Acute targeted induction of gut-microbial metabolism disrupts host circadian rhythm

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1. Summary

The gut microbiota and host circadian 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 (FP). 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 circadian 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.

Keywords: Gut microbiota, circadian rhythm, microbial metabolism, fermentation products

2. Introduction

From regulating our daily sleep-wake cycles to influencing metabolic processes, the circadian rhythm 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 transcription factors oscillating in positive and negative feedback loops [3, 4, 5, 6, 7]. Light works as a trigger to the hypothalamus, the master pacemaker, which transmits time-of-day signals to the rest of the body to affect fluctuations in physiology and behavior [8, 9]. One of the key behavioral rhythms that is regulated by the circadian rhythm 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]. Being synchronous with the 24-hour light-dark cycle increases fitness, and conversely, being asynchronous with the external environment is detrimental to health [12, 13, 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 (Ig) 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, 20, 21].

There is a growing interest in the interplay between the gut microbiota and the host, as it is now thought that not only the host circadian rhythm regulates the microbiota, but also vice versa: that the microbiota influences the host circadian rhythm. The microbiota plays a significant role in the amplitude of host clock gene expression oscillations, and is associated

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with weight gain and reduced longevity in a dysregulated circadian rhythm [22, 23, 24, 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 (FP). FP 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 [27, 28, 29]. FP production by the microbiota triggers satiety signaling in the host through various pathways and is thought to play a role in regulating the host circadian rhythm due to the circadian nature of food intake [30, 17]. However, FP concentrations and microbial metabolism are variable due to the higher order interactions that take place in the complex microbial communities in the gut, making it difficult to evaluate mechanistic interactions between the microbiota and the host circadian rhythm [31].

It has previously been challenging to disentangle the host circadian rhythm and eating patterns from gut microbial metabolism, as metabolic oscillations in the gut microbiota arise from host food intake. In this study, we use an established intervention to acutely increase microbial metabolism in the gut without directly providing calories to the host. This disrupted the normal, host-dependent microbial rhythm, allowing us to evaluate how microbiota activity regulates host circadian clock expression and subsequent eating patterns. We show that microbial metabolism regulates host circadian clock gene expression and that this regulation influences host circadian behavior.

3. Results

The host circadian rhythm influences microbial cyclical fluctuations

The complexity of the gut microbiota and the higher order interactions in the gut can make it challenging to identify mechanisms by which the microbiota interacts with the host circadian rhythm [32]. To overcome this challenge, we colonized germ-free (GF) wild-type C57BL/6 mice with a defined minimal microbiota of three species and bred them for several generations throughout the study. The Easily Accessible Microbiota (EAM) is composed of *Bacteroides thetaiotaomicron* (*B. theta*), *Eubacterium rectale* (*E. rectale*), and *Escherichia coli* (*E. coli*), and can be used to study bacterial interactions in a well-defined system of relevant microbiota members. *B. theta* is the most abundant member of the EAM microbiota, but all three species reach high numbers in the gut of colonized mice (Figure 1A). *B. theta, E. rectale,* and *E. coli* are members of the phyla Bacteroidetes, Firmicutes, and Proteobacteria (recently renamed to Bacteroidota, Bacillota, and Pseudomonadota), respectively, the three most abundant phyla in the human gut [15, 33].

To investigate the rhythmic fluctuations in the EAM population, we measured the bacterial load in EAM mouse feces over two days. Fecal samples were collected every 6 hours and the bacterial load per gram of feces over time was estimated, as well as the relative abundance of each species. All three EAM species vary in total population over time, with the population decreasing at the end of each dark and light cycle (Figure 1B). *E. coli* fluctuates less in relative abundance compared to *E. rectale* and *B. theta,* which both fluctuate throughout the dark and light cycles (Figure 1C). Food intake was measured for each mouse using an isolator-housed TSE Phenomaster system. As a readout for microbial metabolism, hydrogen gas excretion was also measured over time. Hydrogen is a byproduct of butyrate production, and *E. rectale* is the only butyrate producer in the EAM, and therefore the dominant hydrogen producer (Figure S1B) [34, 35]. Hydrogen levels fluctuated in a 24-hour period in the EAM mouse, closely following mouse food intake, which provides the non-host-available fibers that the microbiota can metabolize in the gut (Figure 1D). Germ-free mice did not produce measurable levels of hydrogen despite having the same food intake rhythm (Figure 1E).

Lactulose disrupts the normal rhythm of microbial metabolism

To acutely disrupt the normal rhythm of microbial metabolism in the gut without directly providing nutrients to the host, we administered lactulose to EAM 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 [36, 37, 38]. We administered the lactulose between Zeitgeber times 3 and 4, several hours after the mice had stopped eating and when hydrogen production was decreasing (Figure 2A). To account for the effect of the oral gavage treatment on the microbiota, PBS was administered as a negative control, and all experiments were replicated in GF mice to control for host effects independently of the microbiota. Mice that were treated with lactulose increased hydrogen production two hours after treatment compared to PBS-treated mice, which continued in the normal rhythm of hydrogen production (Figure 2B). There was a significant difference in hydrogen between the mice in the two groups at the endpoint of the experiment, indicating that the microbiota had metabolized the administered lactulose.

Five hours after 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 EAM 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 FP production, which should lead to a decrease in pH in the cecum. There was a significant drop in cecum content pH when EAM mice were treated with lactulose, which was associated with an increase in FP concentration in the cecum (Figure S2A and 2D). Lactulose-treated mice had significantly higher concentrations of acetate, butyrate, and succinate, indicating increased metabolic activity of all EAM species, despite the lack of significant increase in *B. theta* and *E. coli* population densities [39, 40]**.** GF mice that were treated with lactulose showed a small but significant drop in pH in cecum content, likely due to osmotic effects (Figure S2A). In GF mice, FP concentrations did not increase after lactulose treatment (Figure 2E).

In order to disentangle the osmotic from the metabolic effects of lactulose treatment, we measured the water fraction in feces and cecum content after treatment in EAM mice and GF mice in all experiments. The ratio of the dry fecal weight to the wet fecal weight of GF mice decreased with lactulose treatment, indicating an increase in water content in the feces (Figure S2B). However, the dry to wet feces ratio in EAM mice increased with lactulose treatment, possibly due to the increased microbial biomass from lactulose metabolism. We further controlled for the osmotic effect of lactulose by collecting all the feces that were produced by the mice between the treatment and endpoint of the experiment and normalized the dry weight to the dry/wet weight ratio (Figure S2C). The osmotic effect of lactulose was weaker than the metabolic effect in EAM mice feces, indicated by the increase in dry weight in feces. When we measured the water content in cecum content, we found no significant difference between treatments in either EAM of GF mice, indicating that the effect of lactulose largely affected fecal water content rather than cecal water content (Figure S2D). Therefore, we concluded that the osmotic effect of lactulose was minimal in the cecum in this study and osmotic effects therefore are not major contributors to the observed changes in bacterial population sizes and metabolism.

Inducing microbial metabolism alters circadian clock gene expression in GI tissue

To test whether the induction of microbial metabolism changes gene expression driving the host circadian rhythm, we studied the transcription of several core circadian clock genes in the mouse gastrointestinal tract (Figure 3A). The circadian clock regulation begins with the core clock genes (*CLOCK* and *ARNTL),* which activate transcription of the *Period* and

Cryptochrome repressor genes [41, 42]. *CLOCK* and *ARNTL* also activate transcription of the *Reverb* 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, EAM mice were treated with lactulose or PBS between zeitgeber time 3-4 and euthanized roughly 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 EAM mice were treated with lactulose, there was a significant increase in several core clock genes in ileum and colon tissues, but no significant difference in gene expression in cecum tissue, where it is presumed that most of the microbial metabolism occurred (Figure 3B and S3A). These differences in expression reflected the fluctuations seen in peripheral tissues in the normal mouse circadian rhythm [43]. To control for the direct effect that lactulose may have on the host, this experiment was also conducted in GF mice. When GF 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 3C).

Inducing microbial metabolism decreases host food intake in following active cycle

We then tested whether changing host circadian rhythm through specific alteration of gut microbial metabolism has further effects on circadian behavior of the host. We measured the rate and total amount of EAM 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 following dark cycle and compared to the baseline food intake in the previous dark cycle (Figure 4A). EAM 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 EAM mice that were treated with PBS (Figure 4B and S4A) or in GF mice treated with lactulose (Figure 4C and S4B). 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 [44]. However, the decrease in food intake is small in GF mice compared to EAM mice, indicating that actuation of microbiota metabolism is the dominant cause for this of this effect.

Disruption of circadian 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 [45]. However, core clock gene expression was most significantly altered in the ileum when mice were administered lactulose and they 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 4B). To test the systemic effect of microbial metabolism on host food intake, EAM mice were administered a FP mix of succinate, acetate, and butyrate, as these were the FP that were significantly produced by the EAM in the cecum after lactulose treatment (Figure 5A). Orally administered FP were presumed to be taken up by the host in the small intestine, before reaching the cecum [46]. EAM mice reduced their food intake rate and total food intake following the treatment of FP, indicating that the effect of the microbiota on host circadian behavior is systemic (Figure 5D).

One potential mechanism by which the microbiota regulates host food intake systemically is by indirect feeding of the host through fermentation products, which can be used for energy harvesting by the host [47]. To evaluate whether the systemic effect of the microbiota on host food intake was solely due to the supplemental energy provided by the FP, we estimated the

total number of kilocalories that were taken up by the host before and after the treatment of FP, assuming that all FP that were given to the host were used as an energy source (0.19 kilocalories). After accounting for the energy provided by the FP treatment, EAM mice still reduced their food intake, meaning that they reduced their caloric intake by more than 0.19 kilocalories (Figure 5C).

To further evaluate whether this systemic effect of the FP could be observed when they were provided to the host indirectly, we estimated the total maximum number of calories that could be provided to the host by the microbiota after lactulose treatment (Figure 4B). We estimated that the lactulose treatment provided each mouse with a maximum of 0.52 kilocalories (Fig. 5C). This maximum value assumes that the energy available to the host from lactulose feeding is equal to the combustion enthalpy of the fed lactulose. This is an overestimation because the host cannot directly metabolize lactulose and gets energy indirectly after the microbiota has extracted significant amounts of energy already. After accounting for the supplementary feeding of the host through FP, we found that EAM mice reduced their food intake following lactulose treatment by even more than 0.52 kilocalories. This finding indicates that the microbiota has a systemic effect on circadian behavior through a mechanism other than energy homeostasis of the host.

4. Discussion

The gut microbiome and host circadian rhythm are intricately linked, with evidence suggesting that the host circadian rhythm can influence the gut microbiota and vice versa [48, 49]. In this study, we show that the microbiota regulates host circadian rhythm and behavior through metabolic rhythmicity. We first demonstrated that the EAM gut microbiota oscillates in population and in metabolism 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 [50, 51, 18]. We disrupted the metabolic rhythmicity of the EAM by providing a nutrient that is available only to the microbiota during the host's fasting period to evaluate the effect that microbial metabolism has on host circadian rhythm. We observed a significant increase of microbial metabolism using one treatment of lactulose that was comparable to normal production during the host's active state, simulating the host's active state for the microbiota [17]. Consequently, we observed an increase of expression of several core clock genes in the ileum and colon tissue [43].

Most microbial metabolism takes place in the cecum, and interestingly, we found no change in cecum tissue gene expression after treating EAM 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 [52]. The microbiota has a systemic effect on the host immune system that extends even further than GI tissue – FP induce regulatory T-cell production and differentiation in the host peripheral tissues [53]. The systemic effect of the microbiota on the host circadian rhythm influenced host food intake following the lactulose treatment. We observed an increase in FP concentration in the mouse cecum after administering lactulose to EAM 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 FP directly to the host, indicating that microbial metabolism is in part regulating host circadian feeding behavior. One possible mechanism by which this may occur is through microbial feeding of the host [47]. 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 [50]. By treating EAM 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 FP. This finding indicates that providing energy is not the only mechanism by which FP reduce host feeding, and that there is a further mechanism that regulates the host's decrease in total energy harvest.

FP may have a systemic effect on the host through various mechanisms, and there is an association between FP intake and a reduction in food intake [54, 55, 56, 57]. Butyrate dietary supplementation in mice decreases food intake and promotes fat oxidation, and acetate supplementation can decrease appetite directly through the blood-brain-barrier [58, 59]. It has also been found that dietary supplements of fermentable carbohydrates which can be broken down by the microbiota result in an upregulation of GLP-1 and PYY, as well as a downregulation in the orexigenic hormone ghrelin [60, 61]. These hormones involved with satiety signaling are produced in the body in a rhythmic manner [62, 63]. By disrupting the rhythmic fluctuations of microbial metabolism and increasing FP production in the cecum, the microbiota may disrupt the circadian satiety signaling hormone production and therefore disrupt the circadian clock, and subsequently, feeding behavior. It has previously been found that mice with gene deletions in the circadian clock lose rhythmicity in food intake and FP concentration in the cecum, and that restoring rhythmic food intake through restricted feeding restores rhythmicity in FP production, indicating the intricate association between the host circadian rhythm and the microbiota [64, 65, 66].

In conclusion, our work demonstrates that the gut microbiota regulates host circadian rhythm. The microbiota breaks down dietary fibers ingested by the host and the metabolic products of microbial metabolism regulate host circadian gene expression and eating behavior. We speculate that this regulation extends to other factors associated with host circadian rhythm such as endocrine signaling, as the effect of the microbiota on the host circadian rhythm was systemic, and that the microbiota plays a significant role in maintaining regular circadian behavior through its metabolic byproducts.

5. Acknowledgements

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6. Author contributions

G.G., E.S.,and M.A. designed the research. G.G. performed the experiments and data evaluation, and S.O. and D.K. assisted in the experiments. J.L. performed the mass spectrometry. G.G. and M.A. wrote the manuscript, in cooperation with all authors.

7. Declaration of Interest

The authors declare no competing interests.

8. Figure titles and legends

Figure 1: The host circadian rhythm influences microbial cyclical fluctuations. A: Cell counts per grams of feces in the EAM mouse (n=8), calculated by qPCR. **B:** Daily

fluctuations of EAM strains in mouse feces. Feces were sampled over 2 days (n=8). Shaded regions represent the dark cycle in the animal facility, from zt=12 (6PM) to zt=24 (6AM). **C:** Daily fluctuations in relative abundance of EAM strains in mouse feces. Relative abundance was calculated as the proportion of total bacterial population. **D:** Circadian food intake and hydrogen production of the EAM mouse over two days of measurement, where measurements are taken every 24 minutes (n=16). Curve fit was calculated using formula y~s(x; bs="cs). SE is illustrated with grey shading surrounding line. **E:** Circadian food intake and hydrogen production of the GF mouse (n=8).

Figure 2: Lactulose disrupts the normal rhythm of microbial metabolism. A: Schematic illustrating the experimental procedure. **B:** Hydrogen production increases after lactulose gavage, but not after PBS gavage (n=16, 8 in each group). Treatment is indicated by a dotted line at zt=3. An unpaired T-test was performed on final hydrogen measurements ($p<0.0001$). **C:** Bacterial population after gavage (n=22, lactulose=12, PBS=10). *E. rectale* population increases in lactulose treated mice compared to PBS treated mice ($p<0.05$). **D:** SCFA concentration in EAM (n=17, lactulose=10, PBS=7) mice after treatment. Unpaired T-test revealed an increase in acetate, butyrate, and succinate concentration after multiple testing correction ("**" = $p<0.01$, "***" = $p<0.001$, "****" = $p<0.0001$). **E:** SCFA concentration in GF (n=8, 4 in each group) mice after treatment. There is no difference in SCFA concentration after treatment.

Figure 3: Inducing microbial metabolism alters circadian clock gene expression in GI tissue. A: Schematic of the clock gene network in mice. **B:** Clock gene expression in gut tissue after EAM mouse treatment (n=17, lactulose=10, PBS=7) measured via RT-PCR. Unpaired T-test revealed increases in gene expression in the colon and ileum after multiple testing correction ("*" = $p<0.05$, "**" = $p<0.01$). **C:** Clock gene expression in gut tissue after GF mouse treatment (n=16, 8 in each group). No significant differences were found in gene expression.

Figure 4: Inducing microbial metabolism decreases host food intake in following active cycle. A: Schematic illustrating the experimental procedure. **B:** EAM mouse (n=15, lactulose=8, PBS=7) food intake rate in dark phase prior to treatment (control) and following treatment (treatment). Paired T-test displays a reduction in food intake rate after lactulose treatment ($p<0.01$). **C:** GF mouse (n=14, 7 in each group) food intake rate. Paired T-test displays no difference in food intake rate after either treatment.

Figure 5: Disruption of circadian food intake patterns is due to systemic effects of microbial metabolic products. A: Schematic illustrating the experimental procedure. **B:** EAM mouse (n=5) food intake rate in dark phase prior to FP treatment (control) and following treatment (treatment). Paired T-test displays a reduction in food intake rate after FP treatment ($p<0.01$). **C:** EAM mouse total caloric intake after accounting for calories provided by the FP treatment. Paired T-test displays a significant reduction in caloric intake after accounting for calories provided directly by the FP ($p<0.05$). **D:** EAM mouse (data calculated from S4A) total caloric intake after accounting for calories provided by the microbiota after lactulose metabolism. Paired T-test displays a significant reduction in caloric intake after accounting for feeding by the microbiota ($p<0.05$).

9. Methods

5.1 Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Markus Arnoldini (Markus.Arnoldini@hest.ethz.ch).

5.2 Materials availability

All materials are available upon request to the corresponding author.

5.3 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 EAM microbiota and kept under strict hygienic conditions in breeding isolators. The EAM microbiota was derived using strains *Bacteroides thetaiotaomicron* [67], *Eubacterium rectale* ATCC, and *Escherichia coli* HS. All experiments began with 9-15 weeks of age with mixed-sex groups. Mice were fed *ad libitum* for the duration of all experiments and were kept with a dark and light period of 12 hours each. All experiments were approved by the Swiss Kantonal authorities (License ZH120/19 and ZH016/21) according to the legal and ethical requirements.

5.4 Metabolic cages

At the start of the experiment, mice were transferred from the breeding isolators to the isolator-housed TSE PhenoMaster® (TSE Systems, Germany) 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, Switzerland) 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.

5.5 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. The oral gavage was administered between Zeitgeber 3-4. To measure circadian rhythm changes in the host and changes in the gut, mice were euthanized 5 hours after the gavage. To measure the effect of lactulose on host food intake, mice were euthanized the day following the treatment. To measure the effect of FP on host food intake, mice were treated between Zeitgeber 3-4 with 66mg of FP 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.

5.6 Bacterial quantification

Mice were euthanized when a peak in hydrogen production was observed following gavage. Feces or cecum content were collected from mice and weighed for bacterial quantification. Bacterial DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany). qPCR was performed using the FastStart Universal SYBR Green Master Mix (Roche, Switzerland). Primers for each tested strain were diluted to a final concentration of 1µM. DNA was amplified using a QuantStudio 7 Flex instrument (Applied Biosystems, MA, USA) or a StepOnePlus Real-Time PCR System instrument (Applied Biosystems, MA, USA), with an initial denaturation step of 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds. Bacteria were quantified in each run using a standard curve of known bacterial DNA concentration. Germ-free cecum content and water samples were used to generate a detection limit. For each experiment, a contamination control for *Blautia pseudococcoides* was included in the qPCR, and mice with a C_T lower than 26 in cecum samples were excluded from the study.

5.7 Cecal pH measurement

Cecum content was collected in a 2mL Eppendorf tube (Sarstedt, Switzerland) and snap frozen in liquid nitrogen. 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, Ohio, USA) was completely inserted into the cecum content and pH was measured.

5.8 Short chain fatty acid quantification

Cecum content was collected in a 2mL Eppendorf tube, snap frozen in liquid nitrogen, and stored at -80ºC until further analysis. For UPLC/MS, cecum content was thawed on ice, then homogenized in 70% isopropanol and centrifuged. The supernatant was used for FP quantification using a protocol previously described [68]. A 7-point calibration curve was generated with FP 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, San Jose, CA) were used for UPLC/MS analysis. For the FP quantification a Kinetex 2.6 µm XB-C18, 50 × 2.1 mm column (Phenomenex, Torrance, CA, USA) 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 a home-built R code. Limit of blank was calculated as mean of the concentration detected in technical blank samples +- 1.645 standard deviation.

5.9 Dry to wet weight ratio calculation¨

Feces and cecum content were collected at the end of the experiment and the wet weight was measured. Samples were then lyophilized (Alpha 2-4 LD plus, Martin Christ GmbH, Germany**)** 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 and lyophilized. The dry weight of the feces was measured and normalized to the dry to wet weight ratio of each mouse.

5.10 RNA extraction

Mouse ileum, cecum, and colon tissues were collected in RNAlater (Thermo Scientific, CA, USA**)** and snap frozen in liquid nitrogen. Samples were stored at -80ºC 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, CA, USA) and incubated for 5 minutes at room temperature. Samples were then homogenized twice with 0.1mm beads (BioSpec Products, Oklahoma, USA) and 1.0mm silicon carbide beads (BioSpec Products, Oklahoma, USA), for 90 seconds at 30Hz. Further Trizol was mixed with the samples, and centrifuged. Supernatant was mixed with chloroform (Sigma-Aldrich, Switzerland) and centrifuged. Supernatant was then washed in 100% ethanol and centrifuged in collection tubes. RNA was washed in Pre-wash buffer and incubated for 15 minutes at room temperature with 60 units of DNAse. Finally, RNA was washed in Wash buffer and eluted in Nuclease-free water (Invitrogen, Massachusetts, USA).

RNA concentration and quality were measured using a nanodrop machine (ND-1000 Spectrophotometer, Thermo Scientific, CA, USA) 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.

5.11 Circadian clock gene expression

Host circadian clock gene primers were used to estimate expression using RT-PCR. A housekeeping gene (TATA binding protein) was used for normalization. The RT-PCR was carried out as previously described, and sample extracted RNA was included in a reaction with the housekeeping gene to control for DNA contamination. The delta delta C_T method was used to estimate relative gene expression for each experiment [69]**.**

5.12 Data analysis

Phenomaster quality control

For all measurements, a variable of zeitgeber time (h) was created where 0-12 represents the mouse light phase and 12-24 represents the mouse dark phase. 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 for 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. 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 human disruption, as well as leaky water bottles. After the 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 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. Maximum 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 circadian rhythm of 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, it was assumed that the EAM metabolized 100% of the lactulose, and that the entirety of the fermentation products of the microbiota were absorbed by the host for caloric feeding. The combustion enthalpy of lactulose was estimated from the combustion enthalpy of lactose (1346 kcal/mol), and the kilocalories from 132mg of lactulose were calculated to be 0.52 per mouse [70]. For estimating the caloric intake of the EAM mouse in the FP treatment, it was assumed that 100% of FP given to the host were used for energy harvesting. The combustion enthalpies and masses provided to the host of acetate (0.21 kcal/mmol, 19.8 mg), butyrate (0.52 kcal/mmol, 19.8 mg), and succinate (0.36 kcal/mmol, 26.4 mg) were used to calculate the maximum possible calories provided to the host (0.19 kcal).

It was assumed that there are 3.94 kilocalories/gram of standard mouse chow [50]. The total caloric intake was calculated 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 FP 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.

For estimating clock gene expression differences in GI tissue and calculating FP concentration in cecum content an unpaired T-test was carried out to compare both groups. To account for false positives in the data analysis, the Benjamini and Hochberg multiple testing correction method was used on both these datasets. For all further comparisons between treatment groups, an unpaired T-test was used to measure differences. All data analysis was carried out using R V4.2.0.

10. Supplemental titles and legends

Figure S1: *E. rectale* is the principal hydrogen producer in the EAM. A:Circadian food intake and hydrogen production of the EAM mouse over two days of measurement, where measurements are taken every 24 minutes (n=16). Curve fit was calculated using formula y~s(x; bs="cs). SE is illustrated with grey shading surrounding line. Shaded regions represent the dark cycle in the animal facility, from zt=12 (6PM) to zt=24 (6AM). **B:** Circadian food intake and hydrogen production of the incomplete EAM (colonized with *B. theta* and *E. coli*) mouse (n=4)

Figure S2: 132mg of lactulose does not have a strong osmotic effect on the host. A: Cecum content pH of EAM (n=17, lactulose=10, PBS=7) and GF (n=16, 8 in each group) mice after treatment. Unpaired T-test revealed a decrease in pH in EAM mice ($p<0.0001$) and GF mice ($p<0.05$).**B:** Dry to wet feces ratio in EAM (n=15, lactulose=8, PBS=7) and GF (n=8, 4 in each group) mice. Unpaired T-test shows an increase ($p<0.01$) in the ratio in EAM mice after lactulose treatment. **C:** Total wet feces output after normalizing dry feces weight to mean dry/wet feces ratio in EAM (n=8, 4 in each group) and GF (n=8, 4 in each group) mice. Unpaired T-test shows an increase ($p<0.05$) in the total wet feces output in EAM mice after lactulose treatment. **D:** Dry to wet cecum content ratio in EAM (n=17, lactulose=10, PBS=7).

Figure S3: Inducing microbial metabolism alters circadian clock gene expression in GI tissue . A: Clock gene expression in gut tissue after EAM mouse treatment (n=17, lactulose=10, PBS=7) measured via RT-PCR. Unpaired T-test revealed increases in gene expression in the colon and ileum after multiple testing correction ("*" = $p<0.05$, "**" =

p<0.01). **B:** Clock gene expression in gut tissue after GF mouse treatment (n=16, 8 in each group). No significant differences were found in gene expression.

Figure S4: Inducing microbial metabolism decreases host food intake in following active cycle. A: EAM mouse (n=15, lactulose=8, PBS=7) total food intake in dark phase prior to treatment (control) and following treatment (treatment). Paired T-test displays a reduction in food intake rate after lactulose treatment ("**" = p<0.01). **B:** GF mouse (n=14, 7 in each group) total food intake. Paired T-test displays no difference in food intake rate after either treatment.

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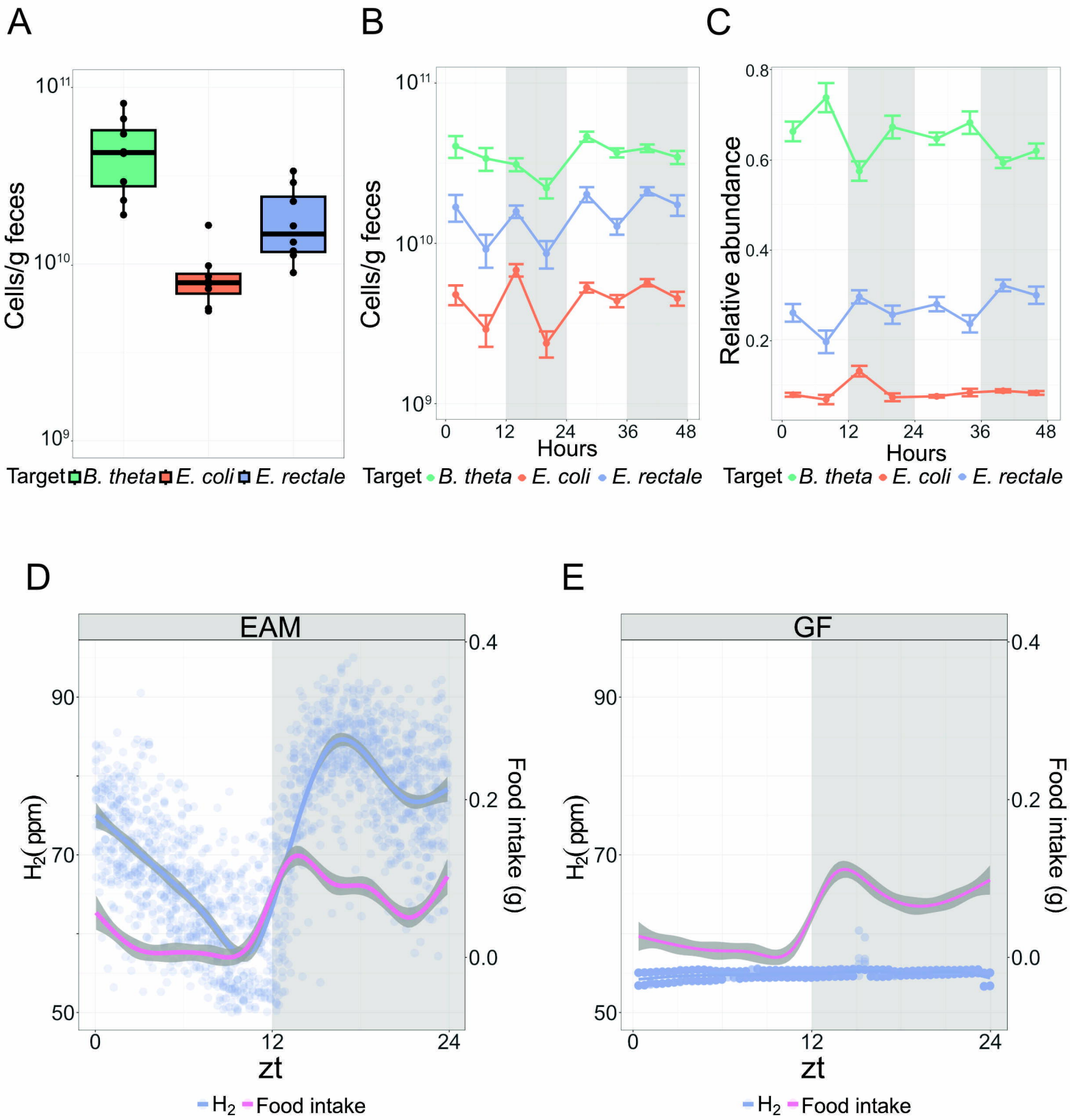


Figure 1: The host circadian rhythm influences microbial cyclical fluctuations. A: Cell counts per grams of feces in the EAM mouse (n=8), calculated by qPCR. **B:** Daily fluctuations of EAM strains in mouse feces. Feces were sampled over 2 days (n=8). Shaded regions represent the dark cycle in the animal facility, from zt=12 (6PM) to zt=24 (6AM). **C:** Daily fluctuations in relative abundance of EAM strains in mouse feces. Relative abundance was calculated as the proportion of total bacterial population. **D:** Circadian food intake and hydrogen production of the EAM mouse over two days of measurement, where measurements are taken every 24 minutes (n=16). Curve fit was calculated using formula y~s(x; bs="cs). SE is illustrated with grey shading surrounding line. **E:** Circadian food intake and hydrogen production of the GF mouse (n=8).

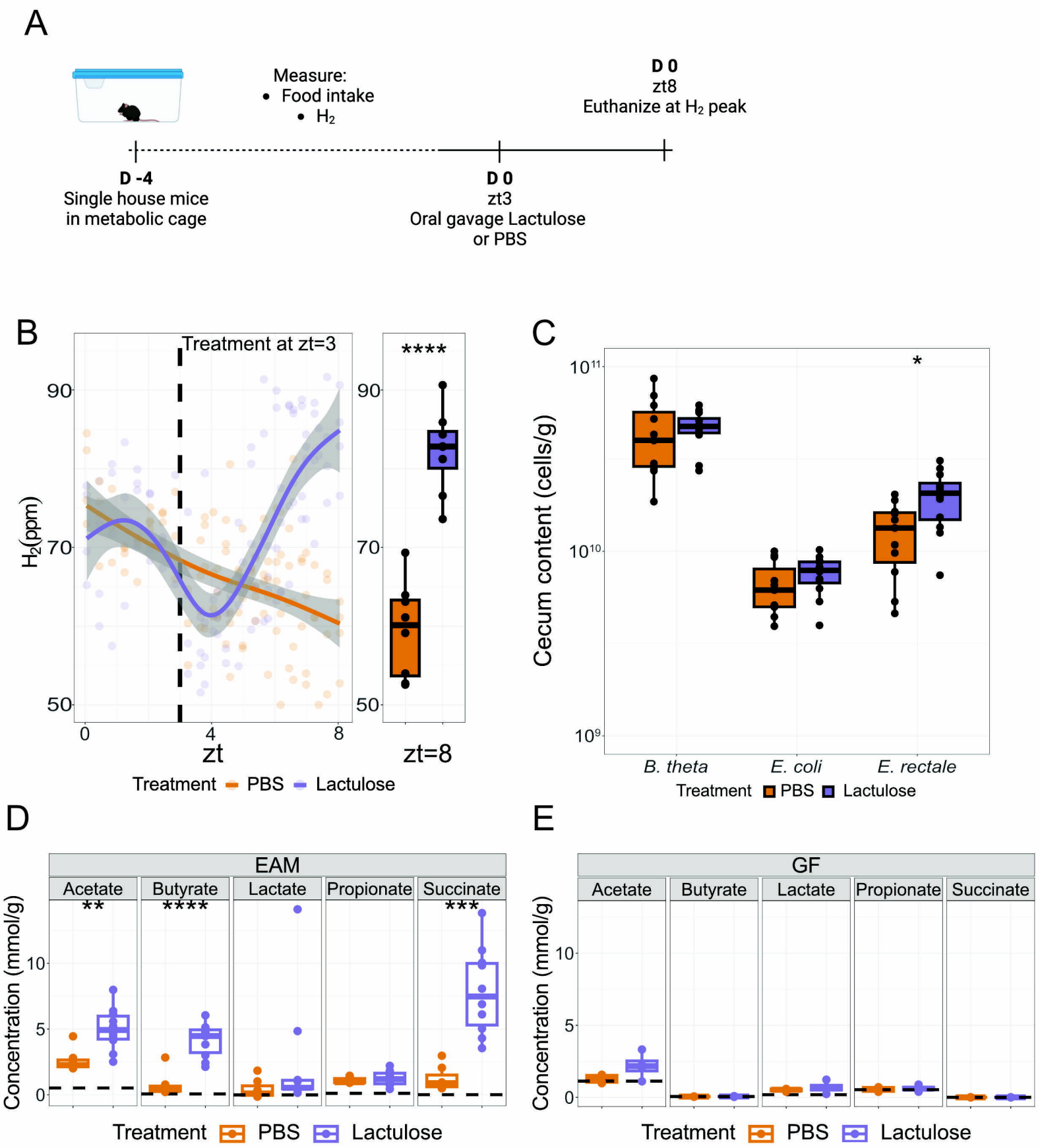


Figure 2: Lactulose disrupts the normal rhythm of microbial metabolism. A: Schematic illustrating the experimental procedure. **B:** Hydrogen production increases after lactulose gavage, but not after PBS gavage (n=16, 8 in each group). Treatment is indicated by a dotted line at zt=3. An unpaired T-test was performed on final hydrogen measurements ($p<0.0001$). **C:** Bacterial population after gavage (n=22, lactulose=12, PBS=10). *E. rectale* population increases in lactulose treated mice compared to PBS treated mice ($p<0.05$). **D:** SCFA concentration in EAM (n=17, lactulose=10, PBS=7) mice after treatment. Unpaired T-test revealed an increase in acetate, butyrate, and succinate concentration after multiple testing correction ("**" = $p<0.01$, "***" = $p<0.001$, "****" = $p<0.0001$). **E:** SCFA concentration in GF (n=8, 4 in each group) mice after treatment. There is no difference in SCFA concentration after treatment.

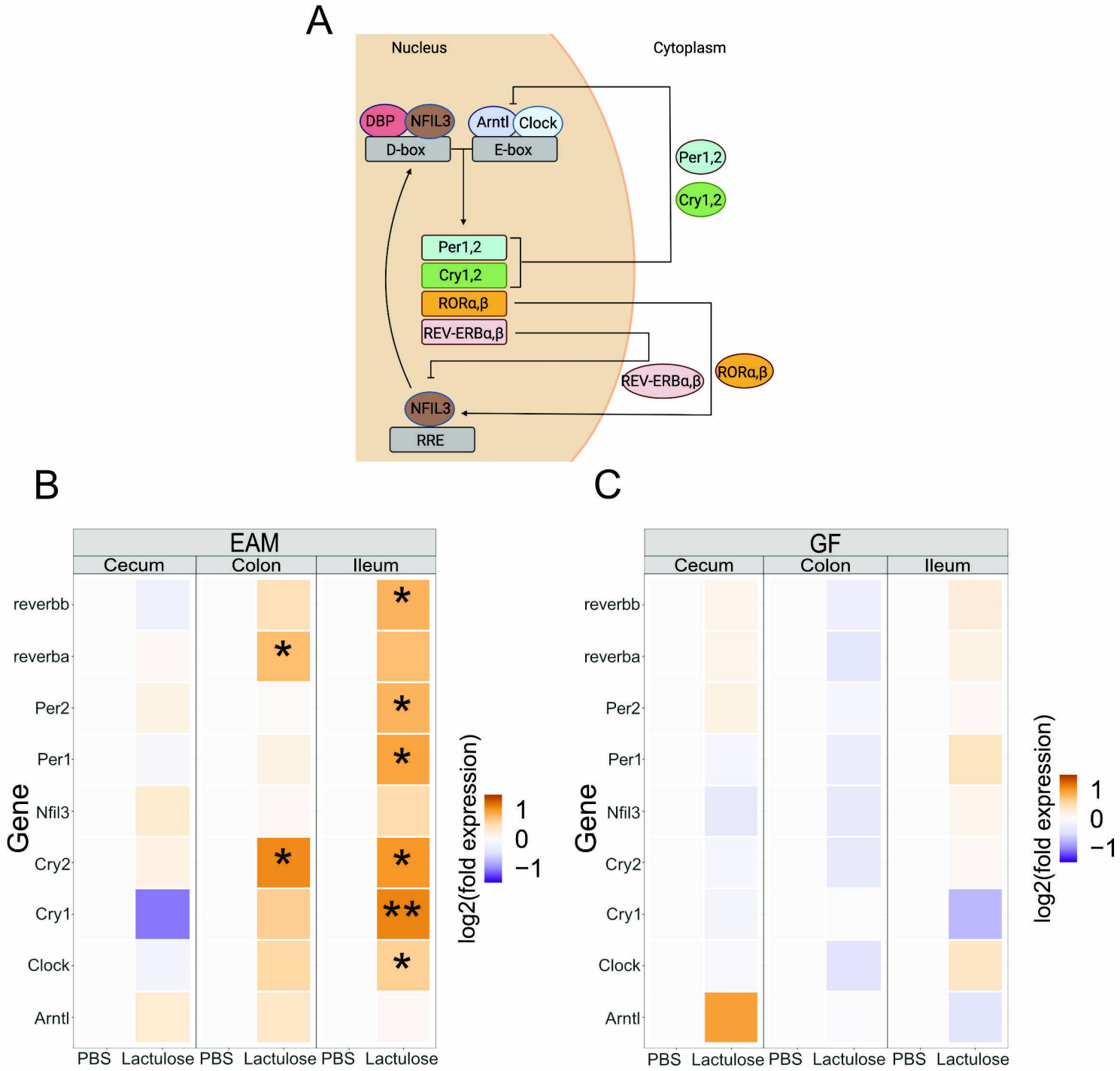
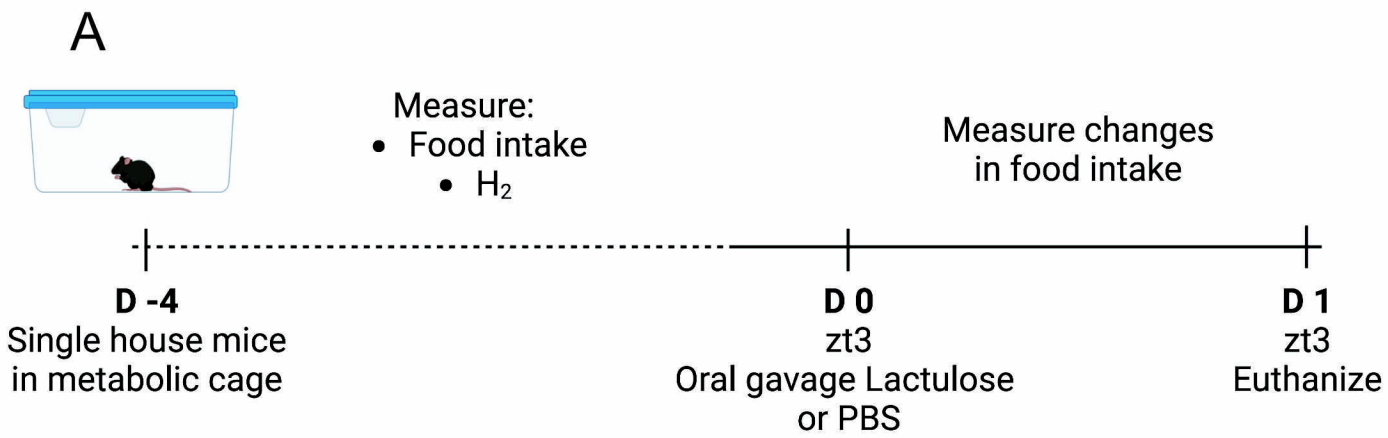


Figure 3: Inducing microbial metabolism alters circadian clock gene expression in GI tissue. A: Schematic of the clock gene network in mice. **B:** Clock gene expression in gut tissue after EAM mouse treatment (n=17, lactulose=10, PBS=7) measured via RT-PCR. Unpaired T-test revealed increases in gene expression in the colon and ileum after multiple testing correction ("*" = $p<0.05$, "**" = $p<0.01$). **C:** Clock gene expression in gut tissue after GF mouse treatment (n=16, 8 in each group). No significant differences were found in gene expression.



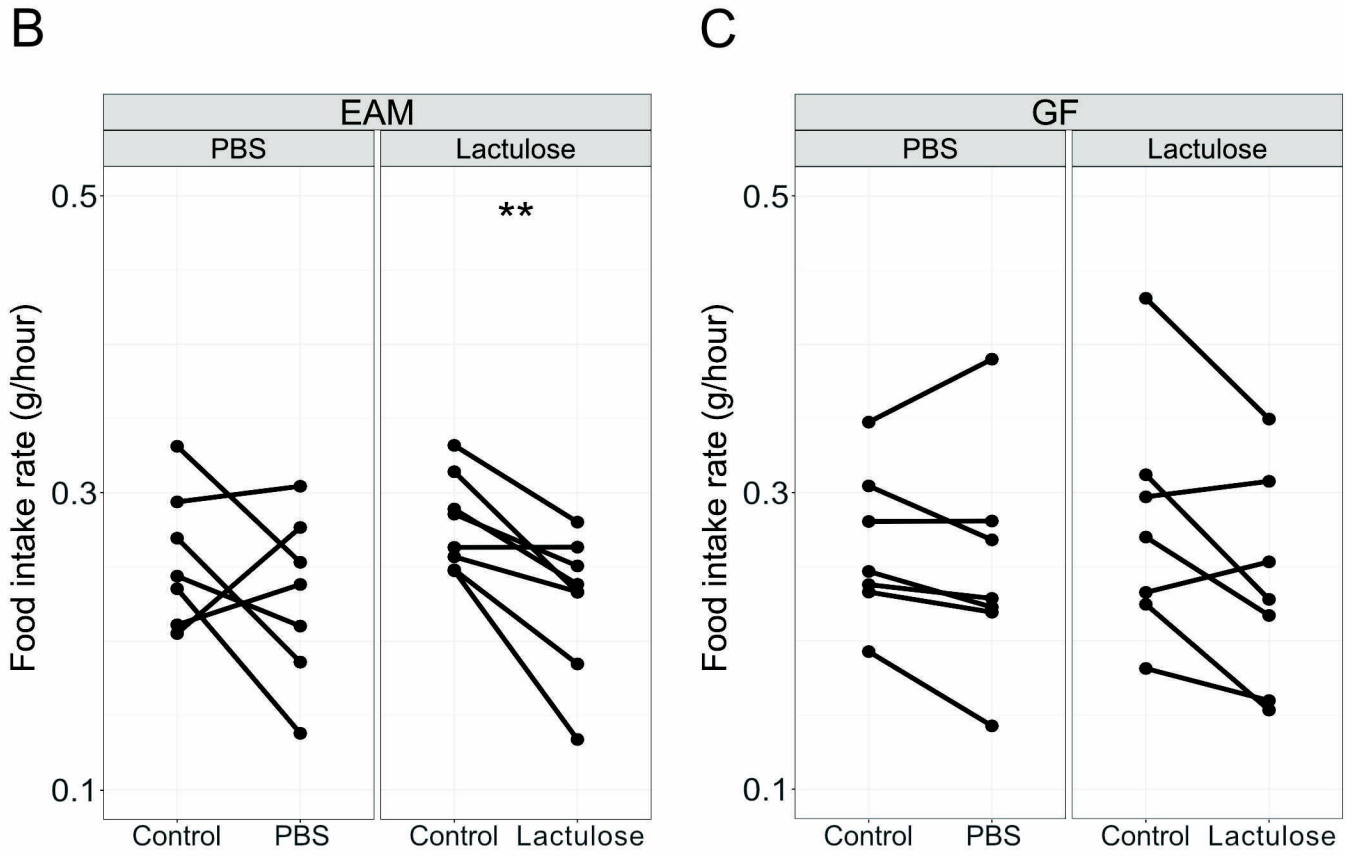


Figure 4: Inducing microbial metabolism decreases host food intake in following active cycle. A: Schematic illustrating the experimental procedure. **B:** EAM mouse (n=15, lactulose=8, PBS=7) food intake rate in dark phase prior to treatment (control) and following treatment (treatment). Paired T-test displays a reduction in food intake rate after lactulose treatment ($p<0.01$). **C:** GF mouse (n=14, 7 in each group) food intake rate. Paired T-test displays no difference in food intake rate after either treatment.

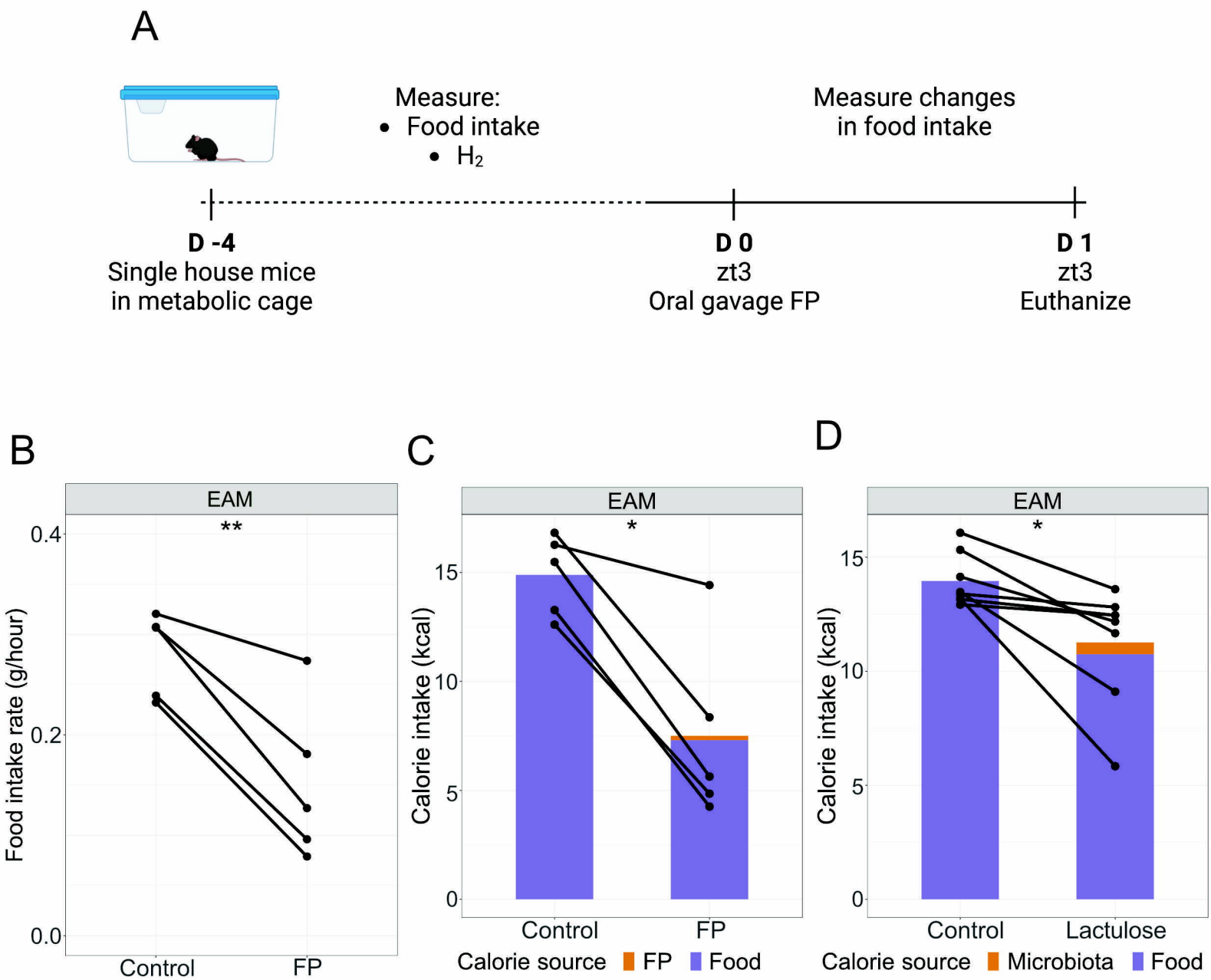


Figure 5: Disruption of circadian food intake patterns is due to systemic effects of microbial metabolic products. A: Schematic illustrating the experimental procedure. **B:** EAM mouse (n=5) food intake rate in dark phase prior to FP treatment (control) and following treatment (treatment). Paired T-test displays a reduction in food intake rate after FP treatment ($p<0.01$). **C:** EAM mouse total caloric intake after accounting for calories provided by the FP treatment. Paired T-test displays a significant reduction in caloric intake after accounting for calories provided directly by the FP ($p<0.05$). **D:** EAM mouse (data calculated from S4A) total caloric intake after accounting for calories provided by the microbiota after lactulose metabolism. Paired T-test displays a significant reduction in caloric intake after accounting for feeding by the microbiota ($p<0.05$).