. We have now changed the metric in Figure S2 (and the new Figure S3) to % water in feces/cecum content, which is easier to interpret.

> *(16) Figure S3 - need to specify the multiple testing correction used.*

Done.

> *(17) Figure S4 title - replace "inducing microbial metabolism" with "lactulose".*

Done.

> *(18) Figure S5 title - modify to weaken the causal claim.*

We have adapted all figure titles to conform to this comment.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *Greter and colleagues present an insightful manuscript investigating the effects of microbial metabolites, such as hydrogen and SCFAs, on feeding behavior and circadian*

> *gene expression at a single time point. Notably, they observed that these metabolites exert a significant influence on behavior in a reduced community model (EAM). However, this effect was not evident in germ-free or SPF mice, highlighting an intriguing paradox. The manuscript is well-written, with experiments that are thoughtfully designed and clearly rationalized. The study contributes valuable data to the poorly understood relationship between the gut microbiome and host circadian rhythms. While I find the manuscript compelling, I believe that providing additional experimental and analytical details would enhance clarity and rigor.*
>
> *Major Comments:*
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> *(1) Additional clarity is needed regarding the stool collection process. Were the samples collected as fresh specimens, or was there a possibility of coprophagia before collection? Clarification on this point is important, as it could impact the results, particularly the dry-to-wet weight ratio analysis. Ensuring the collection process did not introduce this bias is crucial for the validity of these measurements.*

Thank you for pointing out that this was not stated clearly. Wherever we assessed water content in faeces, we used fresh samples that were directly collected from a live animal and immediately frozen in a closed container or analyzed. When we assessed total faecal output, we collected the total bedding from a cage, sorted out the faecal pellets, and only measured dry weight. Total wet weight output was then assessed by using the water content of fresh faeces of animals with the same microbiota and the same treatment as a correction factor. We have now made this clear in the methods section (lines 435ff).

In cases where we performed total output measurements by collecting bedding, there was the possibility for coprophagia. We did not control for this but assumed that coprophagia will have a small effect that is likely similar between groups and should thus not affect the comparison.

> *(2) Caution is advised in the use of the term 'circadian,' which is sometimes used when 'diurnal' might be more appropriate. For example, the title of the first results section could be revised to 'Host Feeding [or Diurnal] Rhythms Influence Microbial Metabolic Fluctuations.' Additionally, line 357 should likely use 'diurnal' instead of 'circadian.' It's important to note that 'circadian' implies that cyclical fluctuations would persist without environmental cues (e.g., feeding). Since it's not clear whether most microbiome compositional or functional fluctuations are truly circadian, 'diurnal' is likely the more accurate term.*

We thank the reviewer for pointing out our imprecise use of the term circadian. We have now adapted this throughout the manuscript.

> *(3) The lack of an osmotic effect of lactulose in EAM mice is quite surprising, given that lactulose is known to be an osmotic laxative. Was this finding specific to EAM mice, or was a similar lack of osmotic effect observed in SPF mice? A positive control is necessary to verify that this unexpected result is not artifactual. If there is no osmotic laxative effect in SPF mice, an explanation is needed as to why this medication is not functioning as expected in these mice.*

This is a good point. While we initially thought that the lack of an osmotic effect was due to the specific lactulose dose we were using, we also did not observe an osmotic effect (measured by the water content of fresh faecal pellets produced after treatment) when we used twice the amount of lactulose in EAM mice (new Figure S3). However, in SPF mice, we did see an increase in faecal water content after treatment. This intriguing result suggests that the effect of lactulose as an osmotic laxative depends on the presence of a complex microbiota. We now show this data in the new Figure S3A and discuss it in the text (lines 127ff).

> *(4) The authors state that 'To account for faulty measurements due to disruptive events and for measurement noise, some datapoints were excluded from the raw datasets.' It would be important for the authors to confirm that this data exclusion was unbiased, meaning it did not disproportionately affect one group over another, and that any exclusions affected groups randomly.*

This is a good point, and we analyzed this for the experiments we show in Figs 4A/S5A, 4B/S5E, 4C, and S5F, and discuss this in the methods part (lines 493ff). The resulting statistics is not fully conclusive: using a Chi-square test to check whether the probability of excluding values differs between experiments, we get significant differences ($p=3.9 \times 10^{-18}$). We would, however, argue that this is not surprising, as different experiments sometimes different in the number of times we needed to do maintenance work on the isolators, which could lead to actuation of the scales measuring feed values, and thus faulty measurements.

We face the same problem within experiments: we found a significant difference in the probability to exclude values between treatment and control in the experiment shown in Figs 4A/S5A ($p=1.7 \times 10^{-6}$), but no significant differences in the experiments in Figs 4B/S5E and S5F (p=0.71, and p=0.10, respectively). While it is hard to strictly exclude an influence of our data exclusion strategy, these findings speak against a systematic effect of treatment.

> *(5) The authors should provide details on the primers and housekeeping genes used in their experiments. It's crucial that the housekeeping gene is not circadian and is stable at all time points (PMID 17878933).*

In the previous submission, the main resources table was omitted by accident. We have now added it (Table S1), including details on the primers and reagents used for all experimental work.

> *(6) The presentation of qRT-PCR data as log2-fold change is confusing. It's unclear what the numerator and denominator represent for this ratio or why such normalization was deemed necessary. Ideally, transcripts should be normalized to a housekeeping gene (as noted in a previous comment), not to a baseline measure of other same genes acquired from other mice. Log2-fold change is typically appropriate when comparing two measures from the same mice; however, in this study, the mice were euthanized, and the denominator is a mean of genes measured from other samples. This approach could introduce bias and might explain why both the positive and negative limbs appear to be activated by the microbial metabolites. It would be more rigorous to present these values as absolute gene expression levels.*

We thank the reviewer for pointing out that this was not described clearly in the previous version of our manuscript. We have now adapted the methods part to explain that all data showing RT-PCR data is normalized to a housekeeping gene. Only after that, we compare the gene expression levels of the treatment group to the control group (or to gene expression of the control group at timepoint 0 in the case of Figure 3C).

> *(7) The use of log-ratios, with a mean as the denominator, could artificially reduce the variability in the data, potentially leading to spurious findings or an increased risk of*

> *Type I error.*

As pointed out above, we have used internal normalization to a housekeeping gene before comparing the resulting values of the treatment and control groups. This method (commonly known as $\Delta\Delta C_t$ method) is a standard way of comparing gene expression values obtained by RT-PCR. The use of a log2 transformation is commonly used to convert the logarithmic data resulting from the RT-PCR measurement to a linear fold-change measurement. We do not see that this data analysis strategy should lead to an increased risk for producing false positives. We now explain this better in the methods section of the manuscript (lines 541ff), and have adapted the labels in Figures 3B, C and S4 to avoid confusion.

> *(8) It is unusual that both the positive and negative limbs of the circadian clock are overexpressed following lactulose administration. The authors should provide data confirming that these genes are in counter phase to each other at baseline. This clarification would help readers better understand the effects of the experimental interventions. As it stands, this critical part of the results is quite confusing.*

We agree that this result does not allow a clear interpretation of the effect of lactulose on the diurnal rhythm of the host. While we agree that this would be interesting to understand in detail, we are merely taking this as a first indication that actuation of microbial metabolism during the inactive phase of the diurnal rhythm can lead to changes in clock genes. This claim is supported by our data.

> *(9) Could the effects of the microbial metabolites be mediated by AMPK, a known nutrient sensor that can influence the post-translational modification of Cry proteins? It would be beneficial for the authors to explore whether these metabolites have a more direct, previously unknown mechanism of affecting the circadian clock, or if their effects are mediated through known signaling pathways such as AMPK.*

We agree that this would be a valuable path to continue investigating the effect of microbial metabolism on clock gene activity.

> *(10) The methods section does not specify how the metabolic hormones (e.g., GLP-1, GIP, leptin, ghrelin) were measured in the experiments. It is important for the authors to confirm that DPP-IV/protease inhibitor tubes were used for hormone measurement, as these proteins can be rapidly degraded by circulating proteases. Without the use of appropriate tubes, this data cannot be reliably interpreted. Additionally, it would have been ideal to collect these hormone levels during both the fasting and fed phases, but it appears this was not done. This represents a significant limitation of the study and should be addressed in the discussion.*

We thank the reviewer for pointing out this omission, we have now added a description of our protocol to measure metabolic hormones to the methods section (lines 469ff).

> *(11) If the samples were appropriately collected in DPP-IV/protease inhibitor tubes, the authors should consider measuring active GLP-1, as this would likely provide a more accurate assessment of GLP-1 activity.*

We agree that this would be a valuable next step, in addition to testing the effect of changes in microbial metabolism on the time traces of hormone levels.

> *Minor Comments:*
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> *(12) Line 350 appears to have an incomplete sentence, as it seems part of the first sentence in the paragraph has been inadvertently deleted. This should be reviewed and corrected for clarity.*

Done.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Major comments:*
>
> *(1) Could the authors provide a deeper description about what they are referring to in the following statement? "...higher order interactions and microbial metabolism are variable..." it is difficult to interpret as written. Do the authors mean cross-feeding interactions?*

We have changed this sentence to clarify the meaning.

> *(2) Could the authors explicitly state their hypothesis in the introduction and provide a brief, but deeper explanation of the intervention prior to the results section?*

This is a good point, we have adapted the text accordingly (lines 53ff).

> *(3) Could the authors include a bit more information regarding the diet provided to the mice? If grain-based chow, please provide insights into the fiber source, etc.*

While we agree that it would be interesting to know what part of the mouse diet is available to the microbes, this is hard for the standard mouse chow that we (and most others doing experiments with mice) feed the experimental animals. We have now added more detailed information on the specific type of chow the mice were fed (lines 347f). We would argue that, because the control and treatment groups were always fed the same chow, the effect of the fiber source and other specifics are controlled for, even if we do not know them.

> *(4) In figure 1A - cells/g does not seem to be the correct unit - # of copies/g feces perhaps?*

Cells/g is the appropriate unit, but it seems like we have not explained the way we arrive at this unit in sufficient detail. In short, we use a qPCR run on a known standard curve of bacterial counts (known cells/g values) to estimate these numbers from qPCR results from faeces. We have now explained this better in the methods section (lines 385f).

> *(5) Figure 1B/C and Figure S1B/C are confusing - the legend states these measurements were taken over two days, however, the plot shows a single 12:12 LD period. Was the data averaged? It might be best to show each day separately (i.e., over a 48-hour period) rather than in one 24-hour plot. Then, the authors could also show the averages in the light period vs. the dark period in a separate, complementary graph.*

We thank the reviewer for pointing this out. The previous figures were indeed averaged over the two days of measurement and projected onto one 24h period for plotting. We have now changed Figures 1B,C and S1B,C to show the full 48h time windows.

> *(6) Line 109 - The reviewer concurs that lactulose is a non-nutritive, synthetic disaccharide, however, in theory, lactulose may have a high heat increment, which could cause the animal to undergo metabolic responses to defend core body temperature (which also exhibits diurnal rhythmicity). Have the authors considered core body temperature rhythms, their connection to microbial metabolism, and the core circadian clock gene network in their model?*

This is an interesting thought. We have not measured body temperature in our experiments. As the heat increment from food is typically associated with metabolic activity, and lactulose is not metabolized, we do not expect its heat increment to be high, at least in GF mice. In mice with a microbiota, we agree that metabolic heat will be produced upon lactulose metabolism by the microbes, which could be a contributor the observed effect.

> *(7) Figure 2A and corresponding text in lines 121 - 123 - indicate at what time the bar and whisker plots were taken (assuming at ZT8, but please be explicit in the figure and corresponding text). Could the authors also include statistics for these waveforms?*

Done.

> *(8) In Figure 2B, the authors state that microbial metabolism had been restored to normal levels by ZT15, however, did this persist into the next light phase? It would be ideal if the authors could present these data in a similar manner to that shown in Figure S2A for SPF mice. Further, what are the statistical considerations here to describe changes in phase, amplitude, periodicity, etc.*

This is a good point. Unfortunately, we do not have H2 measurements for EAM and GF mice over comparable time periods as shown for SPF mice in Figure S2A. However, the food intake data shown in Figure S5BCD is a indicates that the food intake normalized in the second dark phase after treatment.

> *(9) In lines 147 - 149 and in Figure S2B figure legend - assuming these measurements are from individual bacteria? Could this be stated clearly in the text or legend? Also, what are the statistical considerations? Were there significant differences in SCFA production between bacteria?*

We thank the reviewer for pointing out that this was unclear. We have now adapted the legend to explain the way these data were collected and added a statistical analysis.

> *(10) The authors have done a large amount of work to examine the osmotic vs. metabolic influence of lactulose delivery - however, have the authors accounted for the enlarged cecum and increased cecal surface area in germ-free mice? Would an additional control be cecectomy in germ-free mice to be more in-line w/ SPF animals? Further, could the authors tie in these findings more explicitly and state how they pertain to the overall goal of the study? Is this simply to draw the conclusion that microbial biomass is increased w/ lactulose?*

We wanted to make sure that the effect we see with lactulose is due to microbial metabolism and not due to the induction of osmotic diarrhoea or other host-dependent effects, and we have added a statement to that effect (lines 127ff). We have not corrected for the change in cecum size between GF and EAM mice, as EAM size (and many other gnotobiotic mouse models) also have enlarged ceca relative to conventionally colonized mice, but have added a dataset showing how cecum size of EAM and GF mice compare and discuss this in the text (new Figure S2F, lines 135ff).

> *(11) Is Figure 3A necessary?*

It might not be strictly necessary, but it can help with understanding the relation of the genes tested in B and C, and we would therefore like to keep it in.

> *(12) Line 155 - 157 - the authors make the statement that dry/wet feces weight ratio is decreased in GF mice, but this does not appear to be statistically significant. Please adjust to state numerical differences were observed or provide statistics.*

We have changed the statement in the text.

> *(13) Could Figure S3A be moved to the main Figure 3 as this may provide a more logical flow? qRT-PCR data is expressed as - log2 (fold expression), but relative to what? Could the authors provide further info about the control?*

The former Figure S3A (new S4A) and Figure 3B show the same data in slightly different ways. We therefore opted to keep only one of those illustrations in a main figure. The fold changes are always relative to the average of the PBS control group, which we now state explicitly in the figure legend.

> *(14) The authors state that the qRT-PCR data shows that microbial metabolism of lactulose impacts peripheral circadian gene expression, but this conclusion seems simplified. Lactulose treatment only impacted the ileum circadian gene expression. Additional peripheral tissues (liver, adipose tissue, etc.) could be moved to this figure, i.e., move Figure 4C data. Why do the authors think the ileum was most impacted beyond GI hormones as discussed later in the manuscript? Could changes in bile acid deconjugation (i.e., BSH activity?) and/or bile acid resorption by the host in the distal ileum due to lactulose delivery be involved? Or is it simply due to differences in GI transit time (which was not measured in the current study)? Further, lactulose had minimal impact in SPF ileum, and in fact, shifted Cry1 in the opposite direction relative to EAM mice. Could the authors provide more insight into these disparate observations (line 196 - 200)?*

We agree that the statement "lactulose impacts peripheral gene expression" is oversimplified, and we have now adapted the text to avoid the impression that this is our conclusion. No tested tissues other than the ileum showed significant differences in gene expression at the time point tested, which does not rule out that other tissues would react to the treatment at that time point, or the tested tissues would do so at the tested time point. As we don't have a good enough understanding of what mechanism causes the gene expression changes in the ileum, we refrain from speculating in the text, even though we agree that this is an intriguing question.

> *(15) Could the authors provide more insight into the statistical approaches used to assess amplitude, peak, nadir, etc. in Figure 3C? Was the co-sinor waveform tested?*

This is a good question. Even though this was a highly work-intensive experiment using many animals, we would argue that the noise level is too high and the coverage of the time analyzed too sparse to infer meaningful statistics on the fluctuations of gene expression over time. In the new version, we have changed our statistical analysis of this dataset to a two-way ANOVA (treatment, time) to better analyze this dataset.

> *(16) The food intake decrease and interpretation following treatment (Figure 4A and S4A) is curious - all animals were gavaged and in EAM mice, many animals, regardless of PBS or lactulose are trending down in food intake rate/total intake. It seems to be more of an impact of gavage and not of treatment, which the authors somewhat acknowledge in Lines 228 - 230.*

We agree that gavage is a possible factor in future food intake of experimental animals, which is precisely why we used the PBS gavage as a control. Even though the difference is not large, we see a significant change in food intake when lactulose is given, but not when PBS is given (Figure 4A, S4A).

> *(17) Could the authors provide a deeper rationale for their line of thinking for lines 234 - 240? What is the evidence that systemic effects are likely to occur 3 hours after lactulose delivery? Further, as stated in comment 13, could brain and liver data be moved to Figure 3/Figure S3 as an additional example of peripheral tissue clocks?*

We have added an explanation for the rationale we use to justify the 8h time point (it is 3h after the peak of H2 production, as shown in Fig2AB, which happens 5h after lactulose delivery). While we agree that the brain and liver data would also fit into the Figure 3, we have now moved all negative data to the supplementary information (in response to a comment by reviewer 1, and in a general effort to clean up the data in the manuscript).

> *(18) The authors measure PYY and GLP-1 at a single time point and state there are no differences, yet, the goal of the studies is to tie this back to circadian networks. Would it be possible to measure these GI hormones over a 24-hour period to show that the diurnal patterns are altered?*

We fully agree that measuring the metabolic hormones over time would be very interesting. It is possible but would represent a major effort using many animals and a large amount of work. We would therefore argue that it is beyond the scope of this revision, but a good starting point for a follow-up study.

> *(19) The authors state that the administration of fermentation products acutely altered circadian food intake, but the studies do not support that this change is connected to the circadian network. Suggest softening the interpretation of the findings.*

We have changed the language there to soften the interpretation.

> *Minor comments:*
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> *(1) The authors should consider when it is appropriate to refer to rhythms as diurnal vs. circadian, as each has a distinct meaning. Diurnal follows entrainment cues while circadian is endogenously driven (i.e., line 39, line 58).*

We thank the reviewer for pointing out this important difference, we have adapted this in the whole text accordingly.

> *(2) Circadian rhythm should be plural throughout the manuscript (circadian rhythms).*

Thank you, we have changed that where we refer to host circadian rhythms generally.

> *(3) Lines 54 - 63. Fermentation should be capitalized when used at the beginning of a sentence.*

Done.

> *(4) Line 289 - This should be Figure 5D and 5E.*

Done.

> *(5) Line 290 - heart should be cardiac.*

Done.

https://doi.org/10.7554/eLife.100640.2.sa0