Interaction Between Diet and Dominant Fecal Microbiota Along the First Year of Life

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Research Article

Keywords: early diet, gut microbiota, short-chain fatty acids, breastfeeding, complementary food.

Posted Date: November 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1028299/v1>

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Abstract

Extensive work has established the importance of the gut microbiota during the first years of life. However, there are few longitudinal studies describing the role of infants' diet on the evolution of the fecal microbiota and their metabolic activity during this stage. The aim of this work was to explore the impact of diet on the composition of the major intestinal microorganisms and their main microbial metabolites from birth to 12 months. This is a longitudinal prospective study analyzing fecal microbiota, bacterial groups levels were determined by qPCR and short-chain fatty acids (SCFAs) levels by gas chromatography, as well as information from self-administered questionnaires about general characteristics and food frequency from a cohort of 83, Spanish and full-term, infants at 15, 90, 180 and 365 days of age. Results revealed that Enterobacteriaceae and *Bifidobacterium* decrease in weaning period contrary to *Bacteroides* group and *Clostridium* cluster IV. Furthermore, a clustering based on fecal bacterial groups, SCFAs and type of delivery and feeding, gender and living area suggested that the excretion of SCFAs is strongly related to the type of lactation.

Conclusion: our study supports weaning period as a key step for gut microbiota transition and suggests the importance of the consumption of dietary fiber with the increase of certain bacterial groups as *Clostridium* cluster IV, which could be beneficial for the host. Finally, studies specially designed to analyze the production and the excretion of SCFAs in children are needed to understand how diet could influence in this process.

What Is Known

- *The importance of the gut microbiota during the first years of life.*
- *Environmental factors such as the type of delivery, the feeding received, the use of antibiotics, pre- and probiotic consumption, or the maternal diet have a significant influence on the infant's gut colonization.*

What is New

- *The characterization of fecal microbiota and short-chain fatty acids across the first year of life.*
- *The identification of certain dietary components as possible drivers of microbial composition and its activity*

Introduction

The human intestinal tract is the portion of the organism most densely populated by microorganisms that, under normal conditions, have a mutualistic relationship with the host [1]. In contrast to adulthood, with a relatively stable and diverse microbial ecosystem, in early life the composition of the microbiota is less diversified, with a high proportion of some genus such as *Bifidobacterium* until weaning [2]. Newborn digestive tract colonization begins with the delivery process and continues evolving in parallel with the

maturation of the gastrointestinal tract and childhood development [3–5]. The first intestinal colonizers, facultative anaerobes or aerotolerant microorganisms, belong to the phyla Proteobacteria or Firmicutes and degrade the existing oxygen, favoring the subsequent development of strict anaerobic bacteria belonging to the genera *Bacteroides*, *Bifidobacterium* or *Clostridium* [6, 7]. Disturbances in the early gut microbiota, referred to as dysbiosis, have been linked to increased risk of altered immune responses and the development of immune-related pathologies, such as asthma or allergy, and metabolic disorders, including obesity or diabetes [6, 7]. It has been suggested that this initial colonization is also associated with adult microbial composition, and subsequently with the development of several pathologies later in life [8–11].

Several environmental factors such as the type of delivery, the feeding received, the use of antibiotics, pre- and probiotic consumption, or the maternal diet have a significant influence on the infant's gut colonization [12]. Among them, infant's diet plays a key role in microbiota modulation. Extensive research supports the World Health Organization (WHO) recommendation on the importance of exclusively breastfeeding up to 6 months, and mainly as 1 year of age, as the gold standard that provides all the nutritional requirements and growth needs of the infant [13]. In addition to the nutritional components, human milk is a source of bioactive compounds (such as human milk oligosaccharides (HMOs)) and microorganisms, that are transferred from the mother to the infant [14]. Some studies have shown an increased concentration of bifidobacteria and decreased in *Bacteroides* in breastfed infants compared to those receiving starter formulas [15, 16], which is consistent with the bifidogenic effect described for HMOs [17]. Thereafter, complementary feeding (the introduction of solid food between 17 and 26 weeks of age) [18] also influences on the infant's microbiota although its effects on microbiota development are not yet fully understood [19]. Recent research has revealed the existence of crucial time points at which the introduction of certain foods had a substantial impact on microbiota composition [19]. In a general way, it has been observed that *Bifidobacterium* is the most abundant genus in the gut until 6 months of age [20] and then the progressive incorporation of solid foods up to 12 months increased the concentrations of Bacteroidetes and Firmicutes. More specifically, at around 170-190 days of life the introduction of formula and peas to the diet of exclusive breastfeed babies produced an increase in *Bacteroidetes* [19]. These changes in the bacterial ecosystem result in increased concentrations of short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate acids, as a byproduct of the microbial catabolism of carbohydrates [21].

Based on this evidence, a deeper understanding of these interactions may lead to new dietary strategies for early microbiota modulation and deserves further exploration. Therefore, the purpose of this study is to evaluate the impact of the feeding received during the first year of life on the composition of the major intestinal microorganisms and their main microbial metabolites, with special emphasis on the progressive introduction of complementary diet.

Subjects And Methods

Sample recruitment and study design

The children involved in this work belong to EarlyMicroHealth project cohort. The sample population consisted on 83 full-term (37-40 gestational weeks) neonates of which samples from 79 infants were available at 15 days of age, from 83 at 90 days, from 78 at 180 days and from 68 at 365 days of life (Figure 1). Newborns were recruited through the Primary Care Pediatrics Service at the first consultation visit, at 10-15 days after birth. In all cases, written informed consent was obtained prior to enrolment. Parents were notified in writing of the contents, procedures and objectives of the study, as well as the option to withdraw from the study at any time. The study has been evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 12/16, 03/02/2016) and to the Committee on Bioethics of CSIC (Ref. PCIN-2015-233). The procedures have been performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, the Council of Europe Convention on Human Rights and Biomedicine and in the Spanish legislation on bioethics. The Directive 95/46/EC of the European Parliament and the Council of 24 October 1995, on the protection of individuals regarding the processing of personal data and on the free movement of such data, has been strictly followed.

General characteristics

Information on characteristics from the neonates (gender, date of birth), from the mothers (gestational age, pre-gestational weight (kg) and height (m), pre-gestational Body Mass Index (BMI)(kg/m²), education level and living area) and pregnancy factors (type of partum, number of previous pregnancies, pregnancy diseases and smoking habit) were collected. Fecal samples and questionnaires were collected at the recruitment and at 90, 180 and 365 days of age. Families were instructed on the procedure for fecal sampling collection. Stool samples were stored at -80°C until further analysis.

Fecal analyses

For DNA extraction, 1 g of sample was weighed, diluted 1:10 in sterile PBS solution, homogenized in a LabBlender 400 stomacher (Seward Medical, London, UK) at full speed for 3 min and centrifuged (10 000 g, 30 min, 4°C). Then, supernatant and bacterial pellet were separated. Bacterial pellet was used for DNA extraction following the Quality Protocol Q for fecal samples DNA extraction described and recommended by the International Human Microbiome Standards Consortium [22]. Extracted DNA was kept frozen at -80°C until analysis.

The levels of *Bifidobacterium*, *Bacteroides* group, Enterobacteriaceae and *Clostridium* cluster IV were determined by qPCR using methods described elsewhere [23, 24].

The analysis of SCFAs was performed by gas chromatography by using a chromatographic system composed of two 6890N GC (Agilent Technologies Inc., Palo Alto, CA) connected to an FID and an MS 5973N detector (Agilent). Cell-free supernatants from the homogenized feces were treated as described elsewhere [25] and used to determine the concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate, caproate and valerate.

Dietary assessment and nutritional data analysis

Infants' dietary information has been compiled by means of a self-administered weekly food propensity questionnaire adapted from the Pilot study for Assessment of Nutrient intake and food Consumption Among Kids in Europe (PANCAKE) [26] and adapted for Spanish population, thus including traditional regional recipes and typical foods. In addition, food questionnaires were developed using an online tool. Foods were grouped into 11 food categories adapted from European Prospective Investigation Into Cancer (EPIC) classification [27] and 2 additional for human breast milk and processed infant products as follows. Oils (vegetable oils and solid fats); vegetables (bulbs, mushrooms, roots, inflorescences, and stem and leaf vegetables); legumes (lentils, chickpeas, soya, beans and peas); fruits (fresh, dried, and canned fruits); potatoes and tubers (potato and sweet potato); cereals and cereal products (bread, pasta, flours and grains); meat and meat products (white meat (poultry, rabbit and pork loin), red meat (beef, lamb and pork chop), processed meat and others); fish (fish and fish products, crustaceans and mollusks); eggs; human breast milk, processed infant products (infant formulas: starter formulas, special starter formulas, follow-up formulas, special follow-up formulas, growing-up milk; infants cereals and infant purees: fruits, fruit and cereals, vegetables, legumes and pasta, meat, fish and others) [28]; milk and dairy products (milk, yogurt, dairy dessert, fresh, mature, and processed cheeses) and sweet and desserts (sweets, cake, biscuits, chocolate, honey and others).

The questionnaires were filled in by the mother, father or caregiver of the infant who received them via email or mobile phone. Detailed directions for completing the food diary were included at the beginning of each category and the validated picture book developed by the PANCAKE consortium was used for portion size estimation according to EU-Menu recommendations [29]. As dietary information was collected over a prolonged period at different times from birth, adapted different version of the same was used at 15 days and 90 days, due to the absence of complementary feeding. Breastfeeding was collected at all-time points categorically.

For the categorical variable, the type of breastfeeding was classified as breastfeeding (including mixed-feeding), or infant formula. For the quantitative variables, the amount of breast milk consumed was estimated by using the mean values reported in the literature for each stage of age (780 ml for infants until 6 months and 600 ml for infants aged 7–12 months, in the cases of exclusive breastfeeding) [30, 31]. For the infant formulas, the volume reported by the parents was used, assuming that the manufacturer's prescriptions regarding the weight of powdered milk to be dissolved per volume were respected. In mixed-fed infants, based on existing literature, the amount of formula consumed per day was measured and the remaining volume of formula consumed per day was assumed to be breastmilk up to 780 ml from start to 6 months and 600 ml at 12 months [31].

The consumption of foods was converted into energy and macronutrients using the food composition tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID) [32]. Nutritional composition data of human breast milk [33], infant formula, cereal products and infant purees was completed from Gómez-Martín and collaborators' baby foods composition table [28].

Additionally, detailed information regarding type of protein or carbohydrate consumed was extended from the food composition tables published by the United States Department of Agriculture (USDA) [34], the major types of fiber (soluble and insoluble types) from Marlett et al. [35] and the content of the main classes and subclasses of (poly)phenol from the Phenol-Explorer Database [36].

Anthropometric measures

Children height (cm) and weight (kg) were registered to the nearest 0.1 cm and 0.1 kg, respectively, by pediatric nurse with calibrated and approved material. Body Mass Index was calculated as weight, in kilograms, divided by the square of height, in meters. BMI z-score were obtained relative to WHO Child Growth Standards, adjusted by gender and age, by using WHO ANTHRO, Software for Calculating Anthropometry, Version 3 [37, 38].

Statistical analyses

Results were analyzed using the IBM SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, USA) and RStudio software version 1.4.1103. Goodness of fit to the normal distribution was checked by means of the Kolmogorov-Smirnov test. When normality of the variables was not achieved, non-parametric tests were used. Overall, categorical variables were summarized as percentages and continuous ones using means and standard deviations for descriptive purposes. The Mann-Whitney U test and Kruskal-Wallis test were used to evaluate differences in continuous variables. In addition, multivariate analysis adjusted by gender, type of delivery and feeding and living area was used. To explore deeper the associations between fecal bacteria levels and SCFAs excretion with food groups and dietary components, Spearman correlation analyses were performed. A heatmap was generated under RStudio software version 1.4.1103 package corrplot. With the aim to group the sample according to the microbial profile, a clustering based on fecal bacterial groups, SCFAs and the covariates (type of delivery and feeding, gender and living area) was performed, getting two clusters encompassing individuals presenting the most similar characteristics (Supplementary Figure 1). Clusters were generated using a K-means cluster by SPSS and graphically represented by RStudio software version 1.4.1103 by function fviz_cluster (Supplementary Figure 1). GraphPad Prism 8 was used for graphical representations.

Results

Description of the sample

The basal characteristics of the study cohort are described in Table 1. The sample consisted of a total of 83 subjects, 48 male (57.8%) and 35 (42.2%) female. It was observed that most children were born vaginally (78.3%), presented a normal birth weight (BMI z-score of -0.99 to 0.99) and 60.2% live in rural areas. Concerning maternal factors, the average age of mothers was 35 ± 5 years old and the 66.3% presented normal body mass index pre-pregnancy.

Evolution of the microbial groups and short-chain fatty acids

Higher levels of *Bifidobacterium* and *Bacteroides* were observed in breastfed infants, of *Bacteroides* also in vaginally born infants and Enterobacteriaceae and *Clostridium* cluster IV in male infants. Also, living area was related with differences in all bacterial groups along time (data not shown). In consequence, these variables were introduced as covariates for data analyses. *Bacteroides* group and *Clostridium* cluster IV increased progressively over the time (from 6.23 log n° cell/g feces at 15 days to 8.73 at 365 days and from 4.24 at 15 days to 8.06 at 365 days, respectively, p value < 0.001) while *Bifidobacterium* and Enterobacteriaceae only increased significantly until 180 days of life. In addition, in the case of Enterobacteriaceae, a significantly decrease was observed from 180 to 365 days (Figure 2).

To study the changes in fecal SCFAs along time the analyses presented in Figure 3A and 3B were conducted. An increased in the mean levels of all SCFAs from 15 to 365 days was observed (Figure 3A). Relative proportions from the main SCFAs are represented in Figure 3B. The percentage of acetate decreased from 90 days until 365 days (from 80.02% to 67.02%). In consonance, an increase of the proportion of propionate and butyrate was observed from 90 to 180 days (13.59% to 16.46%) and from 180 to 365 days (5.16 to 10.76%), respectively.

Evolution of the diet

Regarding the evolution of the diet throughout the first year of age, as it was expected a pronounced increase in the mean consume of all food group, except for human breast milk and infant formulas was observed (Figure 4A). At 6 months, the predominant food groups were fruits, vegetables, infant formula and human breast milk. However, at 12 months, some of those food groups as infant formula and human breast milk decreased while protein food groups such as meat, legumes, eggs and fish increased and the diet became more adult-like. In turn, energy and lipids increased along the study and the highest changes in macronutrients intake were observed between 180 days and 365 days. The pronounced increase in dietary fiber, soluble and insoluble at 90 to 180 days is noteworthy, as well as the increase in monounsaturated fatty acids and vegetable protein at the same period of time (Figure 4B)

Relationships among bacterial groups, SCFAs and diet

The linear relationship between microbial groups and SCFAs with diet has been explored through the Spearman correlations presented in Figure 5. Breastfeeding has been inversely correlated to acetate, propionate, butyrate and total SCFAs. Moreover, propionate, butyrate and total SCFAs were directly associated with the intake of infant processed products at 180 days (Figure 5A). Propionate and total SCFAs are also correlated positively with energy, lipids, carbohydrates and total protein at 180 days (Figure 5A). On the other hand, at 12 months, *Clostridium* cluster IV was found inversely correlated with intake of some protein food groups such as fish and meat and meat products and also with infant processed products and sweets and dessert (Figure 5B). From nutrients, isobutyrate and isovalerate were positively correlated with insoluble fiber at 12 months of age (Figure 5B).

A cluster based on fecal bacterial groups, SCFAs and type of delivery and feeding, gender and living area was performed. The characterization of the cluster across age is presented in Table 2. The mean of

Enterobacteriaceae was lower in cluster I at 365 days of age whereas this cluster showed higher levels of *Bifidobacterium* at early days (15 days). As for SCFAs, from 15 to 365 days, those children belonging to cluster I presented a higher mean of acetate, propionate and total SCFAs than children belonging to cluster II. In addition, at 90, 180 and 365 days the mean of butyrate was higher in cluster I.

To deepen into the knowledge about the diet of the infants belonging to each cluster and analysis of the dietary intake data from each cluster was conducted (Tables 3 and 4).

A significant difference in energy was observed at 90 days, with the highest intake in cluster I (Table 3). From the early stages of the study up to the 180 days of age, cluster I also showed higher consumption of infant formula as well as lower intake of breastmilk at 90 and 180 days of age comparing to cluster II (Tables 3 and 4). These differences between the two clusters disappeared at 365 days (Table 4). Regarding to complementary feeding, at 180 days of life cluster I showed non-significant lower intake of fruits (91.35 ± 118.81 vs. 137.34 ± 137.30 g/day) banana (17.07 ± 22.13 vs. 35.63 ± 40.74 g/day) and pear (23.82 ± 34.78 vs. 47.77 ± 62.34 g/day) while a significantly higher intake of sweets and desserts (24.61 ± 21.82 vs. 12.78 ± 15.18) and processed meat (10.46 ± 6.85 vs. 4.43 ± 5.12 g/day) was noted at 365 days of life (Table 4).

Discussion

Gut microbiota and SCFAs have been shown to have a wide range of beneficial physiological effects on the host throughout the lifespan [39, 40]. Even though several studies have characterized the microbial profile in adults, there is scarce literature describing the evolution of the bacterial ecosystem during early life beyond weaning period. This work provides a picture of how the bacterial composition and excretion of SCFAs changes upon the introduction of complementary feeding within the first year of life, supporting the importance of breastfeeding in the modulation of the infant's microbiota composition and activity.

From birth, the gut microbiota develops rapidly into a succession of bacterial strains [20] in a transition to an adult microbiota. Throughout this process different factors, related to pregnancy and delivery and the infant's environment, influence the establishment of the different bacterial groups. Among them type of delivery, gestational age, gender, or type of early feeding are known to have an influence and, then, they should be considered when interpreting diet-microbiota relationships [5, 41].

The difference observed in the *Bifidobacterium* mean level between breastfed and formula-fed infants at 15 days of age (9.17 ± 1.46 vs. 8.76 ± 1.33 , data not shown) is consistent with the ability of this bacteria to degrade some HMOs from human milk such as lacto-N-biose [42, 43]. The first colonizers of the newborn's gastrointestinal tract are aerotolerant microorganisms which reduce oxygen levels facilitating an optimal environment for the proliferation of anaerobic bacteria such as *Bifidobacterium* [42, 44]. According with that, bifidobacteria have been shown as the most abundant genus in the sample up to 6 months of age [45] and declined during the weaning process [46]. In line with previous studies in Spanish children the first food groups introduced in the sample during the weaning period were infant cereals (64.1% of the sample), fruits (55.1%), vegetables (44.9%) and tubers (43.6%) [47].

The reason for the changes on the microbiota following complementary feeding are not totally understood yet. Some authors argue that cessation of breastfeeding may play a more important role than the introduction of solid foods [6, 48, 49], but based on our results it seems reasonable to speculate that the incorporation of complex polysaccharides in the infant's diet, may favor the growing of some bacteria groups, such as *Bacteroides* and *Clostridium* cluster IV, which are specialized in the breakdown of fiber [50–52]. In this line, although given the nature of the study we are unable to establish the directionality, our results pointed to a direct association between *Clostridium* cluster IV levels and dietary fiber throughout the first year of life. Some authors have reported that diets enriched in different type of fiber, like inulin, oligofructose, guar gum, arabinoxylan and resistant starch, induce *Clostridium* cluster IV enrichment [51]. Also, the decrease in Enterobacteriaceae and *Bifidobacterium* observed during the first months of life contrary to what occurs with *Bacteroides* and *Clostridium* cluster IV at 12 are in agreement with previous longitudinal studies [53].

Acetate is the main SCFAs in the sample, as occurs in adults [54]. The increase of *Bacteroides* and *Clostridium* groups with the weaning process may probably determine the increment in acetate and butyrate observed from 6 to 12 month [19, 51] since some strains of *Clostridium* genus are able to transform acetate into butyrate [55, 56].

Finally, the classification of infants, at 6 and 12 months, according to their fecal microbiota and short-chain fatty acid resulted in two cluster: being cluster I represented by higher excretory infants in comparison to cluster II. Perhaps one of the mayor findings of this study is the identification of at breastfeeding as one of the major determinants of cluster membership. The higher levels of propionate and butyrate acids showed in formula-fed are in agreement with some of the first studies investigating this relationship [57]. With independence of breastfeeding, it is possible that, undigested proteins and amino acids can pass into the large intestine, being fermented by certain bacteria producing different bacterial metabolites, such as SCFAs [58, 59]. Consistent with this finding, we observed that infants belonging to cluster I (those with higher excretion of SCFAs) had significantly higher total protein intake than cluster II at 90 and 180 days (10.36 g/day vs. 8.75 g/day and 15.17 vs. 13.47, respectively) (data not shown).

Some authors have hypothesized that SCFAs represent the key molecular link between diet, microbiome and health playing an important role throughout the lifespan in protecting the body against deteriorating metabolic control and inflammatory status [40]. In this regard, while some studies related the high excretion of SCFAs with obesity risk [60], we have not observed significant differences in infant body weight among clusters. However, those children belonging to cluster I (higher SCFAs excretion and infant formula intake) experienced more weight gain from birth to 3 and 6 months than those belonging to cluster II (2.71 vs. 2.24 kg and 4.70 vs. 4.20 kg, respectively) (data not shown). Further research is needed to determine the normal range of SCFAs levels and the threshold above which they may have an impact on health.

The study has some limitations related to its observational nature and the collection of dietary data. In interpreting this information, it should be noted that the energy and nutrient content of processed infant foods has been taken into account, a factor that has hitherto been underestimated in the literature. Regarding the quantification of breast milk energy, it is necessary to mention a limitation of the study. Since it was not possible to record the exact volume of milk produced by the mother, an indirect estimation was made using the mean amounts established in the literature for each age range [30, 31]. While the quality of the FFQ depends on the respondent's memory, its ability to accurately classify energy and all nutrient intakes in children is enhanced by the fact that the questionnaires are adapted from the PANCAKE study and have photographs that make them easier to interpret. In addition, it allows comparison with other studies on the European infant population.

Conclusions

This work is one of the first to study how diet could influence microbiota composition in the first year of life. Our results support weaning period as a key step for gut microbiota transition. In turn, this study also suggests the importance of the consumption of dietary fiber with the increase of certain bacterial groups as *Clostridium* cluster IV, which could be beneficial for the host. Finally, studies specially designed to analyze the production and the excretion of SCFAs in children are needed to understand how diet could influence in this process.

Abbreviations

BMI	Body Mass Index
CESNID	Centro de Enseñanza Superior de Nutrición Humana y Dietética
EPIC	European Prospective Investigation Into Cancer
HMOs	Human Milk Oligosaccharides
MUFA	Monounsaturated Fatty Acids
PANCAKE	Pilot study for Assessment of Nutrient intake and food Consumption Among Kids in Europe
PUFA	Polyunsaturated Fatty Acids
SCFAs	Short-Chain Fatty Acids
SFA	Saturated Fatty Acids
USDA	United States Department of Agriculture
WHO	World Health Organization

Declarations

- **Funding** This work was financed by PCIN-2015-233 (Project EarlyMicroHealth from the EU Joint Program Initiative HDHL) (MINECO/FEDER, UE) and by the University of Oviedo (Diet, Rhythms and Early Acquisition Microbiota (DREAMS) Project, REF. 2017/00001/003/005/013). S.A was the recipient of a postdoctoral Juan de la Cierva Contract (Ref. IJCI-2017-32156) funded by MCIU/AEI/10.13039/501100011033 and M. G-M. is the recipient of a predoctoral FPU contract (FPU18/03393) funded by the Spanish Ministry of Science, Innovation and Universities.
- **Conflicts of Interest** The authors declare no conflict of interest.
- **Availability of data and material** Not applicable.
- **Code availability** Not applicable
- **Author's Contributions** SG, MG designed the study. DH-M, MC and TL recruited participants. SS, SA, MG-M analyzed fecal samples. MG-M performed the nutritional assessment and statistical analyses. SG, MG-M: drafted the manuscript. All authors revised the final version of the paper.
- **Ethics approval** The study has been evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 12/16, 03/02/2016) and to the Committee on Bioethics of CSIC (Ref. PCIN-2015-233). The procedures have been performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, the Council of Europe Convention on Human Rights and Biomedicine and in the Spanish legislation on bioethics. The Directive 95/46/EC of the European Parliament and the Council of 24 October 1995, on the protection of individuals regarding the processing of personal data and on the free movement of such data, has been strictly followed.
- **Consent to participate** Informed written consent was obtained prior to enrollment from the children's caregivers
- **Consent for publication** All authors have approved the final submitted version for publication and agree to be accountable for all aspects of the work.
- **Acknowledgments** We thank all the families involved in the EarlyMicroHealth study.

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Tables

Table 1 General characteristics of the study sample

		Total sample (N = 83)	
Infant factors		N	
Gender	Male	48	57.8
	Female	35	42.2
Weight at birth (kg)		82	3.4 ± 0.4
Height at birth (cm)		81	49.9 ± 1.7
BMI z-score at birth	Underweight (−5.99 to <−1)	15	18.5
	Normal weight (−0.99 to 0.99)	55	67.9
	Overweight (1 to 2)	10	12.3
	Obese >2	1	1.2
Maternal factors			
Age (y)		77	35 ± 5
Weight pre-pregnancy (kg)		83	65.0 ± 12.6
Height (m)		83	1.6 ± 0.1
BMI pre-pregnancy (kg/m ²)	Underweight (<18.5)	2	2.4
	Normal weight (18.5–25)	55	66.3
	Overweight (>25)	18	21.7
	Obese (>30)	8	9.6
Education level	Primary	4	4.8
	Secondary	13	15.7
	High school	24	28.9
	University	42	50.6
Living area	Urban	33	39.8
	Rural	50	60.2
Pregnancy factors			
Type of partum	Vaginal	65	78.3
	C-section	18	21.7
Parity, n	0	43	51.8
	1	34	41.0
	2	6	7.2
Pregnancy diseases	Preeclampsia (na= 2)	3	3.7
	HT (na= 3)	2	2.5
	Urinary infection (na= 3)	10	12.5
	Diabetes (na= 3)	14	17.5
Smoking habit	Smoke during pregnancy	9	10.8

Values are presented as mean ± standard deviation and number and % of the subjects. BMI, body mass index; C-section, caesarean section; HT, hypertension; na, no answer.

Table 2 Characterization of the cluster by time

	15 days N = 79		90 days N = 83		180 days N = 78		365 days N = 68	
	Cluster I (N = 19)	Cluster II (N = 60)	Cluster I (N = 31)	Cluster II (N = 52)	Cluster I (N = 36)	Cluster II (N = 42)	Cluster I (N = 14)	Cluster II (N = 54)
Fecal Bacteria (Log n^o cell/g feces)								
<i>Bifidobacterium</i>	9.56	8.85*	9.80	9.64	10.01	9.90	9.26	9.51
<i>Bacteroides</i> group	5.83	6.36	6.32	6.85	7.72	7.57	8.94	8.67
Enterobacteriaceae	7.26	7.60	7.83	7.85	8.27	8.41	6.87	7.71*
<i>Clostridium</i> cluster IV	4.31	4.22	5.48	5.51	6.86	6.72	7.31	8.26
SCFAs (mM)								
Acetate	77.97	31.15**	97.54	44.61**	105.22	51.54**	117.84	66.00**
Propionate	11.65	3.51*	12.65	5.33**	19.34	9.37**	28.33	16.21*
Isobutyrate	1.06	0.93*	1.15	1.06	1.63	1.32	2.21	1.66
Butyrate	2.59	1.98	3.61	2.50*	6.03	3.95*	24.86	9.73**
Isovalerate	1.00	1.16	1.35	1.35	2.05	1.69	2.78	2.17
Valerate	0.99	0.83	0.95	0.91	1.33	0.90	1.60	1.20
Caproate	0.78	0.89	0.80	0.77	0.88	0.77	0.79	0.82
Total	96.04	40.46**	118.05	56.53**	136.48	69.54**	178.42	97.79**

Values presented as mean. Gender, type of feeding and delivery and living area were included as covariates. SCFAs, short-chain fatty acids. Result derived from Mann-Whitney U test (* p value < 0.05, ** p value < 0.001).

Table 3 Characterisation of the diet by cluster from 15 to 90 days of age

	15 days N = 79		90 days N = 83	
	Cluster I (N = 19)	Cluster II (N = 60)	Cluster I (N = 31)	Cluster II (N = 52)
Energy (kcal)	476.71 ± 85.58	479.13 ± 69.16	567.24 ± 73.94	524.78 ± 61.40*
Food group (ml/d)				
Human breast milk	330.00 ± 355.95	513.40 ± 357.65	138.87 ± 278.58	452.12 ± 370.63*
Infant formula	387.89 ± 316.45	210.01 ± 283.22*	712.42 ± 331.90	340.00 ± 396.04*

Values presented as mean ± standard deviation. Result derived from Man-Whitney U test. * p value < 0.05.

Table 4 Characterisation of the diet by cluster from 180 to 365 days of age

	180 days N = 78		365 days N = 68	
	Cluster I (N = 36)	Cluster II (N = 42)	Cluster I (N = 14)	Cluster II (N = 54)
Energy (kcal)	760.58 ± 163.53	725.50 ± 180.65	1041.35 ± 206.16	1038.86 ± 205.46
Oils (ml/d)	4.44 ± 5.04	2.86 ± 4.57	10.00 ± 0.00	10.09 ± 1.53
Vegetables (g/d)	36.03 ± 47.94	34.63 ± 51.33	162.65 ± 85.56	150.56 ± 93.93
Zucchini	4.56 ± 9.92	6.46 ± 11.37	26.05 ± 22.55	26.30 ± 25.04
Pumpkin	1.32 ± 6.42	2.04 ± 7.69	16.48 ± 21.90	8.90 ± 17.68
Tomato	2.09 ± 8.76	2.89 ± 10.99	2.05 ± 5.68	13.70 ± 20.01*
Leek	9.72 ± 12.65	7.19 ± 12.57	28.71 ± 25.13	19.70 ± 22.26
Carrot	10.86 ± 14.48	11.60 ± 20.59	34.85 ± 19.34	33.08 ± 37.00
Potatoes and tubers (g/d)	18.99 ± 28.82	19.63 ± 34.75	57.99 ± 60.92	47.65 ± 34.63
Cereals and cereals products (g/d)	3.48 ± 8.38	3.07 ± 8.84	34.99 ± 22.58	40.56 ± 33.92
Rice	0.16 ± 0.57	0.40 ± 1.31	1.43 ± 1.86	5.26 ± 10.16
Pasta	0.32 ± 1.46	0.10 ± 0.66	1.06 ± 1.78	3.11 ± 6.29
Bread	2.99 ± 7.21	2.56 ± 7.93	31.79 ± 22.84	25.13 ± 28.04
Fruits (g/d)	91.35 ± 118.81	137.34 ± 137.30	228.82 ± 101.33	211.96 ± 125.03
Banana	17.07 ± 22.13	35.63 ± 40.74	46.78 ± 29.56	42.56 ± 30.10
Pear	23.82 ± 34.78	47.77 ± 62.34	61.84 ± 48.94	53.94 ± 47.47
Orange	17.00 ± 36.73	9.43 ± 23.52	37.28 ± 40.94	28.10 ± 40.62
Apple	28.09 ± 41.42	41.25 ± 51.15	63.17 ± 31.70	45.26 ± 38.93
Legumes (g/d)	1.48 ± 6.18	1.46 ± 6.84	16.20 ± 13.64	21.92 ± 18.51
Pea	1.48 ± 6.18	1.26 ± 6.75	2.14 ± 2.61	5.41 ± 10.35
Lentil	0	0.20 ± 1.32	7.10 ± 5.94	7.99 ± 7.24
Meat and meat products (g/d)	4.60 ± 11.33	4.00 ± 14.56	26.27 ± 13.87	29.84 ± 23.43
White meat	2.47 ± 6.28	1.96 ± 6.32	10.17 ± 9.08	14.09 ± 14.64
Red meat	2.05 ± 5.77	2.52 ± 9.82	5.64 ± 5.92	11.24 ± 12.12
Processed meat	0.08 ± 0.48	0	10.46 ± 6.85	4.43 ± 5.12*
Fish (g/d)	0.91 ± 2.69	0.20 ± 1.32	38.86 ± 33.79	30.19 ± 30.70
Hake	0.71 ± 2.61	0.10 ± 0.66	11.61 ± 12.71	15.13 ± 17.44
Sole	0	0	1.61 ± 4.19	6.88 ± 13.30
Salmon	0	0	0.61 ± 1.56	0.99 ± 3.78
Eggs (g/d)	0.51 ± 3.05	0	9.14 ± 6.21	14.05 ± 11.47
Milk and dairy products^a	9.92 ± 36.10	2.13 ± 13.78	205.09 ± 152.98	165.89 ± 192.25
Human breast milk (ml/d)	128.75 ± 272.19	330.48 ± 349.92*	68.57 ± 180.55	126.67 ± 243.84
Processed infant products	715.98 ± 316.23	479.84 ± 379.52*	324.56 ± 226.66	318.38 ± 255.33
Infant formulas (ml/d)	676.53 ± 316.61	455.71 ± 370.64*	295.00 ± 235.89	274.63 ± 233.55
Infant cereals (g/d)	14.73 ± 17.61	9.57 ± 11.24	16.39 ± 12.56	15.89 ± 13.65
Infant purees (g/d)	24.72 ± 62.66	14.56 ± 37.42	13.16 ± 29.84	27.86 ± 73.77
Sweets and desserts (g/d)	1.39 ± 3.34	0.71 ± 2.37	24.61 ± 21.82	12.78 ± 15.18*
Cookies	1.16 ± 3.14	0.71 ± 2.37	16.29 ± 12.55	10.93 ± 13.34*

Values presented as mean ± standard deviation. Result derived from Mann-Whitney U test. ^a Milk and dairy products included: milk (ml/d), yogurt and cheese (g/d). *p value < 0.05

Figures

Assessed for eligibility

Figure 1

Scheme of participant enrollment and study progress

Fig. 2

Figure 2

Differences in the mean levels of microorganisms (log n° cells/g feces) along the study. Unadjusted means are used for graphical representation. Statistical results derived from a multivariate analysis adjusted by gender, type of delivery and feeding and living area are shown. * p value < 0.05; ** p value < 0.001

Fig. 3

Figure 3

(A) Changes in the means of excreted short-chain fatty acids across time. Statistical analyses obtained from a multivariate analysis adjusted by gender, type of delivery, feeding and living area. * p value < 0.05; ** p value < 0.001. (B) Evolution in the proportion of SCFAs presented in feces. Kruskal-Wallis test was used for comparison (different letters indicate significant differences between groups (p value < 0.05))

Fig. 4

Figure 4

Evolution of (A) diet and (B) energy and macronutrients along the study. Results derived from a multivariate analysis. a Milk and dairy products included: milk (ml/d), yogurt and cheese (g/d). MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. SFA, saturated fatty acids. * Differences obtained by comparing with the time-point previous category (p value < 0.05)

Fig. 5

Figure 5

Spearman correlation between fecal bacteria levels and short-chain fatty acids excretion with food groups and dietary components at (A) 180 days (B) and 365 days. Columns correspond to food groups and energy, macronutrients and bioactive compounds; rows correspond to bacteria groups and short-chain fatty acids. Blue and red colors denote negative and positive association, respectively. The size of the circle and the intensity of the colors represents the degree of association between these food groups or dietary compounds and the microbiota matrix. Valerate and caproate were excluded for the analysis. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SCFAs, short-chain fatty acids; SFA, saturated fatty acids. * p value < 0.05; ** p value < 0.001

Supplementary Files

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