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Gut Microbiota Composition and Sleep in Preschoolers;

ELFE Birth Cohort Study

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Term	Definition
ELFE	Étude longitudinale française depuis l'enfance
LCA	Latent Class Analysis
PAM	Partitioning Around Medoids
PERMANOVA	Permutational Multivariate Analysis of Variance
ANCOM-BC	Analysis of Compositions of Microbiomes with Bias Correction
ALDEx2	Analysis of Differential Abundance Taking Sample Variation into Account
DOHaD	the Developmental Origins of Health and Disease
OTU	Operational Taxonomic Units
PCoA	Principal Coordinate Analysis
FDR	False Discovery Rate
DMM	Dirichlet Multinomial Mixtures
BMI	Body Mass Index
PCA	Principal Component Analysis
NRMSE	Normalized Root Mean Squared Error
PFC	Proportion of Fractional Change
DAG	Directed Acyclic Graph

Abstract

Sleep plays a critical role in children's mental health development and physical well-being. Insufficient duration and inadequate quality of sleep are fairly common in children of preschool age (3-5 years) and are associated with an array of health complications in the short and long term. The preschool period is also key in the stabilization of gut microbiota characteristics. This cross-sectional study aimed to assess the gut microbiota composition and diversity in association with the duration and quality of sleep (frequency of sleep onset difficulties and night waking) in children of 3.5 years within the framework of the Étude longitudinale française depuis l'enfance (ELFE) birth cohort study. Gut microbiota profiling was assessed using a 16S rRNA gene sequencing-based method. Two sleep clusters were constructed by Latent Class Analysis (LCA) and represented groups of children with less and more optimal sleep. Two microbiota enterotypes were identified through the Partitioning Around Medoids (PAM) method; enterotype 1 was dominated by *Bacteroides* and *Faecalibacterium* and enterotype 2 was dominated by higher proportions of *Prevotella* and *Bacteroides*. Association between Chao1 and Shannon alpha diversity measures, microbiota enterotypes, and sleep clusters were assessed using binary logistic regression models adjusted for maternal and child health and demographic characteristics and household general status. A stratified analysis was conducted based on child sex. Permutational Multivariate Analysis of Variance (PERMANOVA) was done based on the Bray-Curtis and Weighted UniFrac distance matrices to assess the overall gut microbiota community composition differences. Also, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) and Analysis of Differential Abundance Taking Sample Variation into Account (ALDEx-2) methods were used assess the specific microbiota genus abundances across the sleep clusters. No significant associations were found between the gut microbiota diversity measures or enterotypes and sleep clusters in children, however, in the child sex-specific results, every standard deviation increase in the microbiota richness was associated with a higher probability of belonging to the "less optimal sleep cluster" in boys compared to girls. Neither the overall gut microbiota community composition nor any specific genera abundances were significantly different between the two sleep clusters. Further research is required to validate the results of this study.

Keywords: gut microbiota, diversity, composition, sleep duration, night waking, sleep onset difficulty, preschool.

Résumé

Le sommeil joue un rôle essentiel dans la santé physique et mentale des enfants. Une durée et une qualité de sommeil insuffisantes sont fréquentes chez les enfants d'âge préscolaire (3-5 ans) et sont associées à un moins bon développement mental et physique à court et à long terme. La période préscolaire est également déterminante pour la stabilisation de la composition du microbiote intestinal. Cette étude transversale avait pour objectif d'évaluer la composition et la diversité du microbiote intestinal en association avec la durée et la qualité du sommeil (fréquence des difficultés d'endormissement et des réveils nocturnes) chez des enfants de 3,5 ans de la cohorte de naissance ELFE (Étude longitudinale française depuis l'enfance). Le profil du microbiote intestinal a été caractérisé par un séquençage du gène codant l'ARNr 16S. Deux groupes de sommeil ont été identifiés par « Latent Class Analysis » (LCA) correspondant aux enfants ayant un sommeil respectivement moins et plus optimal. Deux entérotypes ont été identifiés par la méthode « Partitioning Around Mediods » (PAM) ; l'entérotype 1 était dominé par *Bacteroides* et *Faecalibacterium* et l'entérotype 2 était dominé par des abondances plus élevées de *Prevotella* et de *Bacteroides*. Les associations entre les indices de diversités alpha, Chao1 et Shannon, les entérotypes et les clusters de sommeil ont été testées à l'aide de modèles de régression logistique binaire ajustés sur un ensemble de facteur de confusion potentiels dont les caractéristiques sociodémographiques du ménage, les variables relatives à la santé de la mère et de l'enfant et d'autres variables liées au mode de vie de l'enfant. Une analyse stratifiée a été réalisée en fonction du sexe de l'enfant. Une analyse PERMANOVA (« Permutational Multivariate Analysis of Variance ») a été réalisée sur la base des matrices de distance de Bray-Curtis et UniFrac pondérée afin de tester les associations entre la composition globale du microbiote et les clusters de sommeil. Des tests d'abondance différentielle ont été effectués à l'aide des méthodes « Analysis of Compositions of Microbiomes with Bias Correction » (ANCOM-BC) et « Analysis of Differential Abundance Taking Sample Variation into Account » (ALDEx-2) afin de mettre en évidence de potentielle différences d'abondances de genres bactériens spécifiques associés aux clusters de sommeil. Aucune association n'a été mise en évidence entre les indicateurs de diversité du microbiote intestinal, les entérotypes et les clusters de sommeil chez les enfants, cependant, les résultats spécifiques au sexe de l'enfant ont montré que pour chaque écart-type supplémentaire de la richesse du microbiote, il y avait une plus grande probabilité d'appartenir au groupe de sommeil le moins optimal chez les garçons que chez les filles. De plus, la composition de la communauté du microbiote n'était pas différente entre les deux groupes de sommeil. Aucune différence dans l'abondance des genres bactériens n'a été mise en évidence au sein des cluster de sommeils. Des études supplémentaires sont nécessaires afin valider les résultats de cette étude.

Mots clés: microbiote, diversité, composition, durée du sommeil, réveil nocturne, difficultés d'endormissement, âge préscolaire

1. Introduction

1.1. Sleep characteristics in preschoolers

Sleep is a natural physiological condition of unconsciousness that is key to health maintenance and survival (1). Sleep is controlled by neurobiological functions and external factors, including individual lifestyle habits (2). The American Academy of Sleep Medicine (AASM) recommends that healthy newborns (0 to 3 months) need to spend approximately 80% of their day sleeping (14 to 17 hours over 24 hours) (3, 4). Based on the AASM recommendations, 12 to 16 hours of sleep including 2 to 3 naps per day in 3 to 11 months of age, 11 to 14 hours of sleep including 1 or 2 naps in 1 to 2 years, and 10 to 13 hours of sleep with or without naps in 3 to 5 years of age is essential (4, 5). The recommended sleep durations ought to optimize newborn and children's health; inadequate sleep duration and quality can be detrimental to children's mental health development, psychological performance, cognitive function, behavior and academic prosperity, and physical well-being in the short and long term (2, 6).

The sleep architecture evolves over the lifespan, and despite the inter- and intraindividual differences in sleep patterns, sleep duration tends to decrease with age among healthy individuals (2, 6). In children, the duration and quality of sleep undergo significant changes in the first five years of growth and development (6). Nevertheless, due to methodological challenges and ethical considerations, experimental evidence detailing the best and most appropriate sleep duration in children is lacking, and adequate sleep is defined as the required number of sleep hours for optimal functioning in children (2). Evidence from the literature suggests that sleep patterns in children may have geographical variations (7). In a large-scale study among 8,542 children aged 2 to 9 years from 8 European countries, nocturnal sleep duration ranged from 9.5 hours in Estonia to 11.2 hours in Belgium and varied statistically significantly between countries (7). In one study among 1,028 children aged 3 years in France, the average sleep duration was 12 hours and 35 ± 56 minutes a day, including naps in 90% of children (8). The average night sleep duration of French children, as established in the EDEN Mother-Child cohort (8), was between those reported in children from North and South Europe (7).

Despite the significance of sufficient and high-quality sleep in the early years of life, children of preschool age (3-5 years) are fairly susceptible to sleep disorders, including sleep onset difficulties and night waking, with an estimated prevalence of 20 to 30% in children aged 3 years and below (6, 9). Occasional night-waking (≤ 3 times a week) is a normal part of sleep development during the preschool period and higher frequencies are considered abnormal (10). According to the 2004 American National Sleep Foundation Poll among 10,085 children (including 387 preschoolers), 10.2% and 35.6% of children reported experiencing sleep onset difficulties and at least one night waking, respectively (11). In a birth cohort of 11,500 children

in England, night waking prevalence was estimated as 23% at 6 months, 50% at 18 months, and 49% at 3 years (12). Similarly, in the French Mother-Child EDEN birth cohort study of 1,346 children, the prevalence of frequent night waking (>2 nights per week) was 22% between 2 and 5 years and 26% at 3 years (13). In a birth to 6 years cohort study among 2,889 children in Italy, night waking was present in about 35% of toddlers aged 6 to 14 months and 22% of children aged 25 to 48 months (10). Also, in the same study, the prevalence of sleep onset difficulties was reported as 11% in toddlers aged 6 to 12 months, 7.5% in toddlers aged 13 to 24 months, and 4.7% in toddlers aged 25 to 48 months (14). Sleep onset difficulty and night waking are associated with decreased sleep duration if not adequately managed (13). Also, sleep challenges may persist into later childhood, adolescence, and adulthood if remain untreated (13, 15).

It is suggested that the household socioeconomic status, the general health and demographic characteristics of the mother (age at birth, pre-pregnancy Body Mass Index (BMI), history of depression and/or anxiety) and child (sex, breastfeeding duration, main type of care), are associated with the quality and duration of sleep in children (2, 6, 13, 16, 17). In particular, a strong association was reported between maternal depression and night waking frequency (18), and household income, child racial background, bedtime behavior, and sleep onset difficulties in children of preschool age in the literature (18, 19).

1.2. Gut microbiota characteristics in preschoolers

Research on the human gut microbiota had significant advances in recent years through longitudinal cohort studies and deep metagenomic analyses (20). Many studies are highlighting the key role of gut microbiota in human health and physiological traits, including but not limited to the regulation of gastrointestinal homeostasis, metabolic activities, immune system stimulations, and brain-gut axis communication (21). The initial infant–gut microbiota symbiosis in infants is established via vertical transfer from the mother at birth (20, 22). The majority of the gut microbiota composition and function development happens around the age of 3 years, though the age window is under debate upward (approximately 6 years) (23-25). From birth onwards, the gradual development, maturation, and establishment of the gut microbiota are regulated through a complex interplay between the host, perinatal and maternal conditions, and environmental factors (6, 26).

While all body sites are colonized with microorganisms to different extents, the human gut contains one of the highest abundances of the microbiota overall (27). In a multi-national study of the gut microbiota in 903 subjects from infancy up to 3 years of age, a constant and gradual change in the within-sample microbiota diversity and the *Bacteroidetes* and *Proteobacteria* phyla abundances were observed until 30 months of age and from month 31, the diversity and the abundance reached relative stability (23). In another study comparing the composition of

the gut microbiota between children aged 1 and 4 years and adults in the United States of America (USA), children aged 3-4 years still had a lower within-sample microbial diversity anda relatively higher abundance of *Bifidobacterium genus and Actinobacteria, Bacilli*, and *Bacteroidetes* phyla than adults (22)

More extensive and detailed data is available on the gut microbiota variation, composition, and characteristics in infants (<3 years) and adults in the literature, compared to children in the preschool age period (3-5 years) (20). Available studies in preschoolers showed associations between socioeconomic, demographic, and lifestyle factors and gut microbiota characteristics (6, 20, 25, 27, 28). In the first large-scale population-based study of the gut microbiota among 531 individuals, including infants, children (3–17 years), and adults from the Amazonas of Venezuela, rural Malawi, and the USA metropolitan areas, differences in the gut microbiota maturation and diversity were reported with regards to the dietary intake and westernization at the age of 3 years (29). Moreover, results of the Asian Microbiome Project among 303 school-aged children living in the urban and rural regions of five East Asian countries revealed higher microbiota richness among children living in rural than urban sites (30, 31).

The overall gut microbiota composition in children is relatively less stable and resilient in the presence of extreme external stressors compared to adults (20). Therefore, it is important to fill the knowledge gap during this critical phase and intervene and manage health pathologies within this window of opportunity.

1.3. Sleep and gut microbiota in preschoolers

With the development of sequencing and multi-omics technology, there had been significant evolvements in the science of microbiota biology, revealing an extensive bi-directional communication between the gut and brain and a role for the gut microbiota in sleep physiology (32). Also, aligned with the Developmental Origins of Health and Disease (DOHaD) theory, the early three years of life sets the stage for the development of healthy gut microbiota and sleep patterns (33, 34). Despite so, a small handful of studies have focused on this area among human adults and infants, and the data are scarce and inconsistent in the child population. Also, most studies are focused on single measures of sleep quality or duration, whereas both aspects must be considered in the assessment of sleep pathophysiology. In terms of microbiota diversity, one study in adults suggested a positive association between the within-sample gut microbiota diversity and total sleep duration, sleep efficiency, and global Pittsburgh Sleep Quality Index -measured sleep quality (32, 35, 36). In infants, a negative association between day sleep and within-sample gut microbiota diversity was found at 12 months old (37). In another study of 3 months old infants, the within-sample microbiota diversity metrics were not significantly different according to the total sleep duration (38). When considering microbiota composition, the between-sample microbiota diversity was associated with acute

(48 hours) partial sleep deprivation in adults (39). In the only available study among preschoolers in Canada, the between-sample microbiota diversity was linked to the sleep duration at night (40). The possible relationship between gut microbiota activity and sleep is most likely explained by the gut-brain axis activity (39), however, exact mechanisms are yet to be fully understood.

1.4. Research aim and objectives

Emerging evidence from human and animal models supports the bidirectional link between gut microbiota diversity and composition, and sleep (20, 32). Both the gut microbiota and sleep undergo significant changes during the preschool period (20). Despite the significance of this age window in long-term health and the importance of filling the knowledge gap for early interventions, only one study in Canada has focused on the hypothesized microbiota-sleep association in the preschool years so far (40). However, in this study, the potential role of confounders was not taken into account in the statistical analyses. Given the scarcity of data in this research field and the novelty of this research area during the preschool age, here we aim to study the association between the gut microbiota (diversity and composition) and sleep (night and day sleep duration, sleep onset difficulty, and night waking) in preschool-aged children aged 3.5 years within the framework of the Étude longitudinale française depuis l'enfance (the French Longitudinal Study of Children; ELFE) birth cohort study (41), while taking into account the potential known confounders, including the maternal and child health, perinatal conditions, and environmental and lifestyle factors.

2. Materials and Methods

2.1. ELFE birth cohort study

The ELFE study is a prospective nationally-representative birth cohort that was launched in 2011 to characterize the environmental factors and the socioeconomic determinants of child health and development from birth to adulthood (42, 43).

In total, 18,329 newborns born in metropolitan France were recruited from a random sample of 320 maternity hospitals over 5 days, spread over four seasonal waves (15). Infants were eligible to participate if they were born after 33 weeks of gestational age, whose mothers aged 18 years and over, were not planning to move outside of France in the following 3 years, and were able to read French, Arabic, Turkish, or English (15, 42). Participating mothers signed consent for themselves and their infants (15). Fathers signed consent for the infant's participation when present on inclusion days or were informed about their rights to oppose (15). The study received approvals from the Consultative Committee for the Treatment of Information for Health Research (Comité Consultatif sur le Traitement des Informations pour

la Recherche en Santé), the national data protection authority (Commission Nationale Informatique et Libertés), and the National Statistics Council.

During hospitalization at the maternity unit and after child delivery, each mother was interviewed face-to-face by trained interviewers, and information on the mother's health and medical status during pregnancy and the general characteristics of the newborn were collected via standardized questionnaires. Additional information was extracted from the obstetric and pediatric medical files and records.

Telephone interviews with mothers and/or fathers were conducted at the 2, 12, 24, and 42 months postpartum follow-up and collected more details on the overall socio-demographic and health characteristics of mothers, households, and the included children (41). At 42 months, a home visit was also scheduled, during which stool samples were collected for volunteering participants. Figure 1 illustrates the ELFE birth cohort study data collection from the child's birth until 12 years follow-up.

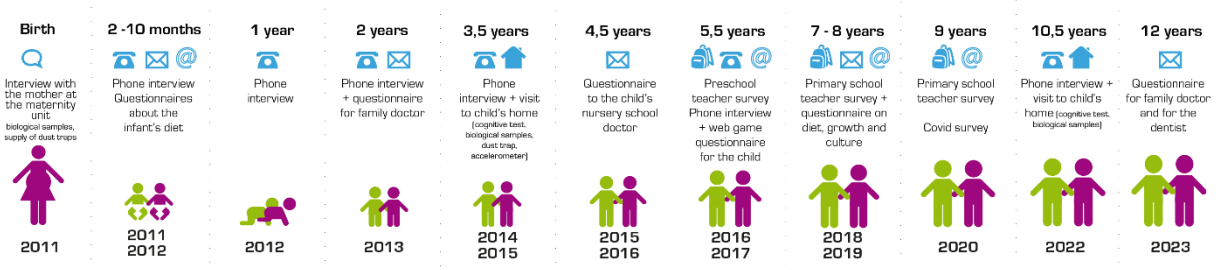

Figure 1; The ELFE birth cohort study data collection and follow-up (n = 18,329)
Adopted from: www.elfe-france.fr, access date: May 2023

2.2. Variables

2.2.1. Outcome variable construction

The main outcome of this study was the sleep characteristics of children aged 3.5 years. Bedtime and wake-up times as well as diurnal napping habits and duration on weekdays and weekends were used to calculate the mean day and night sleep durations over the week (41). Information on sleep onset difficulties and night waking were recoded as yes/no variables (sleep onset difficulty; yes = almost always and often, no = sometimes and never/ night waking; yes = ≥ 3 to 6 nights per week, no = <3 nights per week). Considering that both the duration and the quality of sleep must be taken into account simultaneously to consider sleep in its globality, we used the unsupervised Latent Class Analysis (LCA) method to identify clusters of children that shared similar sleep patterns in terms of night and day sleep duration, sleep onset difficulty, and night waking. LCA allows the detection of latent (or unobserved) heterogeneity in samples (15) and assumes that membership in unobserved classes can cause or explain patterns of scores across survey questions (44). According to the LCA algorithm, sleep clusters were selected based on the minimum goodness of fit measurements (supplementary table 1).

Two sleep clusters were identified described in Table 1. Approximately 25% of the children were classified in the “less optimal sleep cluster” which was featured with shorter night sleep duration compared with children in the “more optimal sleep” cluster (79.2% with 10h30 hours *vs*. 41.4% with 10h54 hours, p<0.001). Also, the sleep quality of children in the “less optimal sleep” was poorer, with a higher frequency of night waking experiences (58.8% *vs.* 0.0%) and sleep onset difficulties (65.3% *vs*. 10.7%) compared to children in the “More optimal sleep” cluster.

Table 1; Distribution of the sleep variables across the clusters of sleep

	Less optimal sleep (n=150)	**More optimal sleep (n=447)**	**Total (N=597)**	**P-value***
	n (%)	**n (%)**	**n (%)**	
Day sleep duration+				0.92
Shorter sleep	79 (53.7)	235 (53.2)	314 (53.3)	
Longer sleep	68 (46.3)	207 (46.8)	275 (46.7)	
Night sleep duration+				<0.001
Shorter sleep	118 (79.2)	185 (41.4)	303 (50.8)	
Longer sleep	31 (20.8)	262 (58.6)	293 (49.2)	
Sleep onset difficulty				<0.001
Sometimes, never	52 (34.7)	399 (89.3)	451 (75.5)	
Almost always, often	98 (65.3)	48 (10.7)	146 (24.5)	
Night waking (per week)				<0.001
<3 nights	62 (41.3)	447 (100)	509 (85.3)	
≥ 3 to 6 nights	88 (58.7)	0 (0)	88 (14.7)	

+Categories based on the median sleep duration; SD = Standard Deviation; Categorical variables expressed as frequency and proportion (n (%));
* Chi-squared or exact Fisher test when the expected frequencies are <5 in some cells

2.2.2. Exposure variables construction

The main exposure of this study was the gut microbiota characteristics in children aged 3.5 years. Stool samples were collected from 630 children according to the operating procedure of the International Human Microbiome Standards (IHMS) (41). Sample collection was done using an Enterom kit, placed in a stabilizing solution, and sent to study biobanks across mainland France (Dijon, Bois-Guillaume, and Annemasse). Within three days, samples were homogenized, aliquoted, and stored at -80°C until analysis. The 16S rRNA sequencing of the gut microbiota was performed, and data became available, providing information on Operational Taxonomic Units (OTUs) and the taxonomy of the gut microbiota. OTUs classify the gut microbiota RNA sequences into clusters based on the similarity of the sequence of the 16S rRNA marker gene (45).

From an experimental perspective, the observed and actual biological abundance and distribution of the microbiota species in each sample might not be essentially identical (41). In other words, the sampling depth across microbiota samples can be different since the 16S rRNA gene sequencing allows for uneven sequencing depth (41). This problem is exacerbated by the fact that the full range of species is rarely saturated, and more bacterial species are

observed with more sequencing depth (41). To adjust for the effect of uneven sampling depth and enable accurate comparisons and statistical analyses, each sample was normalized through rarefaction to the minimum sampling depth of the dataset (46). The rarefied data was used for all downstream analyses except for the differential abundance testing. Rarefaction involves the random discard of sequences in each sample until the number of sequences in each sample equals the number of sequences in the sample with the minimum number of sequences (46, 47). Figure 2 represents the rarefaction plot associated with the rarefaction of samples on the minimum sampling depth, which equaled 10,637 sequences.

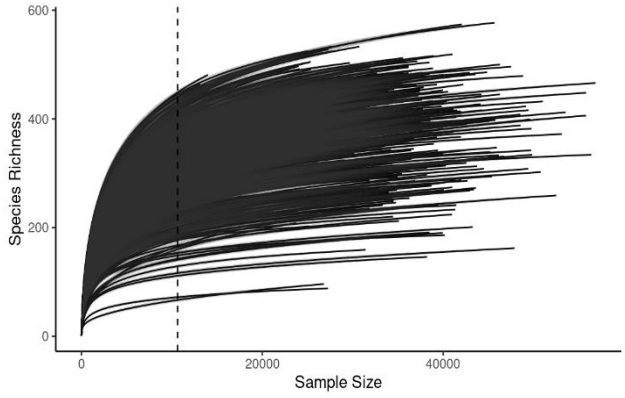

Figure 2 ; Microbiota rarefaction plot (minimum sampling depth = 10,637)

The gut microbiota data were used at the genus taxonomic ranking level to avoid uncertainties of the species level due the resolution of the 16S rRNA gene sequencing approach. Figure 3 illustrates the schematic hierarchy of the microbiota classification.

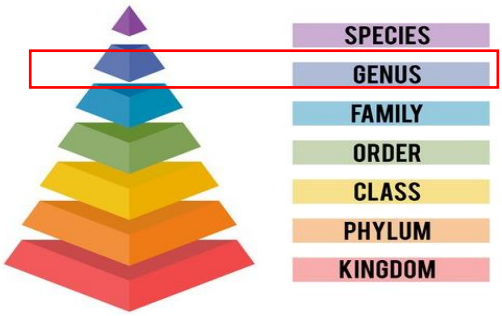

Figure 3; Hierarchy of microbiota classification
Adopted from: www. edu.tbioinfo.com, Access date: May 2023

Within-sample microbiota diversity was estimated by the alpha diversity measures that provide a unique value for each individual (48). These measures reflect the abundance (richness), distribution (evenness), or both in a single sample (49). The richness of the microbiota

community refers to the number of species, and evenness indicates how the species' relative abundance is distributed (49). In the majority of microbiota research, a combination of indices is used to cover the discrepancies (48, 49). In this study, Chao1 and Shannon's measures were used, the most frequently reported in the literature. The Chao1 index considers the abundance of observed and rare species and provides an estimation of richness based on the singleton and doubleton taxa in a sample (50). The Shannon index is based on the abundance of different microbiota taxa and how evenly they are distributed across the sample (48, 49).

An additional diversity metric known as beta diversity was also used in this study. Unlike the alpha diversity measures that are specific to each sample, beta diversity provides a value for each pair of samples and compares them based on distance measurements and dissimilarity matrices (51). Beta diversity distance matrices consider the presence/ absence, abundance, and/or the phylogenic characteristics of the microbiota taxa (49, 51). In this research, Bray-Curtis and Weighted UniFrac distances were used to reflect the different aspects of community heterogeneity. Bray-Curtis distance is based on the taxonomic abundance of species to reflect the community composition, and the Weighted UniFrac considers the presence/ absence, abundance, and phylogenic relationship of species (49). The inter-individual variability of the microbiota was visualized based on the dissimilarity matrices through Principal Coordinate Analysis (PCoA).

Subsequently, the gut microbiota profiles were clustered into enterotypes based on the Partitioning Around Medoids (PAM) method (52). An enterotype refers to the distinct and relatively stable composition of microbial communities, which is characterized by the predominance of certain bacterial taxa (52). Enterotypes were constructed based on the Jensen-Shannon divergence matrix. The optimal number of clusters was selected in accordance with the Calinski-Harabasz index (supplementary figure 1) (53) and validated against the Silhouette width index (supplementary figure 2) and prediction strength (52). In addition to PAM, the Dirichlet Multinomial Mixtures (DMM) clustering method was also used to identify the microbiota enterotypes (supplementary figures 3 and 4), however, the enterotypes found through the PAM method had better interpretability and consistency with the previous studies in the literature and were used here.

We identified two microbiota enterotypes explaining nearly 46% of the variability of the microbiota data. Figure 4 presents the distribution of the microbiota samples according to the enterotypes, showing a clear spatial distinction between the two enterotypes. Enterotype one, in red, included 488 (81.7%) children, and enterotype 2, in blue, included 109 (18.2%) children.

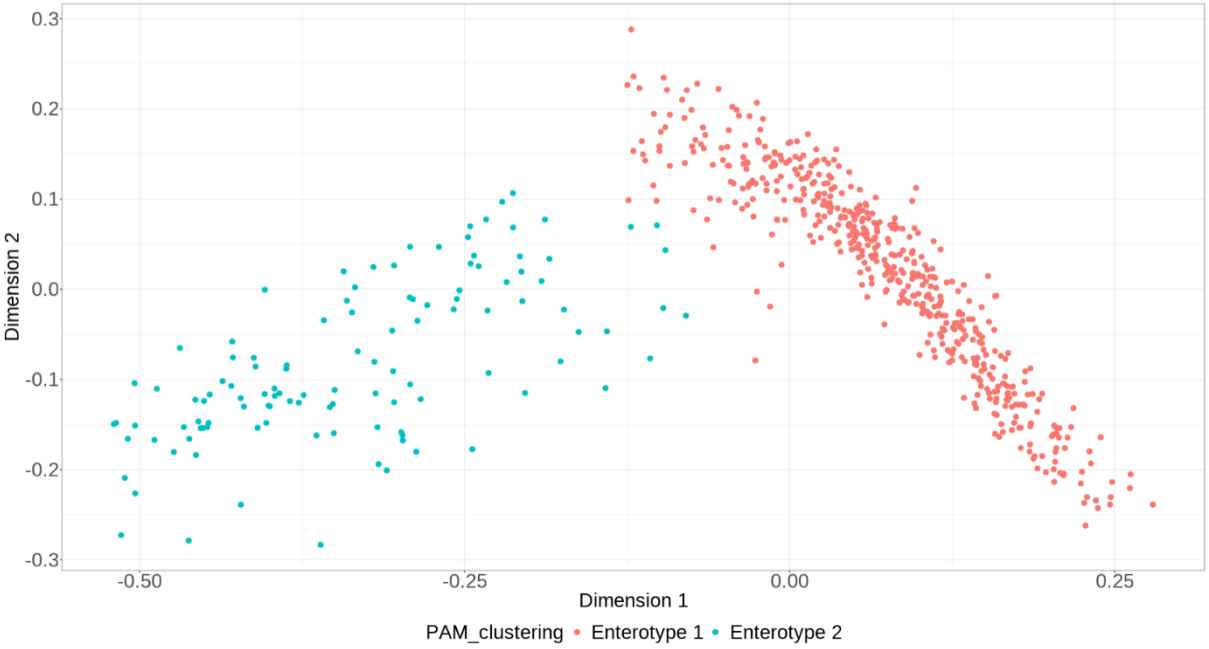

Figure 4; Distribution of the microbiota genera across the two enterotypes

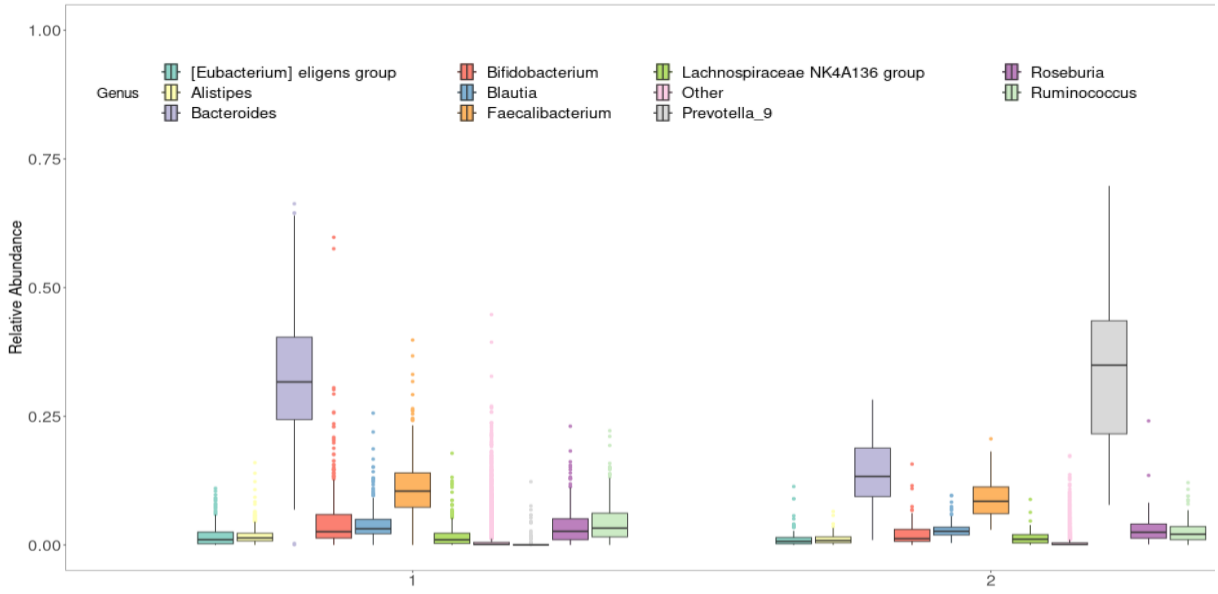

Figure 5; Distribution of the ten most abundant microbiota genera within the enterotypes

The distribution of the ten most abundant microbiota genera within the two enterotypes are presented in figure 5. Enterotype 1 was dominated by the genera *Bacteroides* and *Faecalibacterium*, whereas enterotype 2 was dominated by the genera *Prevotella* and *Bacteroides*.

2.2.3. Covariates

The maternal and household socio-demographic data were collected through face-to-face interviews at the maternity ward or by telephone at 2 months follow-up (41). Information on children's health and general characteristics were retrieved from the 2 months follow-up data due to higher precision and completeness.

The covariables of interest were as follows: maternal birthplace (born in France, Yes/ No), maternal exposure to psychotropic medications during pregnancy (Yes/No, constructed based on responses to the intake of anti-depressant and/or anti-anxiety medication during pregnancy), mother's pre-pregnancy BMI (Kg/m^2) calculated based on the pre-pregnancy weight and height, gestational age (weeks), child delivery mode (recoded to 1=Vaginal (spontaneous vaginal, forceps, spatulas), and 2= vacuum/Caesarean), mother's age at birth (years), child sex (Boy/Girl), sibling (Yes/No), maternal education at 2 months (recoded as 1= <secondary education, 2 = ≤ Baccalaureat +2 , and 3 = > Baccalaureat +2), household income at 2 months according to the age and the number of each co-habitant (consumption unit (CU)) in tertile (<1,500.00 €/month/ CU, 1,500.00 – 1,944.44 €/month/CU, >1,944.44 €/month/CU), pet ownership at 2 months (Yes/ No, constructed based on responses of both parents), breastfeeding duration (in months), main mode of childcare at 2 years (recoded to 1 = Family (responder, responder's partner, grandparents, or paid home help), 2 = Child sitter, 3 = Collective care (crèche or nursery school)), exact child's age at stool collection (months), child tobacco exposure from pregnancy until 3 years (Yes/ No) constructed based on the information collected at the maternity ward and 2 months and 1-3.5 years follow-up, child antibiotics intake between 2 and 3 years (recoded as 0 = Never, 1 = Once, 2 = More than once), and residential setting at 3 years (recoded as 1 = Rural, 2 = Suburban (2,000 to 199,999 inhabitants), 3 = Urban (200,000 to 1,999,999 inhabitants and residents of Greater Paris)). The models were also adjusted for the child's BMI Z-score measurement and diet at 2 years. The BMI Z-score data at 3.5 years contained a high amount of missingness and could have biased the overall statistical analyses, however, it was shown to be significantly correlated with the BMI Z-score measurements at 2 years when assessed by a Pearson Correlation test ($R^2 = 0.73$, $P < 0.01$; supplementary figure 5). Also, the distribution of sociodemographic variables across the BMI measurements at 2 and 3.5 years followed similar patterns (supplementary table 2). Children's diet at 2 years was assessed through a Principal Component Analysis (PCA) of quantitative dietary data included in the 2 years questionnaire (41). PCA is an unsupervised dimensionality-reduction algorithm that summarizes the information content of large datasets to increase interpretability (54). Dietary items used in the PCA and results of the PCA on dietary data are reported in supplementary table 3 and supplementary figure 6. Two dietary patterns were found based on the food loadings; a healthy dietary pattern (positive loading of pasta, fresh fruits, and cooked vegetables and negative loading of fries and sugary drinks) and an unhealthy

dietary pattern (positive loading of meat and ham, fries, quiche, fruit juice, cheese, bread, pastry, and sweets).

The missing data for covariates represented 2.4% (patterns in supplementary figure 7) and were considered missing at random. Imputation of the missing data was performed using the MissForest package in RStudio (55). MissForest is a machine learning-based approach commonly used in mixed-type data (55). MissForest uses a random forest imputation algorithm that imputes missing data by predicting the missing values based on values of other variables in the dataset. It initially imputes all missing data using the mean/mode (depending on the data type) and subsequently fits a random forest on the observed part of each variable with missing values to predict the missing part. The loop of training and predicting repeats in an iterative process until a stopping criterion is met.

In this study, 100 random trees (the default number from the missForest package) were implemented. The missForest imputation accuracy and performance were assessed through the Normalized Root Mean Squared Error (NRMSE) and Proportion of Fractional Change (PFC) indicators. NRMSE measures the average difference between the imputed and actual values while considering the scale of the variables. A lower NRMSE indicates better imputation accuracy. Also, PFC expresses the relative change between the imputed and the actual values and ranges from 0 (perfect imputation) to 1 (poor accuracy). Here, the average difference between the imputed and actual values relative to the range of actual values was 0.076 for the NRMSE, and the PFC equaled 0.196.

2.3. Statistical Analysis

2.3.1. Univariate models

The household sociodemographic and maternal and child health characteristics were presented using count and percentages for discrete variables and means and standard deviation for continuous variables. The normality of the continuous data distribution was assessed through Q-Q Plots. Included and excluded populations were compared using the Independent samples t-test for normally-distributed continuous variables, Wilcoxon Signed Rank test for non-normally distributed data, and Chi-square or Fisher's test for categorical variables.

2.3.2. Microbiota differential abundance testing

To identify the significant differences in relative abundance of specific microbiota genera between the sleep clusters, two differential abundance testing analyses were carried out; Analysis of Differential Abundance Taking Sample Variation into Account (ALDEx2) (56) and Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC) (57). The results of the two analyses are assumed to help with discrepancies (58, 59). ALDEx2 takes into account the compositional nature of microbiota data, where the sum of relative abundances

for all taxa in a sample adds up to 1 (56). This approach uses Bayesian methods to estimate technical variation in the data and identifies differential abundance between groups while accounting for the sparsity and heteroscedasticity in microbiota data (57). ANCOM-BC is a more recently-developed method that also includes a bias correction step to address issues arising from sample size differences (57). The results of both tests are corrected for the False Discovery Rate (FDR) (56, 57). Adjustment for covariates were also performed for these methods (57). Since both methods have specific normalizing functions implemented (57), analyses were conducted on non-rarefied data. ANCOM-BC and ALDEx2 results were interpreted based on the Benjamini–Hochberg-corrected P-values.

2.3.3. Bivariate and Multivariate models

Binary logistic regression models were performed to study the potential association between the gut microbiota alpha diversity, enterotypes, and sleep clusters among children aged 3.5 years. The models were adjusted for potential confounders (maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, sibling, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding duration, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, exact child's age at stool collection, child tobacco exposure from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, and residential setting at 3 years), identified based on the literature and a Directed Acyclic Graph (DAG; dagitty.net software; supplementary figure 8). Additional stratification analysis was performed to assess the potential modifying effect of child sex.

The overall microbiota community composition differences between the sleep clusters were studied through the Permutational Analysis of Variance (PERMANOVA) (60) with 999 permutations, using the beta diversity distance matrices. PERMANOVA is a distance-based method and was used to compare the mean rank of beta diversity measures between the sleep clusters, assuming that a) the cluster variances were homogenous and b) the centroid and dispersion of beta diversity distances in all clusters were equivalent (60). PEMANOVA P-values were corrected for the FDR by the Benjamini-Hochberg Procedure (61). Models were adjusted for the same confounders as the multivariate regression models.

All statistical analyses were performed in RStudio (using R version 4.2.1), with the latest version of the following packages for data management and statistical analyses: *stats, tidyr, eeptools, dplyr, kableExtra, knitr, magrittr, factoextra, tidyverse, skimr, tibble, DataExplorer, epiDisplay, compareGroups, table1, summarytools, reshape2, corrplot, ggpubr, ggfortify, ggplot2, gtools, gtsummary, plotly, RColorBrewer, RColorBrewer, car, naniar, pacman, lattice, permute, phyloseq* (62)*, vegan* (63)*, microbiome* (64)*, fpc (65), DirichletMultinomia (66)l,*

poLCA (67), ALDEx2 (56), ANCOMBC (57), and *missForest (68)*. All packages were downloaded and loaded from The Comprehensive R Archive Network.

Sensitivity analysis was performed through bivariate and multivariate analyses of the complete-case data to assess the impact of missing data imputation (supplementary tables 4 and 5).

3. Results

3.1. Selection of the study population

Out of the 18,329 newborns included in the ELFE cohort study, 12,235 subjects participated in the 3.5-year follow-up. During this follow-up, stool samples were collected from 630 children. Following the exclusion of 33 children that lacked sleep-related data, 597 children aged 3.5 years old with complete data on the exposure and outcome were included for the analysis (Figure 6).

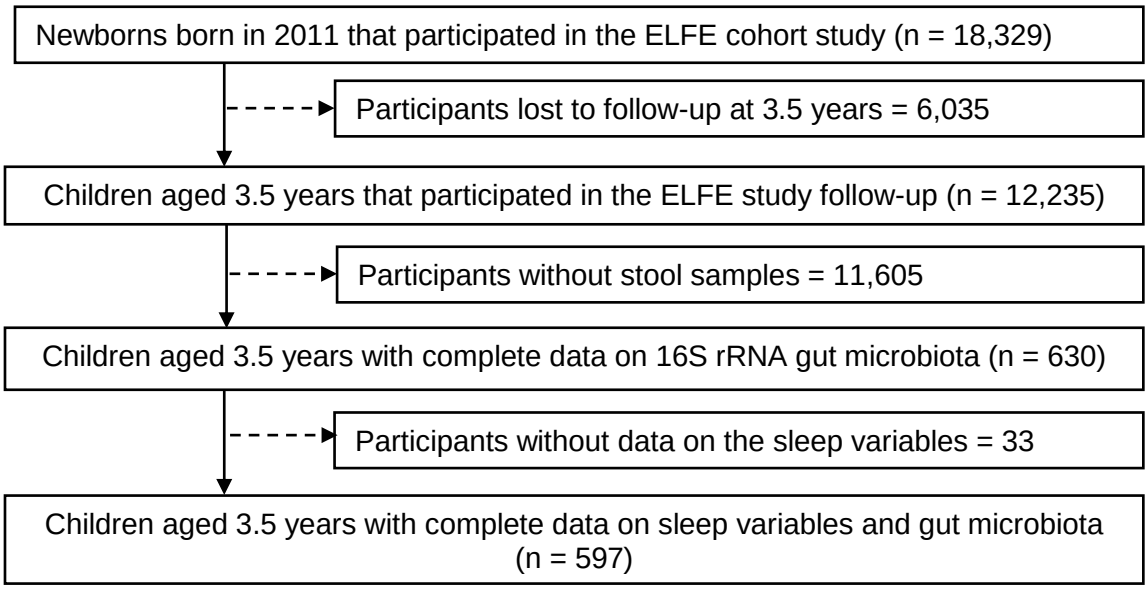

Figure 6; Flowchart of the study sample inclusion

3.2. Description of the study population

Table 2 represents the overall descriptive characteristics of the study population compared to the ELFE population that were not included in the study.

Table 2; Characteristics of the included and excluded population

	Included Population (n = 597)	**Excluded Population (n = 17703)**	**p-value***
	n (%)	**n (%)**	
Maternal characteristics			
Birthplace; France	556 (93.1)	15205 (86.5)	<0.001
Missing	0	119 (0.67)	
Education at 2 months			<0.001
< Secondary level	139 (23.5)	6658 (42.1)	
≤ Baccalaureat +2	155 (26.2)	3428 (21.7)	
> Baccalaureat +2	298 (50.3)	5712 (36.2)	
Missing	5 (2.68)	1905 (10.8)	
Exposure to psychotropic medications during pregnancy	16 (2.68)	304 (2.72)	1.00
Missing	0	6543 (36.9)	
Vaginal delivery	470 (81.3)	13911 (81.2)	1.00
Missing	8 (1.34)	575 (3.25)	
	Mean (SD)	**Mean (SD)**	
Age at birth (years)	31.76 (4.25)	30.78 (5.08)	<0.001
Missing	0	113 (0.62)	
Pre-pregnancy BMI (Kg/m^2)	23.35 (4.68)	23.48 (4.82)	0.69
Missing	7 (1.17)	420 (2.37)	
Gestational age (weeks)	39.54 (1.49)	39.58 (1.36)	0.92
Missing	11 (1.84)	379 (2.14)	
	n (%)	**n (%)**	
Household characteristics			
Income at 2 months (€/month/CU)			<0.001
<1,500	113 (19.5)	5161 (34.6)	
1,500 – 1,944	209 (35.9)	4879 (32.7)	
>1,944	259 (44.6)	4891 (32.8)	
Missing	16 (2.68)	2772 (15.7)	
Pet ownership at 2 months	283 (51.3)	6110 (47.2)	0.07
Missing	45 (7.50)	4757 (26.9)	
Child characteristics			
Sex; Girl	258 (43.2)	8547 (48.8)	0.01
Missing	0	179 (1.01)	
Children with sibling	324 (54.7)	8839 (55.5)	0.74
Missing	5 (0.84)	1781 (10.1)	

CU = Consumption Unit, SD = Standard Deviation; Categorical variables and missing values expressed as frequency and proportion (n (%)), Continuous variables expressed as mean and standard deviation; * Independent samples t-test for continuous variable and chi-squared or exact Fisher test when the expected frequencies are <5 in some cells for categorical variables

Overall, mothers in a higher proportion of the included population were born in France (93.1% vs. 86.5%, P < 0.001) and were older at birth on average (31.8 ± 4.25 years *vs.* 30.8 ± 5.08 years) with higher education level (50.3% *vs.* 30.2% > Baccalaureat +2). Children in the included population were from higher income households (44.6% *vs*. 32.8% >1,944 €/per capita).

When considering the two sleep clusters (Table 3), compared to those from the “less optimal sleep” cluster, higher percentage of children in the “more optimal sleep” were boys (46.5% *vs*. 33.3%, P < 0.01), dominantly taken care of by child sitters (56.8% *vs.* 49.6%, P = 0.07), and from households with a pet (53.8% vs. 44.1%, P = 0.05). Most children in the “more optimal sleep cluster” were living in the suburban setting at 3.5 years of age (37.8 vs. 28.0, P = 0.04). Other covariates were not remarkably different between the two sleep clusters.

Table 3; Characteristics of the study population within the two sleep clusters (n = 597)

	Less optimal sleep (n= 150)	More optimal sleep (n= 447)	P-value*
	n (%)	n (%)	
Maternal characteristics			
Birthplace; France	139 (92.7%)	417 (93.3%)	0.85
Education at 2 months			0.85
< Secondary level	33 (22.1%)	106 (23.9%)	
≤ Baccalaureat +2	38 (25.5%)	117 (26.4%)	
> Baccalaureat +2	78 (52.3%)	220 (49.7%)	
Vaginal child delivery	123 (84.2%)	347 (80.3%)	0.33
Exposure to psychotropic medications during pregnancy	6 (4.00%)	10 (2.20%)	0.25
	Mean (SD)	**Mean (SD)**	
Age at birth (years)	31.7 (4.18)	31.8 (4.27)	0.78
Pre-pregnancy BMI	23.2 (4.58)	23.4 (4.71)	0.76
Gestational age (weeks)	39.5 (1.57)	39.6 (1.27)	0.74
Breastfeeding duration (months)	4.81 (6.20)	3.87 (5.04)	0.24
	n (%)	**n (%)**	
Household characteristics			
Income at 2 months (€/month/CU)			0.79
< 1,500	49 (33.3%)	153 (32.2%)	
1,500 – 1,944	48 (32.6%)	147 (33.9%)	
> 1,944	50 (30.0%)	134 (30.9%)	
Pet ownership at 2 months	63 (44.1%)	220 (53.8%)	0.05
Residential setting at 3.5 years			0.04
Rural	43 (28.7%)	130 (29.1%)	
Suburban	42 (28.0%)	169 (37.8%)	
Urban	65 (43.3%)	148 (33.1%)	
	Mean (SD)	**Mean (SD)**	
Child characteristics			
BMI Z-score at 2 years	0.10 (1.18)	0.04 (1.01)	0.64
Diet at 2 years			
Unhealthy dietary pattern	0.04 (1.62)	-0.01 (1.55)	0.70
Healthy dietary pattern	-0.13 (1.52)	0.04 (1.39)	0.23
Exact age at 3 years	42.3 (1.78)	42.3 (1.71)	0.83
	n (%)	**n (%)**	
Sex; Girl	50 (33.3%)	208 (46.5%)	<0.01
Children with sibling	75 (50.3%)	249 (56.2%)	0.23
Main mode of care at 2 years			0.07
Family	36 (25.2%)	117 (26.7%)	
Child Sitter	71 (49.6%)	249 (56.8%)	
Collective care	36 (25.1%)	72 (16.4%)	
Tobacco exposure from pregnancy up to 3 years	53 (30.1%)	158 (35.9%)	1.00
Antibiotics intake between 2 and 3 years			0.65
Never	47 (31.3%)	150 (34.1%)	
Once	36 (24.0%)	91 (20.7%)	
More than once	67 (44.7%)	199 (45.2%)	

CU = Consumption Unit, SD = Standard Deviation; Categorical variables and missing values expressed as frequency and proportion (n (%)), Continuous variables expressed as mean and standard deviation; *Independent samples t-test for continuous variable and chi-squared or exact Fisher test when the expected frequencies were <5 in some cells for categorical variables

3.3. Association between gut microbiota alpha diversity measures, enterotypes and sleep clusters

In the unadjusted and adjusted models, no significant associations were found between the alpha diversity measures (Chao1 and Shannon), microbiota enterotypes, and sleep clusters (Table 4). Similar results were found with other alpha diversity metrics (supplementary table 6).

Table 4; Gut microbiota Alpha diversity measures, enterotypes, and sleep clusters (n = 597) – The "More optimal sleep" cluster is the reference group

	Crude			Adjusted [+]		
	OR	**95% CI**	**P-value** [b]	**OR**	**95% CI**	**P-value** [b]
Alpha diversity metrics						
Chao1[a]	0.05	-1.27, -0.91	0.56	0.08	-0.13, 0.28	0.45
Shannon[a]	0.02	-0.17, 0.20	0.86	-0.01	-0.21, 0.19	0.91
Microbiota Enterotypes						
Enterotype 1 (ref)	1.00		0.88	1.00		0.92
Enterotype 2	1.04	0.64 – 1.67		0.97	0.59 – 1.62	

OR = Odds Ratio, CI = Confidence Interval, Ref = Reference; [+]Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, sibling, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding duration, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, exact child's exact age at stool collection, child tobacco exposure from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, residential setting at 3 years

[a]Standardized estimates

b Independent samples t-test for continuous variable and chi-squared or exact Fisher test when the expected frequencies were <5 in some cells for categorical variables

3.4. Sex-stratified association between gut microbiota alpha diversity measures, enterotypes and sleep clusters

Table 5 demonstrates the child sex-stratified association between the alpha diversity measures, enterotypes, and sleep clusters within the study population. The proportion of girls and boys within the two sleep clusters was significantly different in Table 2, and the stratified analysis aimed to assess if the child sex has modified the microbiota-sleep association. In the adjusted model among boys only, there was a higher probability of belonging to the "less optimal sleep" cluster with every higher standard deviation of microbiota richness (Chao 1). Although the strength of association did not reach a statistical significance level, the direction was different in girls and boys.

Table 5; Sex-stratified analysis of microbiota alpha diversity measures, enterotypes, and sleep clusters (n = 597) – The “More optimal sleep” cluster is the reference group

	Boys (n= 339)						Girls (n = 258)					
	Crude			Adjusted			Crude			Adjusted		
	OR	95% CI	P-value [b]	OR	95% CI	P-value [b]	OR	95% CI	P-value [b]	OR	95% CI	P-value [b]
Alpha diversity metrics												
Chao [a]	0.14	-0.10, 0.37	0.26	0.26	-0.02, 0.53	0.07	-0.07	-0.37, 0.23	0.65	-0.15	-0.52, 0.21	0.41
Shannon [a]	0.03	-0.20,0.27	0.77	0.001	-0.27, 0.27	0.99	-0.02	-0.33, 0.28	0.88	-0.03	-0.39, 0.32	0.85
Microbiota Enterotypes												
Enterotype 1 (ref)	1.00			1.00			1.00			1.00		
Enterotype 2	0.98	0.54 – 1.78	0.96	1.16	0.60 – 2.24	0.62	1.08	0.48 – 2.43	0.84	0.94	0.36 – 2.39	0.89

OR = Odds Ratio, CI = Confidence Interval; [+] Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother’s pre-pregnancy BMI, gestational age, child delivery mode, mother’s age at birth, child sex, siblings, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding duration, child BMI Z-score at 2 years, main mode of childcare at 2 years, child’s diet at 2 years, exact child’s exact age at stool collection, child tobacco exposure from pregnancy until 3 years, child’s antibiotics intake between 2 and 3 years, residential setting at 3 years.

[a] Standardized estimate

[b] Independent samples t-test for continuous variable and chi-squared or exact Fisher test when the expected frequencies were <5 in some cells for categorical variables

3.5. Gut microbiota community composition differences according to the sleep clusters

Less than 1% of the total variance in the overall gut microbiota community (beta diversity) was explained by the sleep clusters using the Bray-Curtis and Weighted UniFrac distance matrices (Table 6). This finding is consistent with Figure 7, where no clear spatial separation between the two sleep clusters according to the two distances was observed.

Table 6; The gut microbiota community composition differences and sleep clusters (n = 597)

	Crude			Adjusted [+]		
	R^2	P-value	FDR-P-value	R^2	P-value	FDR-P-value
Beta Diversity distances						
Bray-Curtis	0.0014	0.53	0.53	0.0013	0.62	0.71
Weighted UniFrac	0.0024	0.19	0.19	0.0020	0.34	0.76

FDR = False Discovery Rate; [+] Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, siblings, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, child's exact age at stool collection, child tobacco exposure from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, residential setting at 3 years

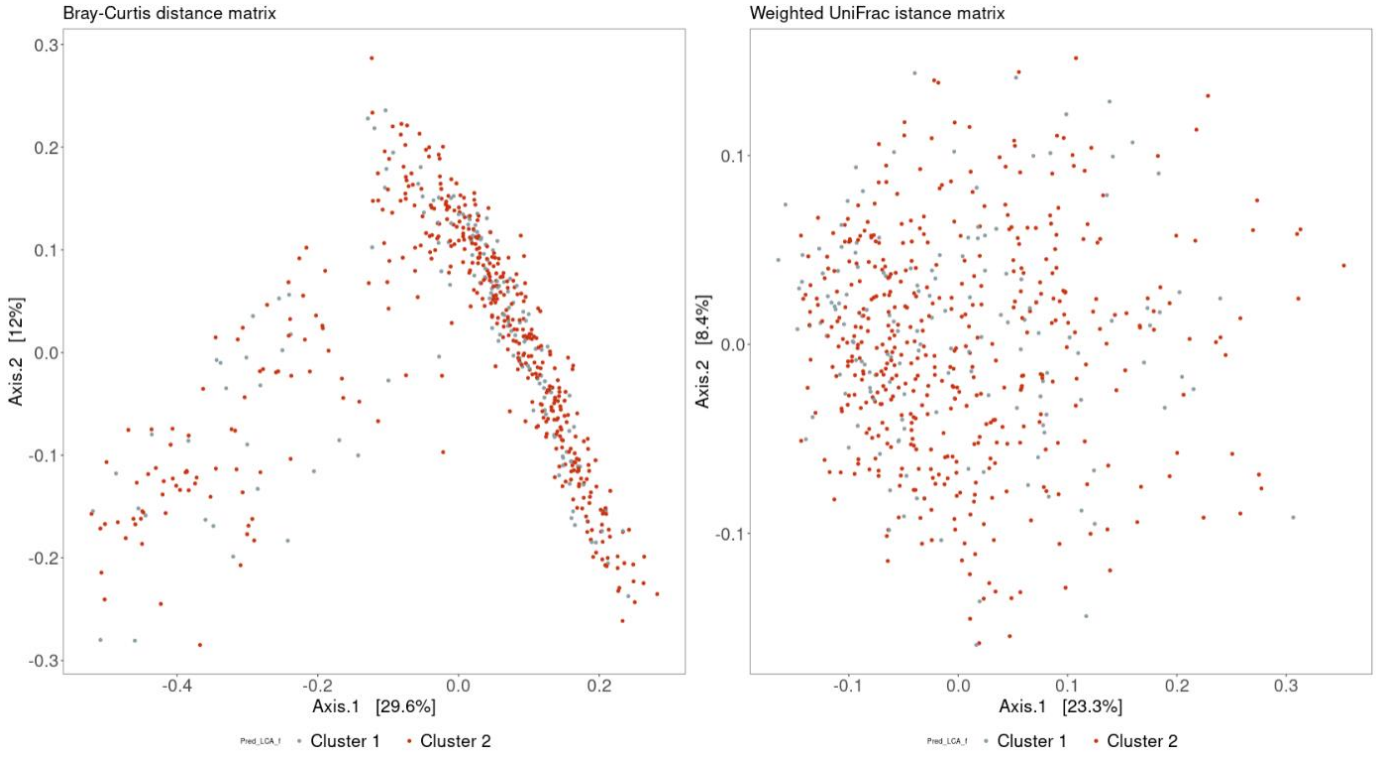

Figure 7; Distribution of the microbiota samples according to the sleep clusters

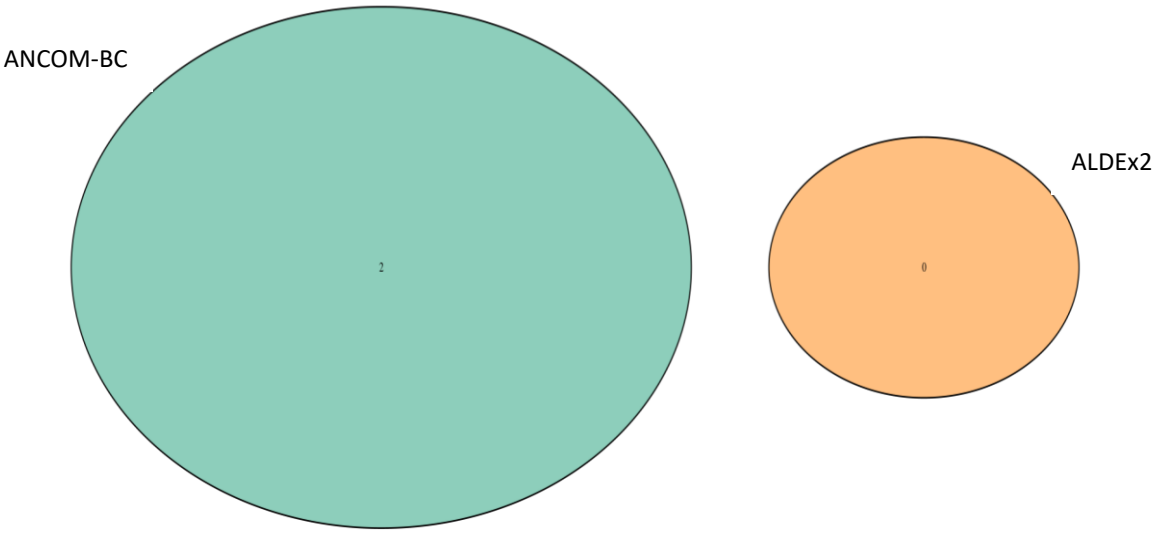

Figure 8; Venn diagram of the ANCOM-BC and ALDEx2 microbiota differential abundance testing

Considering the specific microbiota genera that might be associated with the sleep clusters; no significant results were found overlapping between the ALDEx2 and ANCOMBC methods (Figure 8). Indeed, ANCOM-BC found two genera for which their abundances were significantly different between the two sleep clusters. However, ALDEx2 did not detect any significant genera, preventing any conclusion towards significant differences between the two sleep clusters.

4. Discussion

Interest in the role of the gut microbiota in health and disease has gained ground over the past couple of years (38), yet, few studies have focused on the significance of gut microbiota on sleep health in human subjects, especially children. This study aimed to assess the gut microbiota-sleep relationship at 3.5 years of age. No significant associations were observed between all gut microbiota characteristics studied and sleep clusters in our study population. Nonetheless, considering the gut microbiota richness, there was a potential effect modification by child sex, with boys being more likely to belong to the “less optimal sleep”.

Our study was based on sleep clusters integrating, simultaneously, duration and quality of sleep (sleep onset difficulties and night waking). Previous studies have focused on only one of these facets (duration or quality) and reported inconsistent findings. In a 2016 randomized crossover experimental study in 9 young males, no significant differences were detected in the OTU richness and Shannon evenness indices with partial sleep deprivation (39). Also, a 2022 study among 143 preschool-aged children in Canada did not report any significant differences in alpha-diversity metrices (OTU, Shannon, and Faith Phylogenetic) according to actigraphy-measured night sleep duration and sleep efficiency (40). Consistent with this finding, in a 2019

study on 619 three months old Canadian infants registered in the CHILD birth cohort, microbiota richness and evenness (Chao1, Shannon, and Simpson's) were not associated with sleep duration (38). Aligned with our methodology, the sleep data in the CHILD birth cohort was recorded through parents' self-reported questionnaires and included a combination of day and night sleep habits per 24-hour period (38). In contrast, in the longitudinal assessment of 162 infants at twelve months in Switzerland, a negative association was found between Chao1 and Shannon alpha diversity measures and night sleep (duration, frequency, and regularity) after adjusting for age, sex, and breastfeeding status (37). In this study, sleep data were collected through ankle actigraphy for 11 continuous days, and five sleep patterns were constructed through PCA (37). This study did not report any gut microbiota-night sleep (opportunity and duration) associations (37). Also, no differences in the five sleep patterns were detected between the microbiota enterotypes (37). It is worth mentioning that daytime sleep accounts for approximately 25% of the total sleep time in infancy (34), and also the microbiota composition is undergoing considerable evolvements in this period; therefore, the generalizability of findings beyond this age window may be limited. Some studies conducted on adults concluded that higher gut microbiota diversity promotes healthier sleep (32, 35, 36). In a 2019 research in the USA, microbiota diversity measurements (OTU, Shannon, and Inverse Simpson's) were positively correlated with sleep efficiency in 26 male adults, and the Inverse Simpson's index had a significant and positive correlation with total sleep time measured in a 30-days period (32). Karl and colleagues reported a significant reduction in the microbiota richness among 19 healthy men after applying severe, short-term sleep restriction while controlling for diet and physical activity, suggesting that the experimental alterations of sleep may induce changes in gut microbial composition (35). Also, a recent study of 28 North American adults showed that sleep quality recorded through the Pittsburgh Sleep Quality Index was positively associated with microbial diversity (36). Altogether, it is important to consider the differences in sample sizes and variations in the adjustments for potential confounders when interpreting the discrepancies. Inconsistent results within the literature can be explained by the varying age of the participants, the methods of sleep assessment, the study design (experimental vs. observational), the sample size, and the potential confounders accounted for in the analyses.

There is evidence to support the sex-specific differences in sleep characteristics in children and adults. In a 2015 study of EDEN Mother-Child cohort data among 546 boys and 482 girls aged 3 years in France, boys had a later bedtime and earlier wake-up time compared to girls, resulting in shorter mean night sleep duration (8). This finding was in agreement with a study in the USA among 1-3 years old children, reporting that the likelihood of having a short sleep (< 11 hours per day) was 45% higher in boys than in girls due to earlier waking-up times in the morning (69). Human sex may also affect the gut microbiota composition (70) and therefore,

modulate the microbiota-sleep relationship. In the present study, boys were more prevalent in the “less optimal sleep” cluster. The sex-stratified analysis showed that higher gut microbiota richness (Chao1 index) tends to be associated with a higher likelihood of belonging to the “less optimal sleep cluster” in boys compared to girls. Although the association did not reach a significance level, the observed difference in the direction of association reveals a potential effect-modifying role for child sex. Despite that most studies on the microbiota-sleep relationship among infants and children recruited participants from both sexes, the majority of studies in adults focused on males, and sex-specific outcomes were poorly explained. Schoch and colleagues focusing on infants, controlled for the effect of child sex in their analyses (37), whereas Karl and colleagues and Smith and colleagues (32, 35) only studied young males and discussed the generalizability of the findings as well as the need for further sex-specific research.

In this study, we did not observe any differences in the overall microbiota community composition assessed by the beta diversity metrics between the sleep clusters. This result was consistent with a study among 19 young males in the USA that assessed the beta-diversity microbiota community composition with different sleep deprivation levels (35). Also, a crossover experimental study in young adults in Thailand did not report any genus-level microbial community dissimilarity using the Bray-Curtis distance between two sleep conditions; two-week home sleep extension and two weeks of habitual sleep (71). Nevertheless, in a study of 143 preschoolers in Canada aged 4.5 years, night sleep duration (based on 48-hours actigraphy) was positively associated with the overall gut microbiota community composition assessed by the Weighted UniFrac distance (40).

Finally, we did not observe any differences in the specific microbiota genus abundances across the sleep clusters, which was inconsistent with the majority of studies in the literature performed in the adult population. In a 2020 study using the Pittsburgh Sleep Quality Index and actigraphy-collected sleep data, adults with poor sleep quality had an increased abundance of *Prevotella* and a lower relative abundance of *Blautia* and *Ruminococcus* (36). In this study, the higher relative abundance of *Prevotella* explained more than 25% of the variance in the global Pittsburgh Sleep Quality Index (36). Also, in the study of Benedict and colleagues including 9 adults, partial sleep deprivation led to a significant increase in the abundance of *Coriobacteriaceae* and *Erysipelotrichaceaedecrease* families and a significant decrease in the abundance of *Tenericutes* phylum, compared with participants that also followed two nights of normal sleep (39). In another study among morbidly obese adults, higher proportions of *Coriobacteriaceae* and *Erysipelotrichaceae* were found (72, 73). However, whether changes in these families of gut microbiota were more accentuated in chronic sleep loss conditions remains to be investigated. Finally, in a recent study among 118 middle-aged subjects in Spain (50% obese), gut microbiota composition sequenced through the shotgun

metagenomic method, *Christensenella minuta* (from the *Christensenellaceae* family) was associated with increased Rapid-eye Movement sleep duration, whereas microbiota belonging to the *Enterobacteriaceae* family demonstrated an opposite link (74). Gut microbiota from the *Christensenellaceae* family are generally related to the BMI and serum lipid and glucose levels (75, 76). In the only available study on preschoolers in Canada aged 4.5 years, children with a higher total night sleep possessed a higher relative abundance of *Bifidobacterium*, *Parabacteroides*, and *Turicibacter (40)*. *Bifidobacterium* was previously shown to improve the quality of sleep measured by the Pittsburgh Sleep Quality Index among young adults when administered as a probiotic (77).

To the best of our knowledge, this was the first study to assess the association between the gut microbiota characteristics among children of the preschool period and a holistic clustering metric of sleep using a large nationwide multicentric cohort data. This study has several strengths, namely the inclusion of participants based on a prospective national cohort study, the relatively large sample size and study power to assess the hypothesis within the general French population and the availability of data on gut microbiota and confounders such as dietary information for a large population. Although the addition of potential confounding factors to the multivariate models did not improve the results, identification of the early and contemporary confounders and assessing the potential effect in the regression models can be considered as a methodological strength.

This study also has some limitations that must be acknowledged. In terms of sleep; the data were collected from parents by phone interview through questionnaires (39). Night sleep duration calculation was based on the bedtime and wake-up time and may reflect more time in bed. Also, night waking and sleep onset difficulties were those noticed and reported by parents. Together, this may have led to an overestimation of sleep duration and an underestimation of night waking and sleep onset difficulties (75). Subjective estimation of sleep quality and duration through questionnaires is a pragmatic choice and classical method to provide insight into the important aspects of sleep habits in children in large epidemiological studies (2, 8, 76). Nonetheless, objective methods of sleep data collection such as actigraphy would have provided better estimations of sleep characteristics that were not implemented in this cohort due to cost and logistic challenges (75). Considering the gut microbiota data; stool samples were collected once in the ELFE cohort (61), which prevents controlling for the microbiota composition dynamic throughout early childhood (34). Also, the gut microbiota sequencing was performed through the 16S rRNA gene sequencing approach, which limits the scope of this study to the description of the bacteria composition (77). Alternative methods such as shotgun metagenomics could have allowed for greater sequencing depth and hence, additional information regarding microbiome functional profiles at the species level (77).

Moreover, the studied population was more likely from socioeconomically superior households. Thus, our results might not have adequate external validity for households with lower educational and economic attainments. In addition, the selected families may have been featured with higher health literacy and practices and healthy lifestyle. Indeed, the sleep duration in both clusters (less and more optimal sleep) was within the AASM's recommendations (10-13 hours of sleep within the preschool period) (1), possibly partially explaining the lack of associations observed. Therefore, future studies will focus on the sleep quality variables independently of sleep duration to provide a reasonable effect size.

Finally, the cross-sectional design of this study hinders the establishment of exposure and outcome temporality, causal inferences, and identification of the direction of causation.

5. Conclusion and recommendations

In conclusion, our results did not support the hypothesized association between the gut microbiota diversity and composition and sleep clusters combining sleep quality and duration in French children aged 3.5 years. Further studies are needed focusing more specifically on sleep quality, given that sleep duration included in the clusters was within the international recommendations.

Indeed, both sleep and gut microbiota evolve rapidly up to preschool years. Sleep and gut microbiota can be readily modified at early ages (33). Sleep can be tailored with behavioral and educational interventions (78), and gut microbial composition can be modified by prenatal factors and environmental exposure such as dietary habits and pre- and pro-biotic ingestion (79). Therefore, a well-rounded understanding of these facets of human physiology can enhance our understanding of the bidirectional communication between the host and the gut microbiome. Expanding the body of research in this area can provide new prospects on the necessity and method of intervention in sleep health and may lead to novel strategies tailored to the gut microbiota health-promoting properties to improve sleep quality and duration in children. Also, the statistical methodology used here can generate new insights into the selection of variables and statistical models to assess the microbiota-sleep relationship in prospective studies. Finally, the planned publication of our results will help with the possible challenges of publication bias in the area of microbiota-sleep. Considering the nature of scientific publishing, it is important to discern if the underreporting of negative or statistically non-significant findings can explain the scarcity of research in the microbiota-sleep literature. Our results can help with minimizing the risk of underreporting statistically non-significant results in the academic literature.

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Appendices

Supplementary table 1; The goodness of fit measurements in the LCA analysis

	Model	log-likelihood	resid. df	BIC	AIC
1	Model2	-1389.22	6.00	2835.97	2796.44
2	Model3	-1387.64	1.00	2864.76	2803.28
3	Model4	-1386.60	-4.00	2894.65	2811.20

Supplementary table 2; The study population characteristics according to the BMI Z-score measurements at 2 and 3.5 years

	BMI Z-score at 2 years (n = 443)	**BMI Z-score at 3.5 years (n = 337)**
	n (%)	**n (%)**
Maternal characteristics		
Born in France	417 (94.1)	322 (95.5)
Education at 2 months		
< Secondary education	97 (22.0)	63 (18.9
≤ Baccalaureat +2	112 (25.5)	95 (28.4)
>Baccalaureat +2	231 (52.5)	176 (52.7)
Exposure to psychotropic medications during pregnancy	8 (1.8)	8 (2.4)
Vaginal delivery	353 (80.6)	286 (82.7)
	Mean (SD)	**Mean (SD)**
Age at birth (years)	31.8 (4.09)	31.8 (4.26)
Pre-pregnancy BMI (Kg/m^2)	23.2 (4.49)	23.2 (4.70)
Gestational age (weeks)	39.6 (1.29)	39.6 (1.36)
Breastfeeding duration (months)	3.92 (5.01)	4.25 (5.78)
	n (%)	**n (%)**
Household characteristics		
Income at 2 months (€/month/CU)		
<1,500	137 (31.7)	105 (32.1)
1,500 – 1,944	153 (35.4)	116 (35.5)
>1,944	142 (32.9)	106 (32.4)
Pet ownership at 2 months	214 (51.6)	163 (52.6)
	n (%)	**n (%)**
Child characteristics		
Sex; Girl	198 (44.7)	147 (43.6)
Children with sibling	239 (54.3)	163 (48.8)
Main mode of care at 2 years		
Family	115 (26.4)	73 (22.3)
Child sitter	243 (55.7)	187 (57.2)
Collective care	78 (17.9)	67 (20.5)
Tobacco exposure from pregnancy until 3 years; at some point	150 (34.3)	110 (33.4)
Antibiotic intake between 2 and 3 years		
Never	152 (33.6)	109 (31.7)
Once	96 (21.9)	75 (22.5)
More than once	195 (44.5)	153 (45.8)
	Mean (SD)	**Mean (SD)**
Exact age at stool collection (months)	42.3 (1.69)	42.2 (1.73)
Diet at 2 years		
Healthy dietary pattern	0.12 (1.43)	0.15 (1.43)
Unhealthy dietary pattern	<0.1 (1.40)	<0.1 (1.58)

Data reported based on the available data of BMI Z-scores at both ages; CU = Consumption Unit, SD = Standard Deviation; Categorical variables and missing values expressed as frequency and proportion (n (%)), Continuous variables expressed as mean and standard deviation

Supplementary table 3; The PCA factor loading of the food items reported in the 2-years child dietary questionnaire

	Factor loading	
Food Item	**Unhealthy dietary pattern**	**Healthy dietary pattern**
French fries	0.52	-0.41
Meat and ham	0.51	0.23
Quiche	0.49	-0.24
Pastry	0.46	-0.21
Egg	0.40	0.05
Charcuterie	0.39	-0.33
Fruit juice	0.39	-0.11
Cheese	0.38	0.16
Bread	0.37	0.24
Sweets	0.36	-0.20
Fresh fruit	0.33	0.36
Pasta	0.29	0.56
Cooked vegetable	0.18	0.74
Fruit compote	0.05	0.30

Supplementary table 4; Complete-case analysis of the gut microbiota-sleep association (n = 374)

	Crude			Adjusted		
	OR	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Alpha diversity metrics						
Chao1	0.15	-0.08, 0.39	0.20	0.18	-0.08, 0.45	0.18
Shannon	0.13	-0.11, 0.36	0.29	0.05	-0.22, 0.31	0.73
Enterotypes						
More optimal sleep (ref)	1.00			1.00		
Less optimal sleep	1.28	0.67 – 2.42	0.44	1.22	0.60 – 2.46	0.58

OR = Odds Ratio, CI = Confidence Interval; [+] Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, sibling, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding duration, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, child age at stool collection, child tobacco exposure from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, residential setting at 3 years

* Standardized estimates

Supplementary table 5; The PERMANOVA analysis of variance on the complete-case data (n = 374)

	Crude			Adjusted +		
	R^2	P-value	FDR-P-value	R^2	P-value	FDR-P-value
Beta Diversity metrics						
Bray-Curtis	0.002	0.50	0.50	0.003	0.45	0.75
Weighted UniFrac	0.004	0.16	0.16	0.003	0.22	0.62

FDR = False Discovery Rate; + Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, sibling, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding duration, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, exact child's age at stool collection, child tobacco exposure from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, residential setting at 3 years

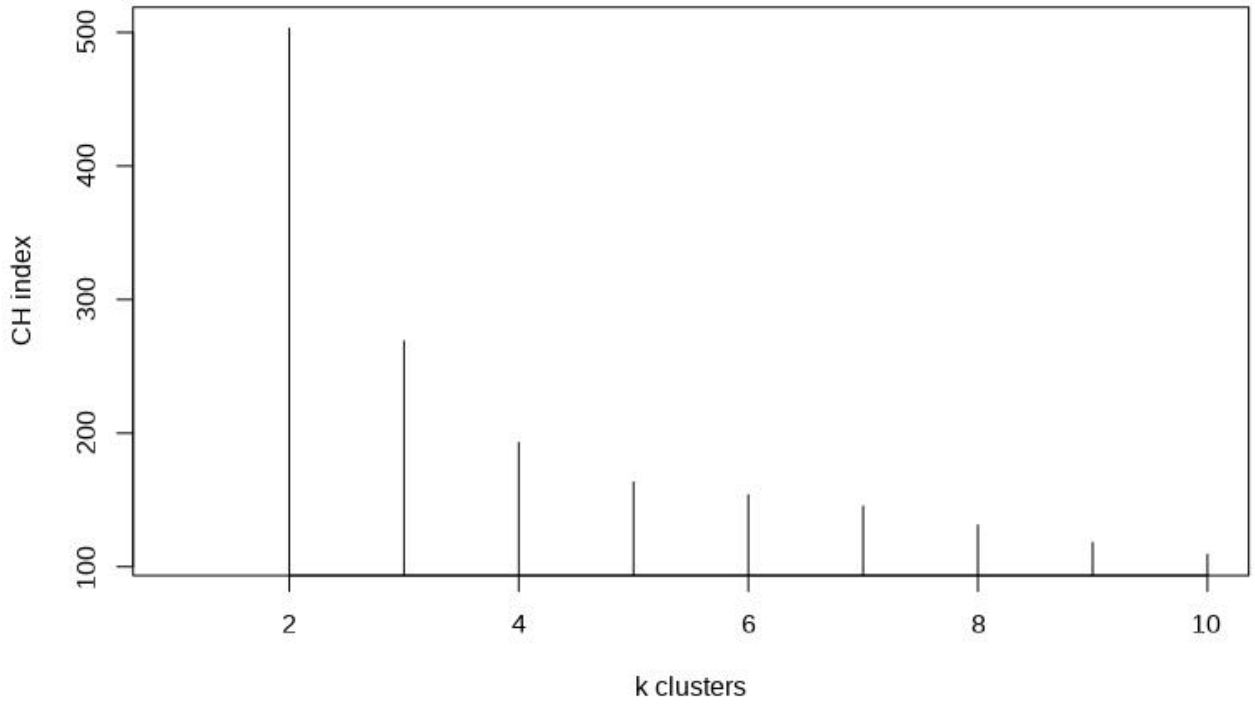
Supplementary table 6; Additional microbiota alpha diversity measures, DMM clustering enterotypes, and the sleep clusters (n = 597)

	Crude			Adjusted		
	OR	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
Alpha diversity metrics						
Observed	-0.07	-0.25, 0.12	0.47	-0.21	-0.48, 0.06	0.14
Simpson's	-0.03	-0.22, 0.15	0.72	-0.06	-0.33, 0.21	0.98
Inverse Simpson's	0.01	-0.17, 0.20	0.91	0.02	-0.24, 0.27	0.74
Enterotypes						
Enterotype1 (ref)	1.00			1.00		
Enterotype 2	1.50	0.90 – 2.49	0.12	1.75	1.01-3.02	0.04*
Enterotype 3	1.38	0.80 – 2.39	0.25	1.63	0.90 – 2.97	0.11
Enterotype 4	1.35	0.75 – 2.45	0.32	1.39	0.73 – 2.62	0.31
Enterotype 5	1.36	0.70 – 2.68	0.37	1.46	0.71 – 3.01	0.30

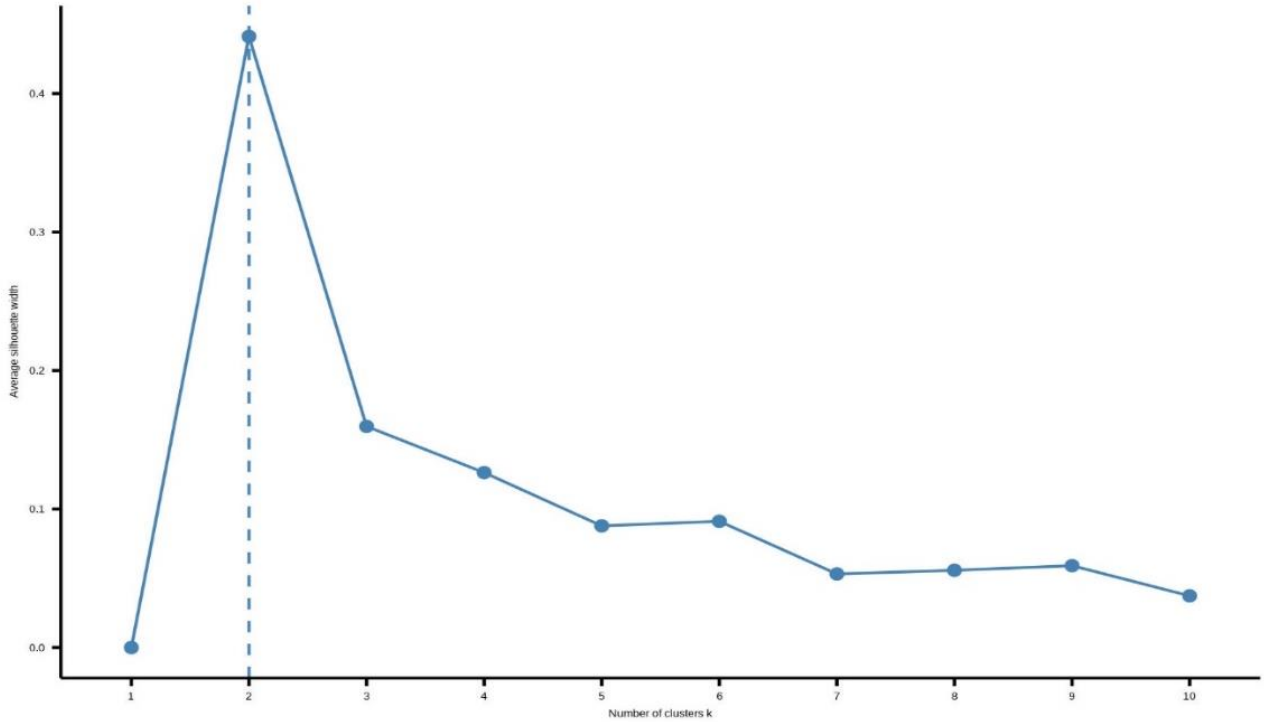
OR = Odds Ratio, CI = Confidence Interval; + Model adjusted for maternal birthplace, maternal exposure to psychotropic medications during pregnancy, mother's pre-pregnancy BMI, gestational age, child delivery mode, mother's age at birth, child sex, sibling, maternal education at 2 months, household income at 2 months, pet ownership at 2 months, breastfeeding, child BMI Z-score at 2 years, main mode of childcare at 2 years, child's diet at 2 years, exact child's age at stool collection, child tobacco from pregnancy until 3 years, child's antibiotics intake between 2 and 3 years, residential setting at 3 years

* Standardized estimates

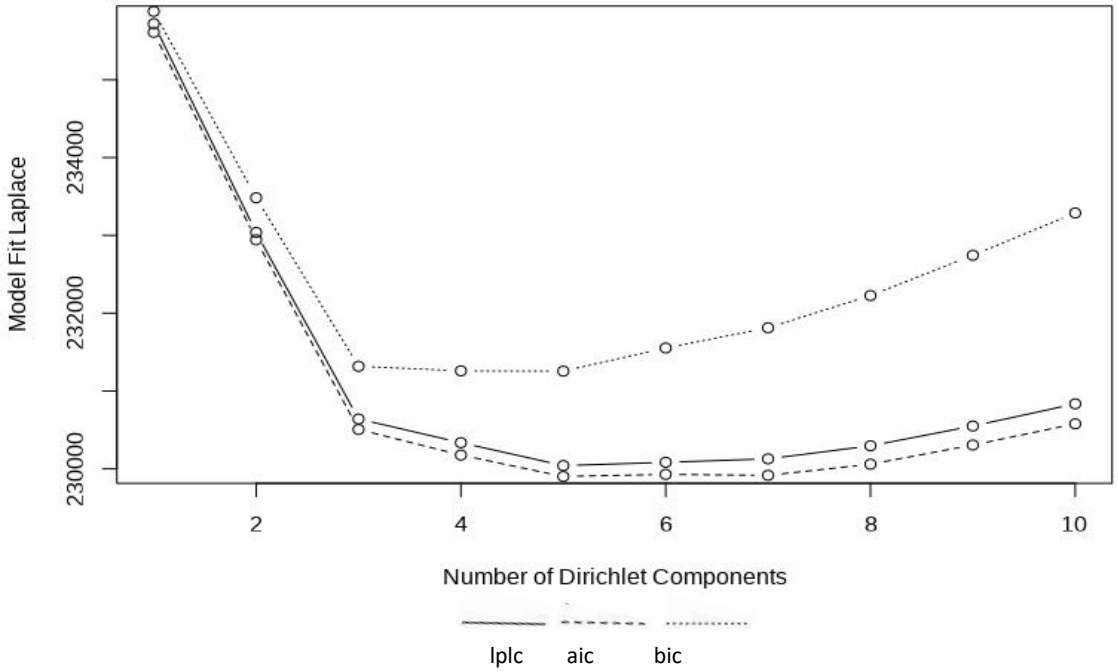
Supplementary figure 1; The Calinski-Harabasz Index in the PAM clustering microbiota enterotypes



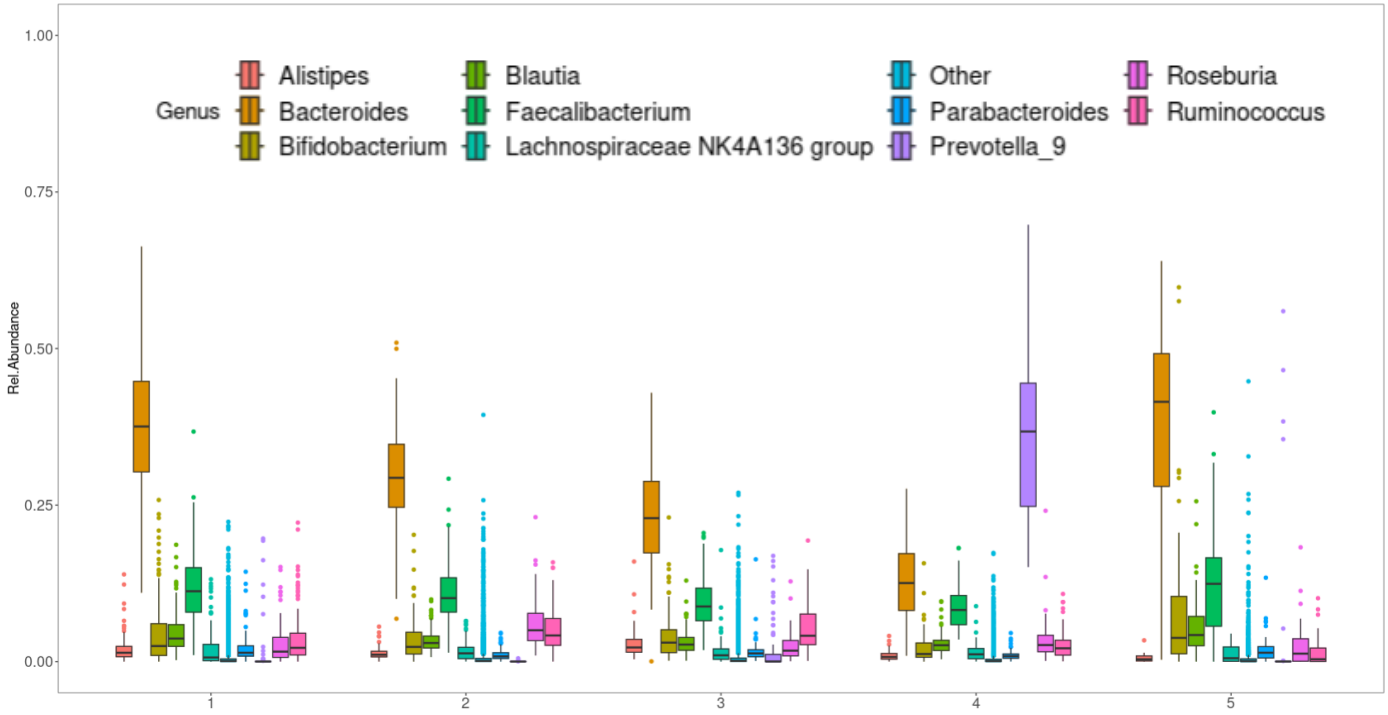
Supplementary figure 2; The Silhouette Index in the PAM clustering microbiota enterotypes



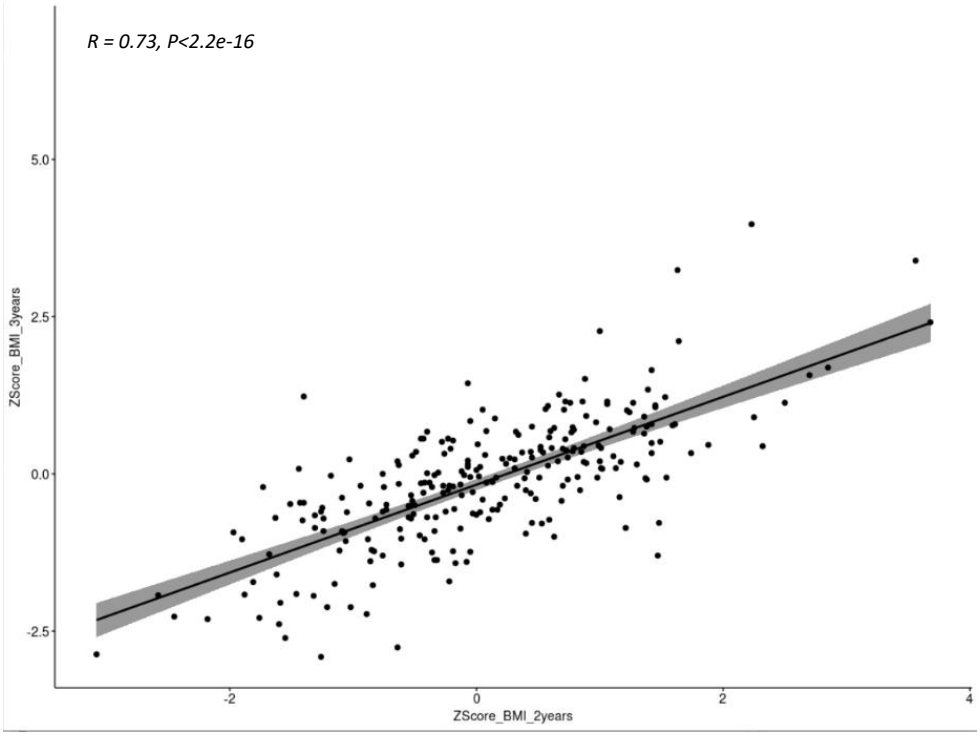
Supplementary figure 3; Comparison of the Goodness of fit measurements in the DMM clustering microbiota enterotypes



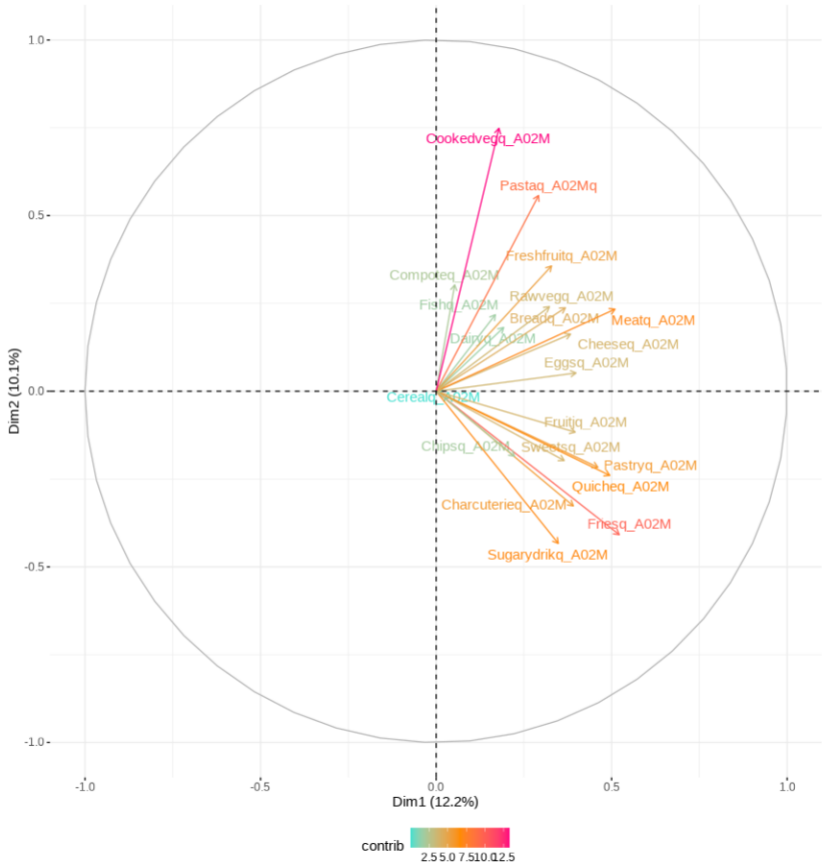
Supplementary figure 4; Distribution of the ten most abundant microbiota genera across the five enterotypes identified through DMM clustering



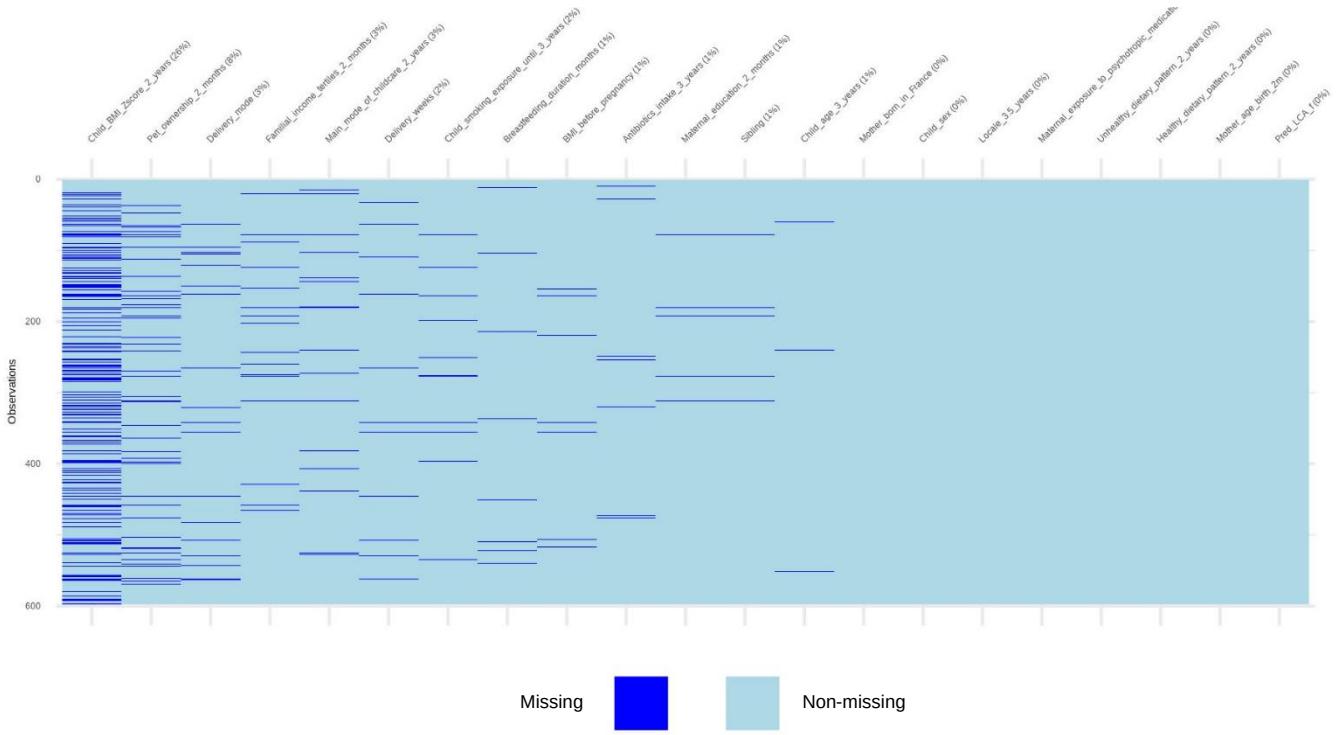
Supplementary figure 5; Pearson correlation of BMI Z-score measurements at ages 2 and 3.5 years



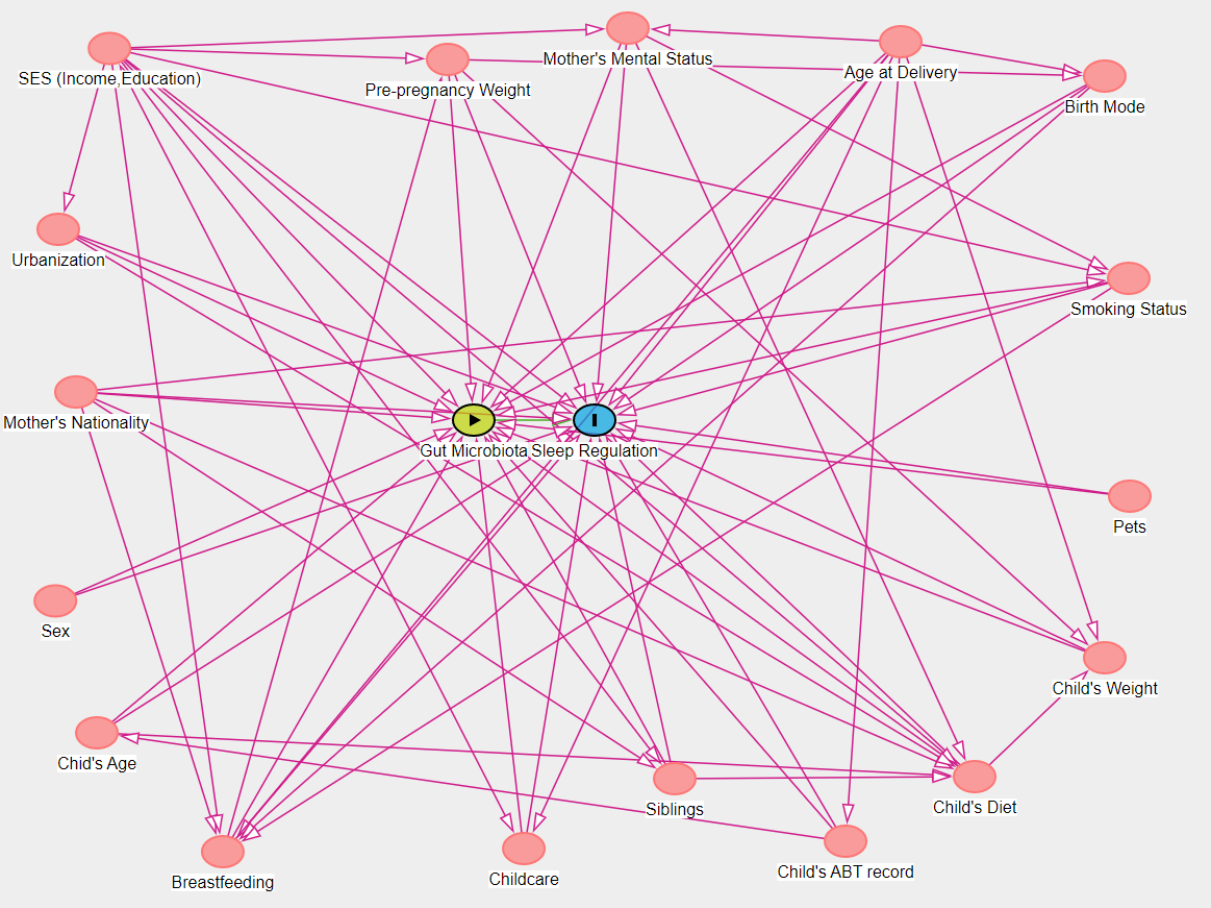
Supplementary figure 6; Contribution of the recorded food items to the child diet PCA model



Supplementary figure 7; Pattern of missing data for the covariates



Supplementary figure 8; Identification of the covariates through DAG



به راه بادیه رفتن به از نشستن باطل

و گر مراد نیابم به قدر وسع بکوشم...