Rhythms of Early Life: Gut Microbiota Rhythmicity and Circadian Maturation in infants

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Abstract

Background

The human gut microbiota undergoes daily fluctuations, yet its interaction with sleep-wake patterns during infancy remains largely uncharted. This study aims to elucidate the relationship between gut microbiota rhythmicity and the development of sleep patterns in infants over the first year of life. We continuously monitored 162 healthy infants across multiple days at 3, 6, and 12 months of age using ankle actigraphy and 24-hour diaries. The Circadian Function Index (CFI) was computed as a proxy for sleep-wake rhythm maturation. Stool samples were collected to profile gut microbiota composition via 16S rRNA gene amplicon sequencing, and microbial oscillations were assessed through sine and cosine fitting to detect 24-hour patterns.

Results

Our findings revealed that the abundance of bacterial taxa exhibited rhythmic patterns, with 26 zOTUs (1.74%) following a sine pattern and 100 zOTUs (6.69%) displaying cosine rhythmicity. Cosine rhythmicity became more pronounced with age, showing strong maturation: 7 zOTUs at 3 months, 2 zOTUs at 6 months, and 86 zOTUs at 12 months. Notably, 105 zOTUs (7.02%) were associated with CFI, demonstrating a significant relationship between gut microbiota rhythms and sleep development. Among these, 27 zOTUs with sine dynamics and 96 zOTUs with cosine dynamics were linked to CFI, with this association strengthening as infants aged.

Conclusions

These results highlight the increasing synchronization between gut microbiota rhythmicity and sleep-wake cycles during infancy, pointing to a critical window for potential health interventions. This novel observation, previously reported in rodents and adults, underscores the role of gut microbiota in early human development, offering new avenues for enhancing developmental outcomes through targeted interventions.

Introduction

Infancy represents a critical developmental phase of the human brain. During early childhood sleep undergoes pronounced maturation, a process essential for cognitive and neurophysiological development (Timofeev et al., 2020). Core electrophysiological features of sleep, such as slow waves and spindles, have been identified as biomarkers for the maturation of neuronal connectivity and function (Jaramillo et al., 2023; Kurth et al., 2012). Sleep furthermore affects immuno-regulation (Besedovsky et al., 2019), cardiovascular function (Korostovtseva et al., 2021), and metabolic balance (Stich et al., 2022), underscoring the necessity of adequate sleep to both neurological development and physiological health. Early human life is characterized by notable variability in individual sleep behavior (Iglowstein et al., 2003; Schoch, Huber, et al., 2020). Despite the substantial health implications, such as effects on cognitive development and obesity risk (Li et al., 2022; Morales-Muñoz et al., 2021), the underlying drivers of this variability remain largely obscure. The complexity of capturing sleep maturation through continuous monitoring presents a substantial challenge, hindering a thorough understanding of infant sleep patterns. During infancy, neurophysiology and behavior are highly modifiable, allowing for effective re-shaping of developmental trajectories through timely interventions (Sinthong & Ngernlangtawee, 2024). For example, early sleep interventions have been shown to extend nighttime sleep duration, reduce night wakings, and promote self-soothing behaviors, all of which contribute to improved sleep quality and support healthy development. This highlights a promising approach to enhancing sleep regulation and fostering positive developmental outcomes.

Given the multifaceted factors influencing sleep, recent research has uncovered a bidirectional relationship between sleep and gut microbiota—the microorganisms inhabiting the gastrointestinal tract (Sen et al., 2021). Modifying the gut microbiota through antibiotics can disrupt sleep patterns, such as reducing non-rapid eye movement sleep (NREMS) and increasing REM sleep episodes in mice, or decreasing slow-wave sleep (SWS) in humans and rats, suggesting a role for microbiota in maintaining healthy sleep architecture (Brown et al., 1990; Nonaka et al., 1983; Ogawa et al., 2020). Conversely, sleep disruptions have been found to alter gut microbiota composition (Bowers et al., 2020; Thaiss et al., 2014). In adults, a fraction of variability in sleep quality and duration is explained by gut microbiota profiles, underscoring the interconnectedness of sleep to microbial signatures (Grosicki et al., 2020; Smith et al., 2019). The sleep-gut connection is not limited to adulthood, but is observed in adolescence, childhood, and infancy; likely it primes health outcomes across the lifespan (Carpena et al., 2024; Schoch et al., 2022; Wang et al., 2022; Xiang et al., 2023).

Similar to sleep, variability in individual gut microbiota during infancy is large (Hill et al., 2017), and defines critical health aspects such as metabolism (Altaha et al., 2022), immune function (Lubin et al., 2023), and neurodevelopment (Sordillo et al., 2019). Colonization of the infant gut begins at birth, and across the first three years of life microbial diversity, stability, and complexity increase. The succession of microbial communities in a healthy infant gut is influenced by environmental and dietary factors, such as mode of birth, antibiotic usage, and dietary habits (Catassi et al., 2024; Mercer et al., 2024; Pantazi et al., 2023). Furthermore, gut microbiota have recently been found to undergo “diurnal” dynamics, demonstrating variance in abundance and function across the day (Thaiss et al., 2014; Voigt et al., 2014). Animal models show that diurnal microbial dynamics are tied to the host’s circadian rhythm, as bacterial diurnal variation is partially regulated by their host’s clock genes (Per1/2, Bmal1) (Heddes et al., 2022; Thaiss et al., 2016). However, this interaction between the host’s circadian rhythm and bacterial rhythm appears to be bidirectional: in germ-free mice, the absence of microorganisms disrupts the intestinal epithelial cell clock, decreasing the expression of core clock genes such as Bmal1 and Cry1, while increasing Per1 and Per2. This disruption alters metabolic homeostasis and rhythmic expression in the intestinal cells (Mukherji et al., 2013). Further evidence supporting the role of the gut microbiome as a “bottom-up” regulator of the circadian clock is provided by observations that microbial metabolites modulate the peripheral clock in mice (Tahara et al., 2018). This suggests that the interaction between the gut microbiota and the host's biological clock extends beyond mere temporal coordination, but also includes but also includes regulatory effects on the host’s circadian clock function.

In addition to these observations in adult animals, novel studies suggest that diurnal variations also occur in the gut microbiota of infants (Heppner et al., 2024). However, their connection to the infants’ circadian rhythm remains unknown. Addressing this phenomenon requires longitudinal data across infancy, collected from large, well-controlled cohorts with objective and continuous assessments of sleep. Ankle-actigraphy captures physiological acceleration, thereby providing reliable data that captures the large individual variability in infant rest-activity patterns (Schoch, Kurth, et al., 2020; Werner et al., 2008). Applying the Circadian Function Index (CFI) (Ortiz-Tudela et al., 2010), which effectively differentiates sleep and circadian disorders (Rodriguez-Morilla et al., 2019), can serve as a proxy for circadian maturation; facilitating the investigation of its relationship with gut microbiota dynamics.

We hypothesized that the maturation of infant circadian rhythm, as quantified by an increased CFI, is associated with individual gut microbiota profiles. This relationship would indicate that as the circadian rhythm matures, distinct shifts in gut microbiota composition can be observed. Additionally, we predicted a maturation of diurnal rhythm in gut microbiota composition across the first year of

life. Specifically, we anticipated that the proportion of rhythmic zOTUs would increase with age. Third, we expected that the diurnal microbial rhythm is linked to infants' sleep regulation, quantified by the CFI. A linkage would indicate that as the circadian rhythm develops and strengthens, the rhythmic expression of the gut microbiota also becomes more pronounced.

Methods

Study Population

This study was conducted in Switzerland and included 162 healthy infants assessed at ages 3-12 months from 2016 to 2019 (Schoch et al., 2022). Infants included in the study were term-born, showed no signs of acute or chronic illness, were delivered vaginally, and received at least 50% of their nutrition from breast milk at the time of enrollment. Exclusion criteria encompassed the use of antibiotics or sleep-affecting medications, chronic illnesses, or disorders of the central nervous system. The cantonal ethics committee of Zurich, Switzerland, approved the project (BASEC 2016-00730), in line with the principles of the Helsinki Declaration. Before study enrollment, parents received detailed explanations about the study procedures before providing written consent.

Study design

Families and infants were longitudinal assessed at 3, 6, and 12 months of age. For each assessment, actigraphy data was collected for 11 consecutive days (9.16 ± 1.23 days) using a GENEactiv sensor (Activinsights LtD, Kimbolton, UK, 43x40x13mm, MEMS sensor, 16g, 30 Hz frequency) attached to the infant's left ankle (either with a paper strap or in a modified sock). Concurrently, caregivers completed a 24h paper-pencil sleep diary to document sleep and wake periods, any temporary removal of the device, and external movements during sleep (*e.g.*, such as when the infant was in a moving vehicle) (Schoch et al., 2019; Werner et al., 2008). Additionally, caregivers collected at least one infant stool sample during each assessment, for which clock time was documented. Demographic data and complementary information was collected with online questionnaires at each assessment timepoint (SoSci Survey; (Leiner, 2021).

Data processing

Actimetric activity counts were processed in Matlab (R2016b), by using binary files extracted through GENEactiv PC Software (version 3.1). Transformation involved applying a 3–11 Hz bandpass filter and compressing the signal into 15-second bins. Acceleration data from all three axes was combined using a sum of squares. To enhance data accuracy, actigraphy output was integrated with information

provided by parents in the 24-hour diaries, particularly regarding instances when the actimeter was not worn, instances of external movement during sleep (e.g., while in a car), as well as cases of poor fit between actimetry data and diary records. The acceleration signal was compressed to one data point per minute through data summation (Schoch, Huber, et al., 2020). This compressed acceleration data was then used to compute the CFI as a proxy for circadian maturation.

Circadian Function Index

CFI measures the regularity of activity-rest patterns with a score from 0 to 1 (Ortiz-Tudela et al., 2010). The computation of CFI relies on 3 measures , computed through the R package "nparact" version 0.8 (Blume et al., 2016): Intradaily Variability (IV), Interdaily Stability (IS), and Relative Amplitude (RA). IV determines the frequency and extent of transitions between rest and activity within a 24-hour day, such that a lower IV represents more consolidated rest-activity periods, whereas a higher IV suggests frequent transitions and thus fragmented sleep patterns. IS quantifies the similarity of activity patterns across days. A higher IS represents a more stable day-to-day activity-rest pattern. RA measures the amplitude between the most active and least active periods within a 24-hour cycle. A higher RA indicates a more pronounced difference between peak activity and rest periods.

For each infant, these three scores were averaged across days of continuous actigraphy data collection, covering an average period of 9.16 ± 1.23 days across all ages (3, 6, and 12 months). Specifically, at 3 months, infants were assessed over an average of 9.28 ± 1.17 days. At 6 months, the average was 9.14 ± 1.46 days. By 12 months, the average assessment period was 9.04 ± 0.99 days. The CFI was then calculated with the following formula:

$$\mathrm{CFI} = \frac{(\mathrm{IS} + (2 - \mathrm{IV})/2 + \mathrm{RA})}{3}$$

This formula combines each component to provide an overall measure of circadian maturation, with higher CFI values reflecting more regular and stable activity-rest patterns.

DNA extraction from stool and processing of gut microbiota

Gut microbiota profiles were computed from stool samples collected at infant ages 3, 6, and 12 months. Upon collection, samples were stored in the participants' fridge (approximately 4°C) before being transported at a consistent temperature within 96 hours to the laboratory. Upon arrival, samples were divided into 200 mg aliquots and preserved at -50 °C until processing. Total DNA extraction was performed using a PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA), following the manufacturer's guidelines with small modifications (for details see (Schoch et al., 2022). 16S rRNA gene amplicon libraries were prepared using specific primers targeting the V3 region

173 (Ovreås et al., 1997), equipped with adapters compatible with the Nextera Index Kit® (Illumina, CA,
174 USA): NXt_338_F: 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG ACW CCT ACG GGW GGC
175 AGC AG -3' and NXt_518_R: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAT TAC CGC GGC
176 TGC TGG -3'. Subsequent steps for amplification profiling, barcoding, purification of amplicon
177 libraries, and sequencing were carried out as previously described (Krych et al., 2018). Initial quality
178 assessment of the raw datasets (2x151bp, paired-end) was conducted using FastQC58. Overlapping
179 reverse and forward reads were trimmed using SeqKit59, and reads were merged with FLASH
180 (v1.2.11) (Magoč & Salzberg, 2011), allowing for overlaps between 15 and 300, with a maximum
181 mismatch density of 0.25. Cutadapt (v1.12) (Martin, 2011) was utilized for primer region trimming,
182 maintaining an error rate below 0.01. Prinseq (Schmieder & Edwards, 2011) was used for quality
183 filtering by discarding reads containing ambiguous nucleotides or with an average quality score below
184 20. Zero-radius Operational Taxonomic Units (zOTUs) were identified using usearch (UNOISE3
185 v10.0.240) (Edgar & Flyvbjerg, 2015), and taxonomic classifications were determined based on the
186 Greengenes database (DeSantis et al., 2006).

187 To mitigate batch effects during stool sample processing, batch run was incorporated as a control
188 variable in all statistical models. Samples with fewer than 50,000 reads were excluded from the study
189 (n=6). The remaining samples were rarefied to the lowest observed read count of 50,268. This
190 process resulted in 1430 amplicon sequencing variants. Autoclaved water samples, subjected to the
191 same experimental procedures, served as negative controls and exhibited read counts ranging from 2
192 to 150,785. zOTU2 (Enterobacteriaceae), identified in 30% of the negative controls and 10% of the
193 stool samples, showed no significant impact on the studied gut microbiota markers. Only bacterial
194 taxa present in at least 20% of the samples from all collection points (3-, 6-, and 12-months) and
195 accounting for at least 1% of total bacterial counts were included in subsequent analyses. This
196 selection process resulted in 1451 zOTUs included in the analysis.

197 Rhythmicity Analysis

198 To assess diurnal rhythmicity in the abundances of specific bacterial taxa (zOTUs), we adapted a
199 cosinor analysis approach. Cosinor analysis utilizes sinusoidal models to capture periodicity in time-
200 series data and is thus particularly well-suited for biological rhythms (Cornelissen, 2014). We
201 transformed the clock times of stool sample collection into numerical values representing the time of
202 day, and then applied sine and cosine transformations to these values. By fitting sine and cosine
203 functions, we could evaluate whether zOTU abundance exhibited patterns conforming to a 24-hour
204 cycle. This method allowed us to detect and quantify cyclical variations in the abundance of microbial
205 populations.

Statistical Analysis

Data processing, modeling, visualization, and statistical analysis was performed using R version 4.2.1 and packages ggplot2 (Wickham, 2016), phyloseq (McMurdie & Holmes, 2013), and lme4 (Bates et al., 2015). We applied linear mixed-effects models to compute associations between the abundance of zOTUs (dependent variable) and CFI, clock time of stool sampling (fitted to sine and cosine functions), and age group, allowing us to examine microbial rhythmicity. We further incorporated interactions between sine and cosine fits with CFI to assess the combined effects of infant circadian rhythm and microbial zOTU rhythmicity. For rhythmic zOTUs, we reported the relative proportions of phyla. Control variables in the models included sex (0 for male, 1 for female), breastfeeding status (0 for not or rarely breastfed, 1 for occasionally, regularly, or daily breastfeeding) and sequencing run (categorical, with levels 1 to 5). Random intercepts for each participant were included to capture individual-level variability. The alpha level was set to $P < 0.05$, and to address multiple comparison concerns, we applied False Discovery Rate (FDR) correction to the p-values.

Results

Circadian maturation across infancy

Across the first year of life, CFI increased with infant age, with mean CFI values of 0.586 ± 0.0659 at 3 months, 0.645 ± 0.0574 at 6 months, and 0.704 ± 0.0530 at 12 months. CFI increased significantly from 3 months to 6 months ($p < 0.001$, 95% C.I. = 0.044, 0.080; Tukey post-hoc tests), from 3 months to 12 months ($p < 0.001$, 95% C.I. = 0.101, 0.134), and from 6 months to 12 months ($p < 0.001$, 95% C.I. = 0.0416, 0.0747).

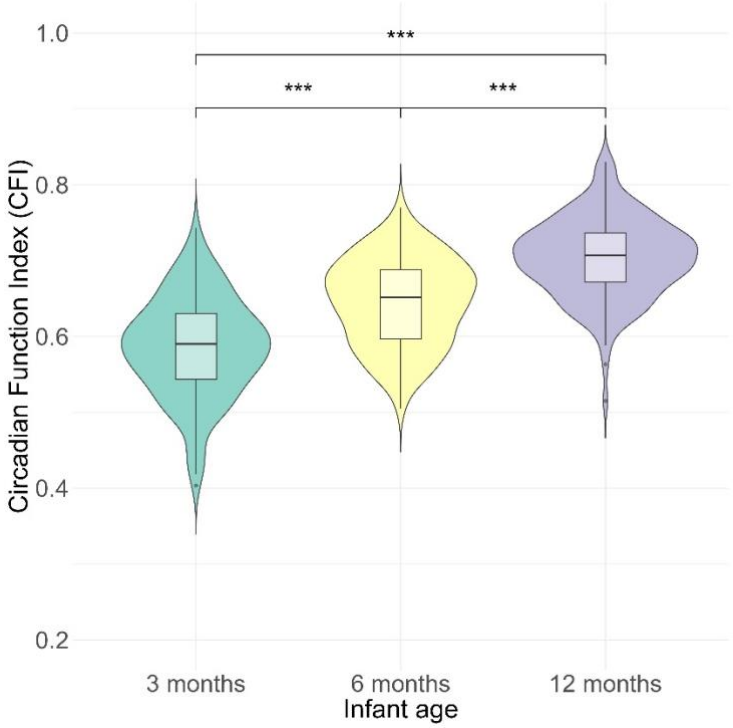

Figure 1. Age-related increase in sleep regulation across infancy assessed with Circadian Function Index (CFI) (Tukey post-hoc tests, *** $p < 0.001$). Central horizontal lines refer to median, the box spans the interquartile range (IQR), covering the middle 50% of data from the 25th to the 75th percentile. Whiskers extend to the highest and lowest values within 1.5 times the IQR from box edges. Points beyond the whiskers represent outliers.

<u>Association between circadian maturation and overall gut microbiota composition</u>

To define whether infant CFI underlies differences in overall gut microbiota composition, we analyzed associations between zOTUs abundance and CFI (rhythmicity of zOTU abundance is presented subsequently). This identifies the bacterial taxa linked to the infants' circadian maturational status. We observed both positive and negative correlations between zOTUs and CFI. A total of 105 zOTUs showed significant associations with CFI, representing 7.02% of all identified zOTUs across samples. The strength of this association increased with age: at age 3 months, only 10 zOTUs were linked to CFI, increasing to 17 zOTUs at 6 months, and 87 zOTUs at 12 months. Thus, over the first year of life, a progressively stronger association between infant gut microbiota and sleep rhythm emerges.

Among all 105 zOTUs associated with CFI, 74 showed positive correlations (Figure 2A), indicating that infants with higher CFI had increased abundance of these bacterial taxa. At 3 months, among the 3 zOTUs positively correlated with the CFI were 1 Actinobacteria, 1 Bacteroidetes and 1 from unknown phylum (Figure 2B). At 6 months *Actinobacteria* was the primary phylum associated with CFI, while at 12 months it was mainly *Actinobacteria* and *Firmicutes.*

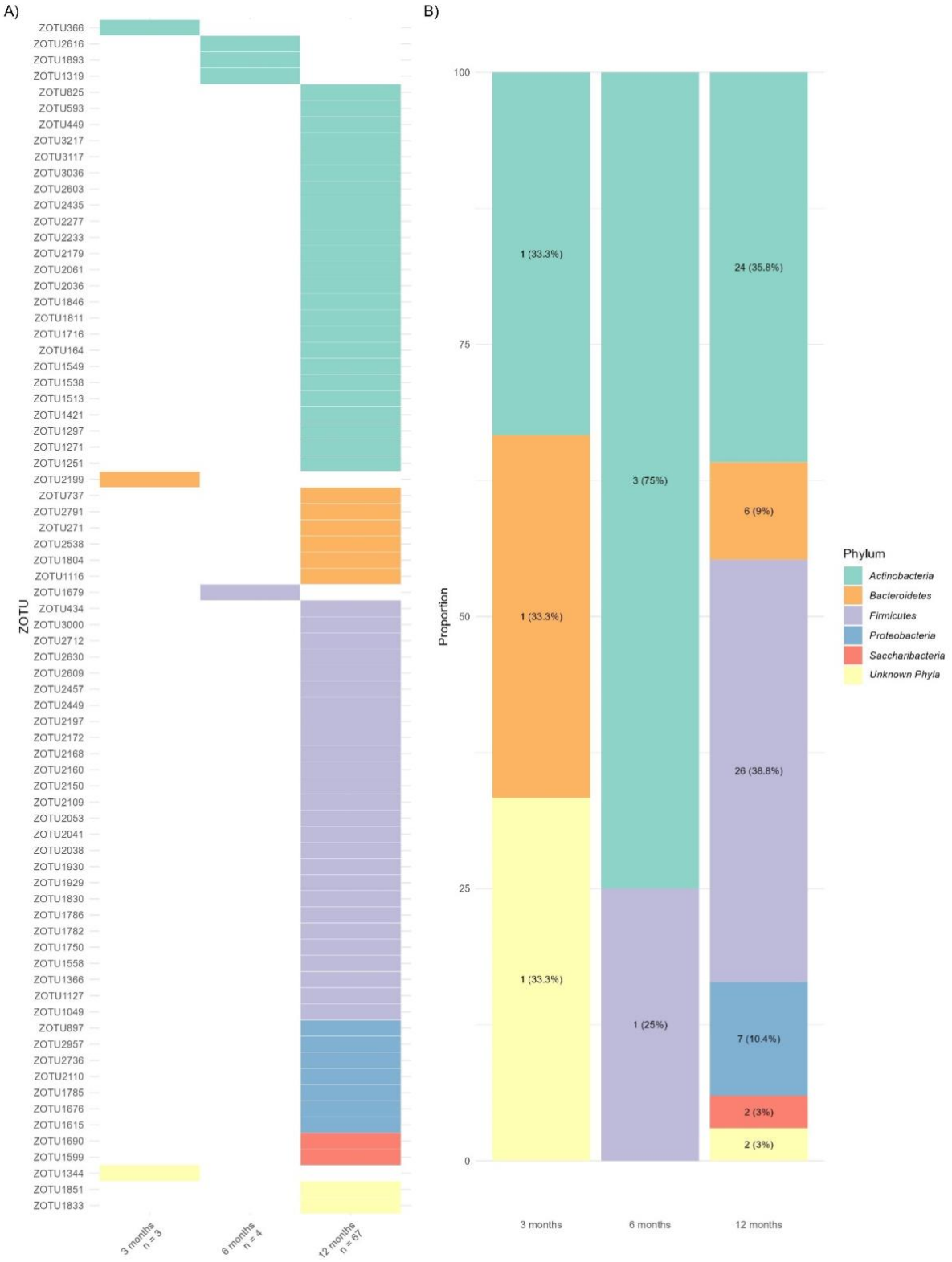

Figure 2. A) Heatmap of zOTUs (n = 74) with abundance positively associated with infant CFI ($p < 0.05$), specified for each age group. Values below age groups indicate the number of zOTUs. B) Distribution of phyla positively linked to infant CFI.

252 24 zOTUs were negatively linked with CFI, indicating that more mature child sleep regulation
253 decreased the abundance in these zOTUs (Figure 3A). No negative association between CFI and gut
254 microbiota abundance was observed at 3 months, while it increased to 6 zOTUs at 6 months and 18
255 zOTUs at 12 months. At 6 months, Actinobacteria was the most frequently associated phylum with
256 CFI, while at 12 months it was Firmicutes (Figure 3B).
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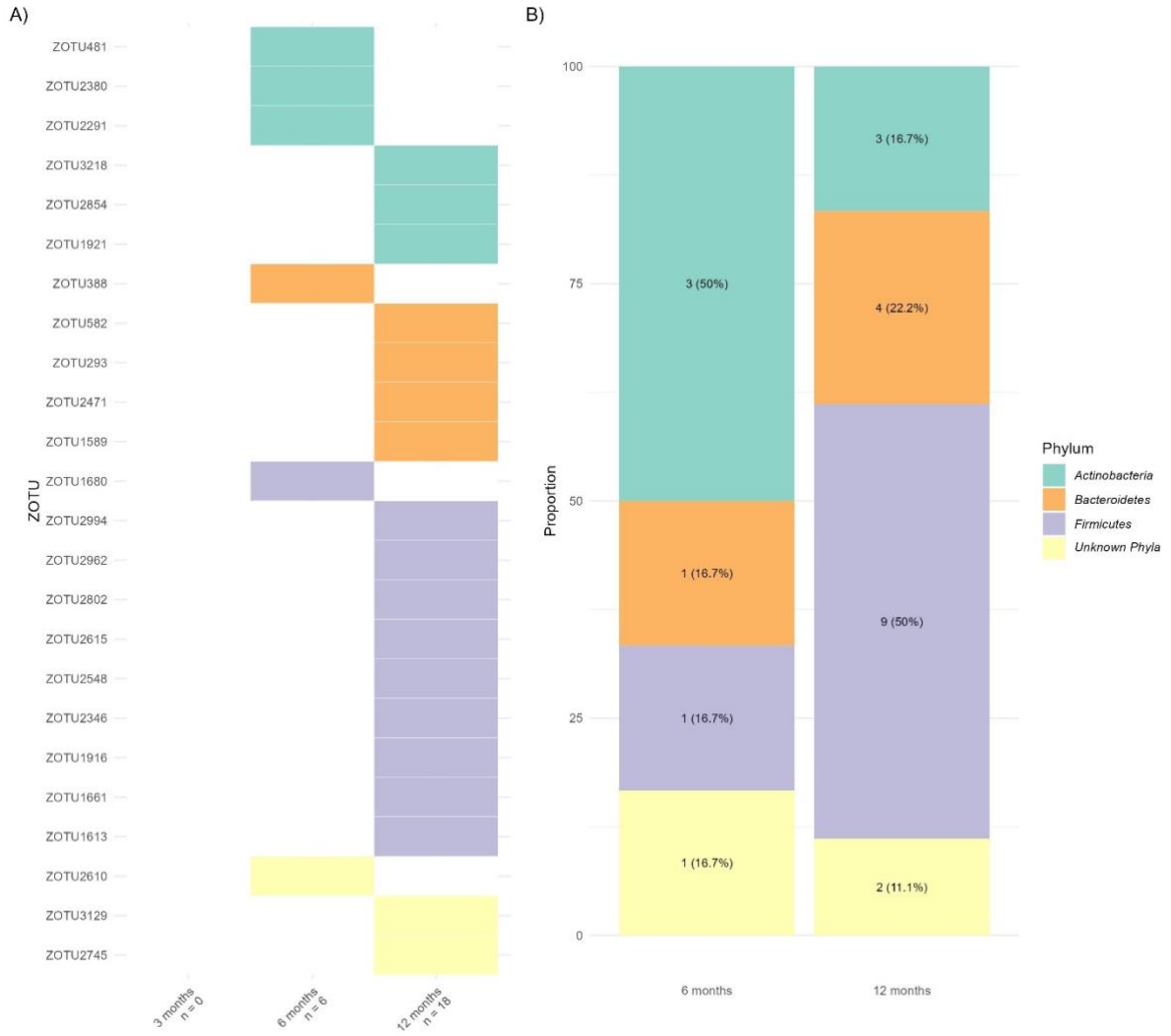
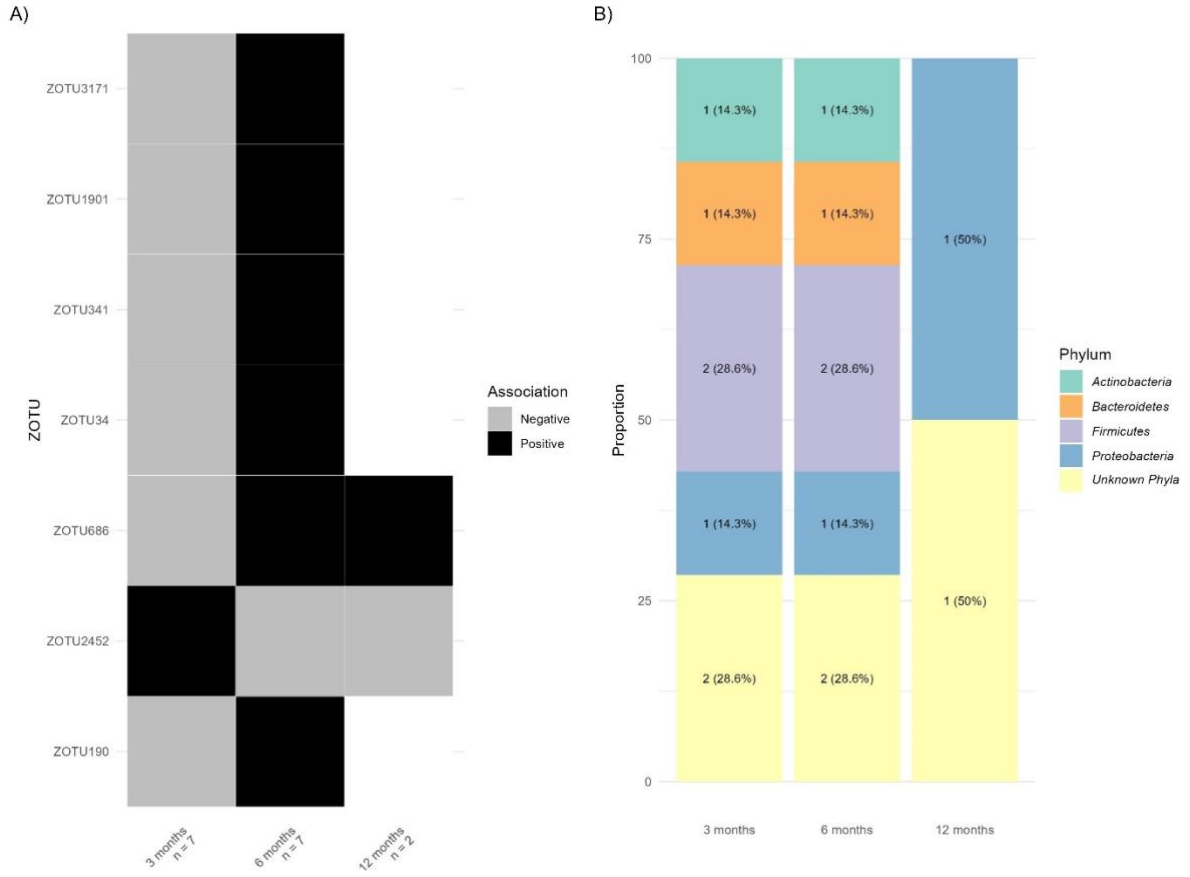


258
259 **Figure 3.** A) Heatmap of zOTUs (n = 24) with abundance negatively associated with infant CFI ($p <$
260 0.05), illustrated for each age group. Values below age groups represent the number of associated
261 zOTUs. B) Distribution of zOTUs phyla negatively linked to infant CFI.
262
263 Interestingly, for 7 zOTUs, the direction of the association between zOTUs abundance and CFI was age
264 dependent (Figure 4A). Specifically, at 3 months 6 zOTUs showed negative associations, which shifted
265 to positive associations at 6 and/or 12 months. Conversely, one zOTU exhibited the opposite: showing
266 positive correlation at 3 months shifting to negative at 6 and 12 months. Among these 7 zOTUs, at 3

267 and 6 months, there were 2 from the phylum *Firmicutes*, 1 from *Actinobacteria*, 1 from *Bacteroidetes*,
268 1 from *Proteobacteria* and 2 from unknown phyla. By 12 months, there was 1 zOTU from
269 *Proteobacteria* and 1 from an unknown phylum (Figure 4B).



270

271 **Figure 4.** A) Heatmap of zOTUs (n = 7) with their abundance positively (black) or negatively (grey)
272 associated with infant CFI ($p < 0.05$). Values below age groups represent the number of zOTUs
273 identified. B) Distribution of zOTU's bacterial phyla linked to infant CFI, showing changes dependent
274 on age.

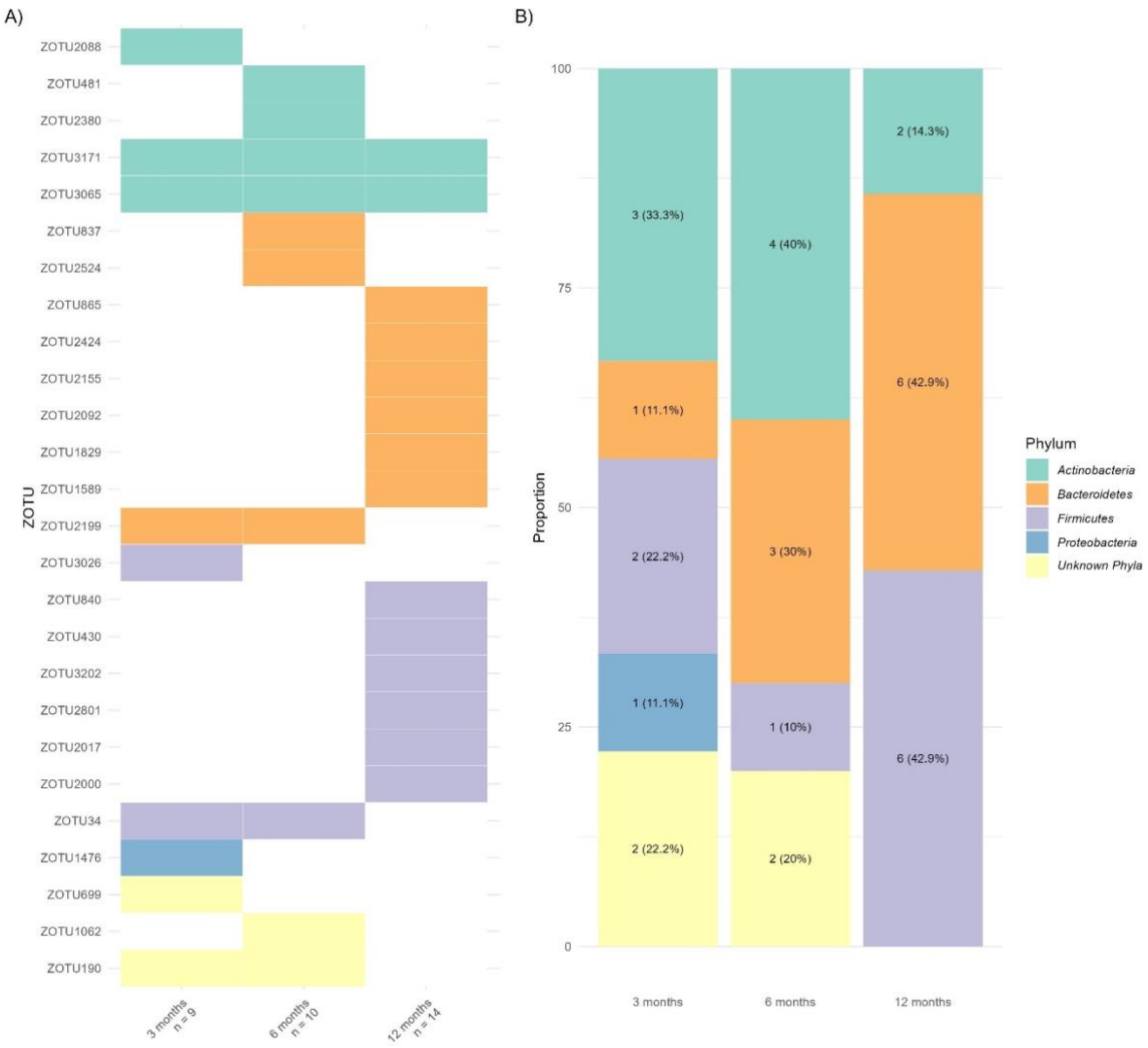
275

276 <u>Rhythmic patterns in gut microbiota abundance</u>

277 Subsequently, we investigated whether rhythmicity in gut microbial abundance was present during
278 infancy, by considering the influence of clock time of stool sampling. The clock time of stool sampling
279 was fitted to sine and cosine curves capturing potential diurnal effects.

280 Overall, the abundance of 26 zOTUs (i.e., 1.74% of total identified zOTUs) followed a sine pattern,
281 indicating rhythmic diurnal variation in their abundance. The number of rhythmic zOTUs remained
282 relatively constant across age (9 rhythmic zOTUs at 3 month, 10 rhythmic zOTUs at 6 months and 14
283 rhythmic zOTUs at 12 months; Figure 5A). *Actinobacteria* was the main rhythmic phyla undergoing a

284 sine pattern at 3 months (33.3%) and 6 months (40%; Figure 5B). *Bacteroidetes* and *Firmicutes*
285 represented rhythmic phyla at 12 months (each 42.9%).



286

287 **Figure 5.** A) Heatmap of zOTUs (n = 26) with rhythmic abundance following a sine pattern ($p < 0.05$).
288 Values below age groups represent the number of rhythmic zOTUs abundance. B) Distribution of
289 zOTUs phyla following a sine rhythm.

290

291 Next, we applied the cosine approach to zOTU abundance. Compared to sine fitting, cosine fitting
292 revealed that a greater number of zOTUs (100 in total, accounting to 6.69% of all zOTUs) exhibited
293 rhythmic dynamics (Figure 6A). More pronounced than sine, rhythmic cosine dynamics unraveled an
294 age-dependent increase, starting with 7 zOTUs at 3 months, increasing from 12 zOTUs at 6 months to
295 86 zOTUs at 12 months. The main cosine rhythmic zOTUs bacteria at 3 months were of unknown
296 phylum, while for 6 and 12 months the majority were assigned to *Firmicutes* (respectively 41.7% and
297 45.3%, Figure 6B).

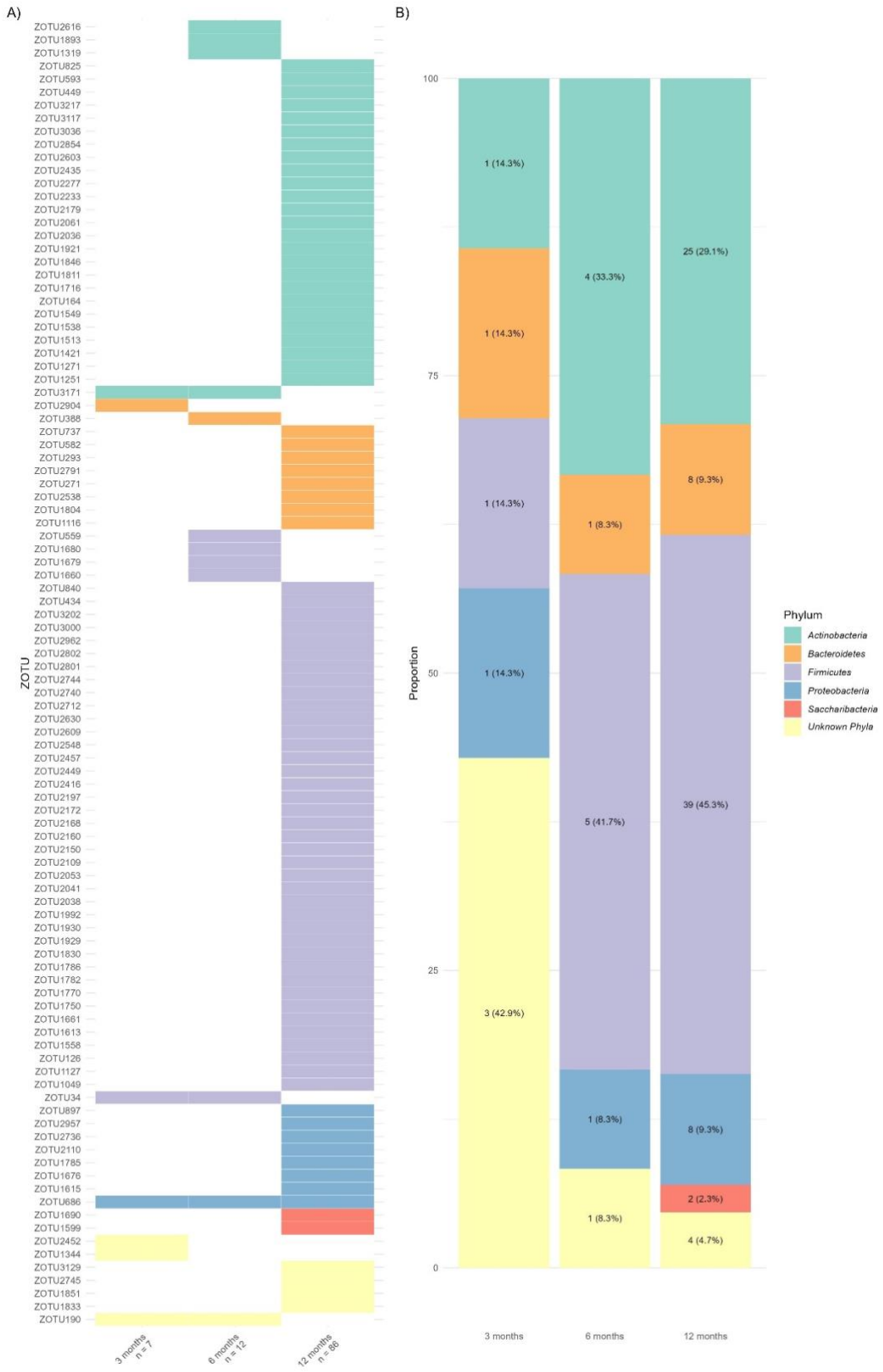
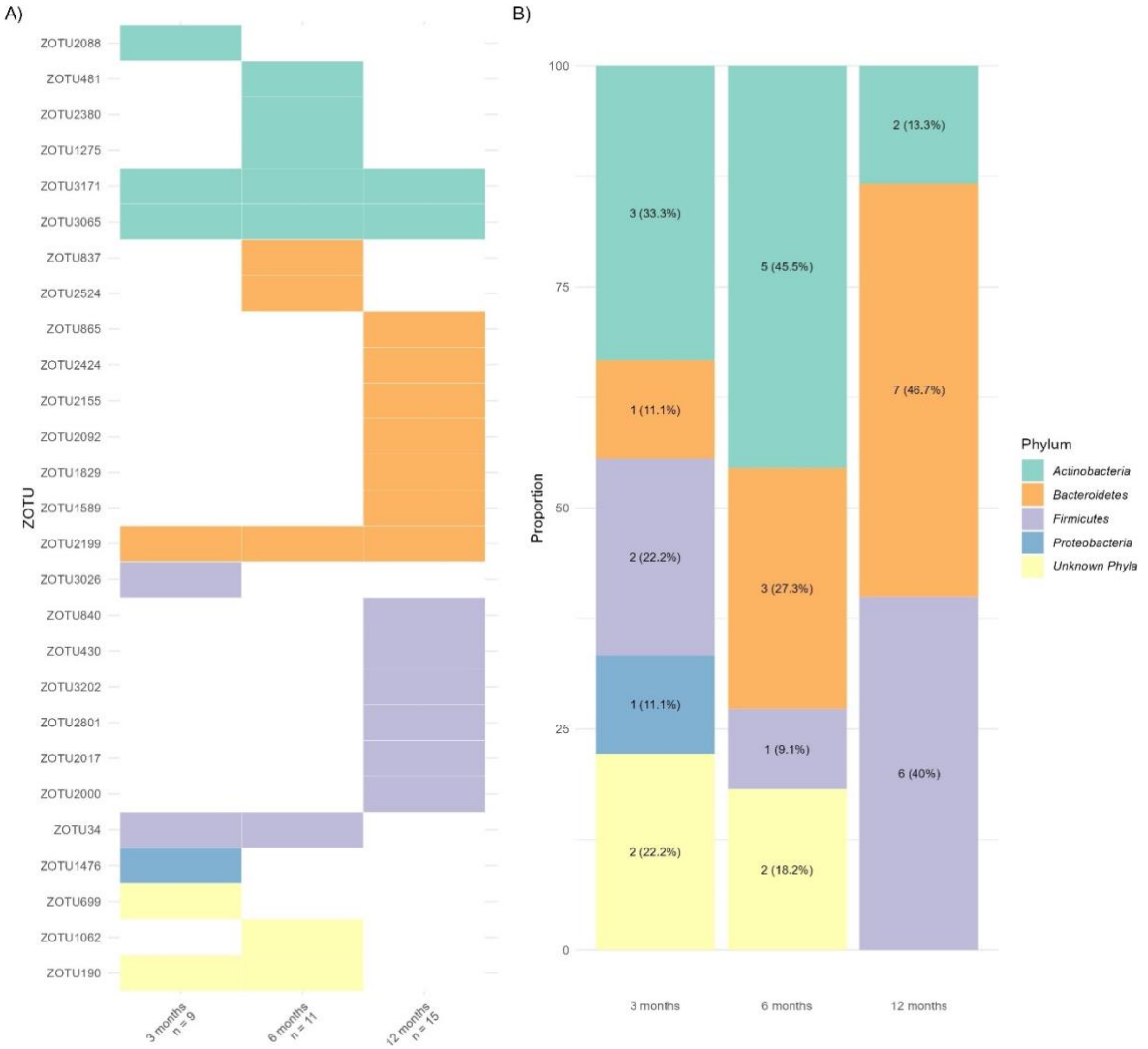


Figure 6. A) Heatmap of zOTUs (n = 100) whose abundance followed a cosine rhythm ($p < 0.05$). Values below age groups represent the number of zOTUs identified. B) Distribution of zOTU phyla expressed as a cosine pattern.

302

303 Gut microbiota rhythmicity and infant CFI

304 Finally, we addressed whether gut microbiota rhythmicity relates to infant circadian maturation by
305 testing interactions between zOTU sine and cosine fitting with CFI. Significant interactions would
306 suggest that CFI relates to the rhythmic expression of bacterial composition. Indeed, this interaction
307 was found in sine fitting for 27 zOTUs (1.81% of total zOTUs), with the number of rhythmic zOTUs
308 linked to CFI increasing slightly across 3, 6 and 12 months to 9, 11 and 15 zOTUs, respectively (Figure
309 7A). We observed that the interaction of sine rhythmicity with CFI was mainly represented within
310 *Actinobacteria* at 3 and 6 months (33.3% and 45.5% respectively, Figure 7B), and *Bacteroidetes* at 12
311 months (46.7%).



312

313 **Figure 7.** A) Heatmap depicting zOTUs (n = 27) with significantly associated sine rhythmicity and CFI
314 ($p < 0.05$). The numbers below age groups indicate the number of zOTUs associated. B) Distribution
315 of zOTUs phyla whose rhythmic sine patterns were linked with CFI.
316
317 Cosine microbial rhythms were associated with CFI in 96 zOTUs (corresponding to 6.43% of total
318 zOTUs). At 3 months cosine rhythmicity in 9 zOTUs was linked to CFI, increasing to 13 zOTUs at 6
319 months, and 81 zOTUs at 12 months (Figure 8A), indicating an increasing number of zOTUs exhibiting
320 cosine rhythmicity interacting with circadian rhythm maturation across the first year of life. At 3
321 months, most zOTUs linked to CFI were assigned to unknown phyla, while at 6 and 12 months mainly
322 Firmicutes was involved (38.5% and 43.2%, Figure 8B).

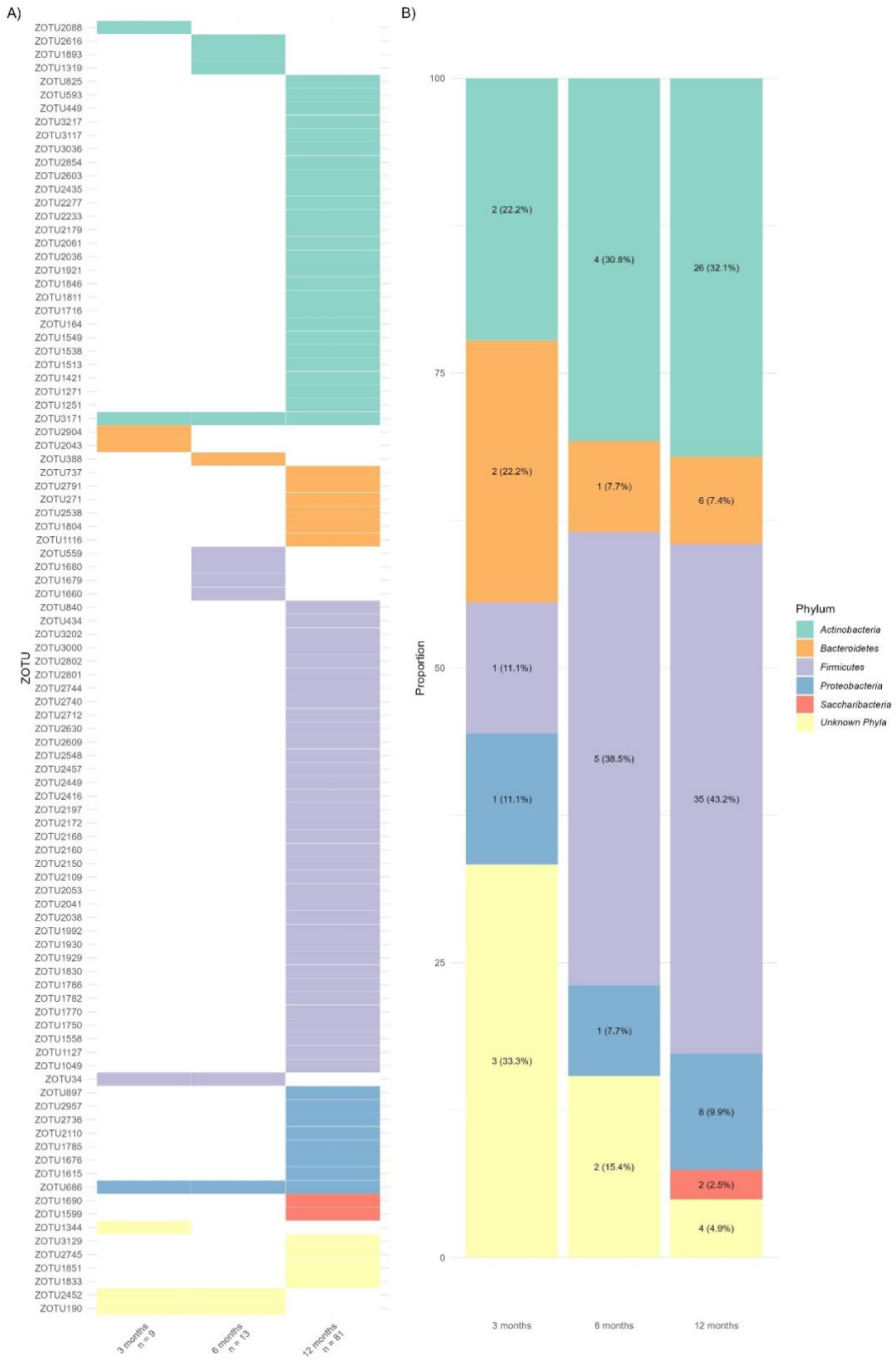


Figure 8. A) Heatmap indicating zOTUs (n = 96) with significantly associated cosine rhythm and CFI ($p < 0.05$). Values below age groups represent the number of corresponding zOTUs . B) Distribution of zOTUs phyla with cosine rhythmicity linked to CFI.

In summary, these results establish associations between circadian maturation in infants, quantified by the Circadian Function Index (CFI), and overall gut microbial composition, involving 7.02% of zOTUs. Crucially, microbial composition exhibits diurnal variation, with 1.74% of zOTUs following a sine rhythm, and 6.69% adhering to a cosine rhythm. The expression of both, sine and cosine zOTU rhythmicity, gradually increases across infancy. Finally, the interaction of sine and cosine-fitted microbial rhythm with CFI was found in 1.81% of zOTUs for sine rhythm, and 6.43% of zOTUs for cosine rhythm, the latter experiencing a gradual strengthening with age.

Discussion

In this longitudinal study across the first year of human life we examined the interaction between sleep rhythm maturation (CFI) and diurnal variation of gut microbiota. We demonstrated that CFI increases with age, confirming the measure as a reliable proxy for circadian maturation when using infant ankle actimetry. Interestingly, infant CFI was associated with overall gut microbiota composition, a link that strengthened with age. Specific Zero-radius Operational Taxonomic Units (zOTUs), primarily from the phyla *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, manifested rhythmic fluctuations across the day, following distinct sine or cosine patterns in their relative abundance. Age-related augmentation in rhythmicity was predominantly observed in cosine fitting, suggesting a gradual development of microbial diurnal rhythm with bacterial abundance peaking around midnight and reaching a nadir around midday. Remarkably, gut microbial rhythm was connected to CFI, underscoring a relationship between host circadian rhythm and gut microbial rhythmicity. Particularly cosine microbial rhythm maturation was accompanied with CFI. These findings demonstrate that sleep rhythm and gut rhythmicity are linked in infancy and highlight the potential that infant sleep may influence or be influenced by microbial dynamics, which may ultimately foster microbial colonization and developmental health.

This study employed CFI computed from ankle actimetry, which revealed a progressive increase in behavioral sleep rhythmicity. This finding aligns with developmental research indicating that sleep-wake patterns mature during the first year towards adult patterns (Zornoza-Moreno et al., 2011). The progressive increase in CFI from 3 to 12 months highlights a critical developmental window where sleep-wake patterns gradually evolve from fragmented to consolidated forms. This maturation is critical, as sleep quality, timing, and duration are linked to various aspects of developmental health, including cognitive functions like executive control, neural connectivity, and synaptic stability, all of which are crucial for neurodevelopment and cognitive maturation (Beaugrand et al., 2023; Kurth et al., 2012; LeBourgeois et al., 2019; Paavonen et al., 2010). Previous research on the

development of sleep-wake patterns used actigraphy to objectively assess periods of sleep, capturing various metrics such as total sleep time, sleep efficiency, and wake after sleep onset (Schoch, Huber, et al., 2020). In this study, we demonstrate that CFI, integrating continuous sleep and wake activity, provides a novel perspective on the maturation of infant sleep rhythms. As CFI has previously been used successfully to detect sleep and circadian disorders in adults (Rodriguez-Morilla et al., 2019), our findings deepen the understanding of sleep regulation development and underscore the CFI as a reliable measure for pediatric circadian rhythm research.

This study confirmed the hypothesis that the circadian maturation during the first year of life is associated with gut microbiota composition. Specifically, we found that the number of bacterial taxa associated with CFI increases across infancy, more specifically showing that increased CFI is linked with higher abundance of *Bacteroidetes* and *Proteobacteria* and decreased abundance of *Firmicutes*. These results add to the evidence that circadian rhythm is associated with microbial colonization and the reciprocal interaction between gut microbiota rhythms and the host's sleep pattern (Heppner et al., 2024; Thaiss et al., 2016). Our findings expand on this perspective by demonstrating that the relationship between gut microbiota and host rhythms encompasses not only sleep behavior but also the broader spectrum of activity-rest cycles extending beyond a single 24-hour cycle. This underscores an integral role of daily activity patterns in shaping gut microbiota composition, which likely impacts development. Conversely, bottom-up signals through gut microbiota may influence behavioral rhythmicity, which, in turn, likely affects long-term developmental outcomes (Carlson et al., 2018; Diaz Heijtz et al., 2011).

This study provides evidence for diurnal dynamics in the gut microbiota throughout infancy. Using sine and cosine fitting, we identified rhythmicity in specific bacterial taxa (zOTUs), with 26 showing rhythmicity in sine fitting and 100 in cosine fitting. Notably, the rhythmicity of gut bacteria becomes more pronounced with age, which aligns with recent research highlighting diurnal fluctuations in microbial and metabolic profiles in infants (Heppner et al., 2024). The increased rhythmicity of gut microbiota with age may be driven by several developmental changes. For instance, the transition from breastfeeding to solid foods significantly impacts gut microbiota composition, potentially increasing the abundance of rhythmic bacterial taxa (Bäckhed et al., 2015). Moreover, regular feeding schedules, which tend to become more consistent during the first year of life, are linked to greater microbial diversity, as shown in our previous research (Mühlematter et al., 2023). Feeding regularity may not only influence the composition of the gut microbiota but also support its rhythmicity, a relationship previously demonstrated in animal models (Thaiss et al., 2014). The effects of feeding rhythms on microbiota and host health may extend beyond infancy. In adults,

studies have shown that meal timing plays a crucial role in aligning internal circadian rhythms, with benefits for metabolic health. For example, restricting meals to daytime has been found to prevent circadian misalignment and reduce glucose intolerance, a finding relevant for conditions like night shift work (Chellappa et al., 2021). Disruption of microbial rhythmicity has also been linked to metabolic health issues in adults, such as type 2 diabetes, suggesting that interventions supporting microbial rhythms could benefit long-term metabolic health (Reitmeier et al., 2020). Recognizing the importance of diurnal rhythms in gut microbiota could lead to new clinical and preventive strategies across the lifespan, especially in metabolic and immune health. Targeting microbial rhythmicity through dietary modifications or probiotic interventions may hold promise for supporting health outcomes from infancy through adulthood.

Our findings show that certain bacterial taxa in the gut microbiota exhibit daily rhythms in their abundance, which align with indicators of circadian maturation in infants. This suggests that as infants' circadian rhythms develop, these microbial taxa adjust their daily patterns, potentially coordinating with the infant's sleep-wake and activity cycles. As infants' sleep becomes more structured over the first year, certain microbial taxa accompany this host behavior, notably within the Bacteroidetes and Firmicutes phyla, which show increasing proportions as CFI scores rise. Bacteroidetes and Firmicutes are known contributors to SCFA production, including butyrate, acetate, and propionate, each of which has distinct roles in metabolic and neural pathways. For instance, butyrate, a major SCFA produced by Firmicutes (Vital et al., 2014), has been shown to influence sleep architecture in animal models by promoting non-rapid eye movement (NREM) sleep and affecting the regulation of neural circuits involved in sleep (Szentirmai et al., 2019). Additionally, studies in older adults suggest that higher levels of butyrate and propionate are associated with improved sleep efficiency and longer sleep duration (Magzal et al., 2021). In the context of our findings, the increased abundance of SCFA-producing taxa such as Firmicutes in infants with higher CFI scores may indicate that the metabolic byproducts of these microbes support the maturation of sleep patterns through mechanisms involving SCFAs. Beyond SCFAs, the gut-brain axis may serve as a communication pathway through which gut microbiota influence sleep and circadian rhythms. The vagus nerve provides a direct link between the gut and brain, while intestinal epithelial cells interpret microbial signals, including those from SCFAs, to regulate host circadian rhythms (Cryan et al., 2019; Eshleman & Alenghat, 2021; Valles-Colomer et al., 2019). While the importance of microbial rhythmicity in these pathways warrants further exploration, the implications of these findings may open avenues for stabilizing gut microbial rhythms as a potential approach to managing sleep disturbances and promoting developmental health. Further research identifying the specific bacterial strains within the Firmicutes and Bacteroidetes phyla that contribute to SCFA production could pave the way for

probiotic or dietary interventions tailored to enhance circadian maturation and optimize sleep. Since our findings suggest that higher abundance of SCFA-producing taxa is associated with increased circadian function in infants, targeting these strains may offer a strategy for promoting sleep quality, physiological resilience, and mental health during critical periods of development. Such interventions could leverage SCFA pathways to support the natural maturation of circadian rhythms, potentially providing benefits beyond infancy and into later developmental stages (Aburto & Cryan, 2024).

To further elucidate human microbial rhythms and their health implications, future research must rigorously investigate the role of environmental contexts (Weng et al., 2024), particularly diet. Diet is a major driving factor of gut microbiota composition; for instance, high-fat diets are associated with shifts toward increased Firmicutes and decreased Bacteroidetes, highlighting the significant impact of dietary patterns on microbial communities (Murphy et al., 2015). Even though categories of feeding were accounted for, our analysis does not include precise information on diet or the transition to solid food. This study used comprehensive longitudinal data with cross-sectional data underlying bacterial rhythm computation, which should be further addressed with serial stool sampling within the same subjects, ideally also including nighttime. Without nighttime sampling, the identified sine and cosine patterns represent trends across the observed period; additional night samples would provide further support for these diurnal fits. Beyond the identified 24-hour period for microbial rhythms, cyclicity over shorter or longer periods may also exist. Interactions between diet, microbiota rhythmicity, and infant sleep patterns remain crucial in fully comprehending these complex systems during critical developmental windows and later stages. Future research will pinpoint bacterial strains and metabolic activity that promote host rhythm, offering strong potential for enhancing pediatric therapy.

This study reveals rhythmic dynamics in gut microbiota across infancy and demonstrates the association between infant circadian rhythm and gut rhythmicity, emphasizing the importance of microbial rhythms in pediatric, neuromaturational, and immunological development. Considering gut microbiota dynamics in early life could lead to targeted interventions for at-risk pediatric groups. For instance, dietary modifications aimed at promoting SCFA-producing bacteria or probiotic supplements containing specific bacterial strains associated with circadian support (e.g., certain Firmicutes or Bacteroidetes strains) could potentially enhance sleep regulation and overall developmental health. Tailoring early-life interventions in this way holds promise for managing health risks and supporting optimal growth trajectories through microbial health.

Declarations

Ethics approval and consent to participate

The cantonal ethics committee of Zurich, Switzerland, approved the project (BASEC 2016-00730), in line with the principles of the Helsinki Declaration. Before study enrollment, parents received detailed explanations about the study procedures before providing written consent.

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Availability of data

Data available on request due to privacy/ethical restrictions

Declaration of AI-assisted technologies

During the preparation of this work the authors used chatGPT and perplexity in order to improve language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM and SK conceptualized the study. CM, SS, and JCM curated the data. Formal analysis was conducted by CM, SS, JCM, and JCW. SK and SS obtained funding. CM conducted the investigation. Methodology was developed by CM, SK, JCW, and DN. SK managed the project and provided resources together with SS. SK supervised the study. Validation was performed by CM, JCM, JCW, and SK. CM created visualizations. CM and SK wrote the original draft. All authors reviewed and edited the manuscript and approved the final version.

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