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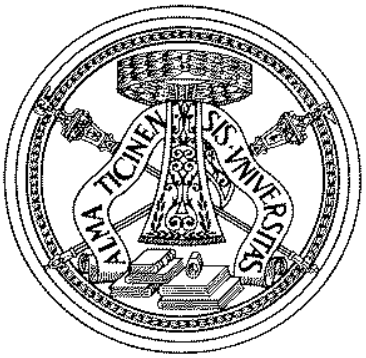

PhD in Psychology, Neuroscience and Data Science

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Early life microbiota colonization.

The A.MA.MI Study

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INDEX

ABSTRACT

Background. The intestinal microbiome is relatively dynamic during the first years of life, becoming relatively stable throughout a lifetime, thus dictating adult life's future health. There are many factors influencing gut microbiome composition, including maternal pre-pregnancy, body mass index (BMI), gestational weight gain (WG), type of feeding, weaning timing, and birth/family environment.

Objectives. The study aims to investigate the intestinal microbiome development of infants (ages 0-12 months) and to analyze how prenatal and postnatal factors, including maternal BMI and weight gain during pregnancy, type of feeding, time and type of weaning, and the presence of siblings in the family, could influence the infant gut microbiome composition at one year of age.

Methods. 63 dyads were enrolled at Neonatal Unit, IRCCS Policlinico San Matteo, Pavia. Anthropometric parameters were assessed before discharge (T0), at 30 days from birth (T1), at 6 months (T2) and 1 year (T3); validated questionnaires were used to evaluate mother's dietary habits and physical activity at each time. Mothers were interviewed about family environment and infants feeding/supplementation at each time. For each child a stool sample was collected at each time and analyzed using metagenomics 16s ribosomal RNA gene sequence-based methods. Cluster and correlation analysis were used to assess changes in the microbial composition across different follow-up times.

Results. At one years of age, pre- and perinatal variables were not significantly associated with any bacterial taxon, while postnatal variables showed the major contribution in shaping microbiota. Feeding and weaning were the main influencing factors.

Conclusion. Our findings highlighted that microbial colonization during the first year of life is likely affected by various factors resembling a simultaneous effect of multiple variables. Mainly, different variables play a significant role at different time. Thus, these data contribute to add evidence concerning the complex multifactorial interaction of GI-microbiota depending on the various stimuli during the early stages of life. This study will provide the starting point for a future prospective and observational study assessing the potential association between maternal diet and lifestyle with newborns microbiome composition, their influence on childhood obesity development and the associated increased risk of NCDs in adulthood.

1. INTRODUCTION

1.1 *"Fetal Origins of Adult Disease (FOAD)" and* "Developmental Origins of Health and Disease"

Over the last century, epidemiologic research has steadily shifted its focus from communicable to non-communicable diseases, such as cardio-metabolic coronary diseases, type 2 diabetes, asthma, chronic pulmonary condition, and allergies strengthening interest in lifestyle and social factors as determinants for these conditions. The shift has also increased the focus on more distal determinants, both socio-culturally (i.e., socioeconomic region) and temporally (i.e., events during earlier stages of life).

The XX century was signed by one of the most revolutionary and fundamental observations in the epidemiological discipline, called "Fetal Origins of Adult Disease" (FOAD), also known as "*Barker's hypothesis*". According to this theory, the origin of chronic diseases in adults refers to the fetal and pediatric years [1].

During intrauterine development, the fetus is vulnerable to various factors, mainly affected via maternal tissues. Maternal mental and physical status, environment to which women are exposed, physical activity, and nourishment habits can permanently affect the growing child's health and physical status. This process was called "fetal programming" [2].

The fetal development begins from the ninth week after conception and represents the start point of rapid growth that continues until the end of the pregnancy. The main feature of fetal growth is the cellular division that leads rapidly to the formation of different tissues and organs. These "critical periods" are very shorts and take place in the uterus at different times for different organs and apparatuses. The central part of organic development ends in the first 1000 days after conception [2]. David Barker, an English epidemiologist, developed this theory by observing that in the poorest countries of England and Wales, there was a higher mortality rate for cardiovascular diseases [1]. However, this relationship seemed very strange to Barker because this kind of disease is typically correlated to overnutrition and physical inactivity, the opposite of what we would expect in the context of poverty. Afterwards, with further analysis, he observed a significant geographic correlation between the mortality rate for ischemic heart disease between 1968 and 1978 and child mortality between 1921-1925. [1] He hypothesized that the development of these diseases in adult age

could be influenced by the mother's diet during the prenatal period, after birth, and childhood [1].

According to Barker's theory, in a poor nutrition environment, the nutrients intake is steered to the brain than to the other tissues in the fetus. This long-lasting condition could lead to an unbalanced distribution of nutrients that may predispose to chronic-degenerative diseases in adult life. In subsequent studies, Barker proves that adults with the lowest weight at birth had the highest death rate for cardiovascular diseases [3] [4].

Instead, the death rate gets lower with the increase of weight, cranial circumference, and body mass index (BMC= weight/height^2) [5]. This relationship was also confirmed for other diseases, as insulin resistance and type II diabetes [6] [7]. Barker hypothesized that the fetus could set up not only the functioning and the structure of its organs and apparatuses but also its metabolism depending on external stimuli: when the nutrient intake isn't sufficient, the fetus, in response to an immediate need, builds a "saving machine" able to spend as little nutrients and energy as possible ("thrifty phenotype") [8].

Such “fetal programming” supports survival in the short term, but when environmental conditions change, promotes the development of diseases. External signals could modify gene expression in favour of the phenotype that guarantees survival and adaptation to environmental conditions. If the resulting phenotype is appropriate for the surrounding environment, the organism will remain healthy and will not develop the disease. When adaptive mechanisms are improper, the risk of disease in later times increase. This event is called improper “Predictive Adaptive Response” (PAR) [9].

By triggering mechanisms of adaptation to the environment (e.g. the decrease in the vascular bed, the reduction in the number of nephrons, alterations in insulin secretion, etc.), the fetus limits its size and store the little energy available to cardiac functions and neuronal development [10]; in that way, it would be able to respond, also in the future, to a poor ad hostile environment. However, during childhood and consequently adult life, the environment could change and become drastically different from the prenatal one. Nevertheless, the body has already lost the ability to adapt to a “healthier” environment. This gap between the uterine and extra-uterine environment, lays the groundwork for the predisposition to develop diseases in adult life, because the physiological setting established during the plastic and predictive phases, wouldn’t be proper for the environment in which the adult organism lives [9].

Moreover, the relationship between cardiovascular diseases and low weight at birth seemed to be independent of the adult lifestyle (alcohol consumption, cigarette smoke etc…) and

existing in the whole population, in any social class, for each obesity level, especially in males [11]. The adult lifestyle wasn't considered as a trigger factor, but surely it could increase the risk of developing diabetes and cardiovascular diseases in those "fragile subjects" [7].

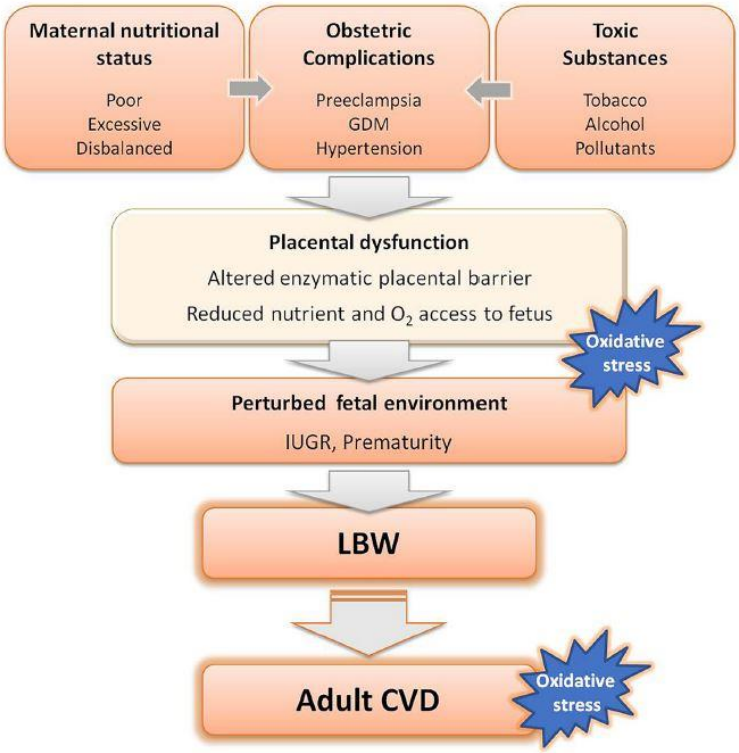


Figure 1. Factors that influence the development of cardiovascular diseases

This theory leaves behind the old concept, that the onset of a degenerative disease is determined by the interaction between genes and environment in adult life, and introduces a new model that contemplates planning by the fetal environment and recognizes the maternal feeding as one of the most important factors that influence this kind of planning [12].

In the last years, more studies extended these hypotheses to the theory of the "*Developmental Origins of Health and Disease*" (DOHaD). The name emphasizes the fundamental role of both the pre and postnatal environment in shaping the different development trajectories that will affect long-term health [13]. The "*DOHaD theory*" involves a different branch of knowledge, as evolutionistic medicine, anthropology, clinical practice and public health, and analyse how environmental factors, act during the plastic phase of development and interact with genotypic variations to modify the adaptability to the environment in adult life.

The same window of plasticity and critical development was proposed also for the formation of the human microbiota. The same environmental stimuli responsible for the PARs seem to have an important role also in the modulation and the development of human microbiota, from conception until childhood [12].

1.2 The Human Microbiome

The human microbiome has emerged as an area of great interest and since the last two decades, many studies have highlighted its impact on the health and development of diseases [14]. Every mammal, since its first appearance on Earth, has formed vital symbiotic alliances with the microorganisms throughout a long and steady process of coevolution. Every human being hosts an enormous number of microorganisms organized in communities and placed all over the body: on the skin, in the mouth, inside the intestine and on the mucous membranes.

It is a real "super organism". Counting the different types of human cells, we can appreciate that, while we have almost 3×10^{13} human cells, we also have about 3.8×10^{13} bacterial cells, most of which are in the gastrointestinal tract. In a typical 70 kg adult, the total bacteria mass represents about 0.3% of the overall body weight, which means approximately 0.2 kg of bacteria [15]. The totality of microorganisms that peacefully coexist with their human host is called "microbiota" [16] [17] The totality of bacterial genes is defined as "microbiome" and is made of approximately 10 million genes, a 400 times greater number than the human genome (about 23.000 genes) [18]. During the last decade, many studies have shown how these symbionts take on a fundamental role in the regulation of the most important physiological and metabolic functions of their hosts, contributing to homeostasis throughout life. Intrinsic and extrinsic factors of the human being can induce important alterations in this balance, causing a condition known as dysbiosis, which recent studies have associated with the etiopathogenesis of many alterations, especially metabolic ones (obesity, type II diabetes, metabolic syndrome), immunological ones (allergies, asthma), inflammatory ones (inflammatory bowel diseases) and neurological ones (dementia), at every stage of life, from childhood to old age, from primitive tribes to modern societies [19]. Important changes in this ecosystem can be responsible even for alterations in the human genome: intermediate metabolites produced by the microbiota can act as gene expression modulators, turning the gene transcription on or off [20]. The gut microbiota evolves steadily throughout life from infancy to early childhood, from puberty to adulthood, and then to the

elderly years, and during periods of special physiological status, such as pregnancy [20]. Although the mechanism and the drivers for the changes or adaptations remain unknown, the main factors involved are host genetics, evolutionary history, diet, geography, and medical intervention, as well as the first microbial colonizers [21].

The intestinal microbiota performs essential functions in protecting their host's health, playing a metabolic, structural, and protective role.

The molecular analysis has shown that commensal bacteria modulate the expression of genes involved in many crucial intestinal and extra-intestinal functions, including the metabolism of xenobiotics, intestinal maturation after birth, the absorption of nutrients, and the support of the mucosal barrier. Its primary roles are:

- Protective. The totality of the bacterial communities represents the first obstacle against the pathogenic microorganisms' colonization through a variety of mechanisms such as the regulation of nutritional substances used by pathogens, the secretion of bacteriocins, and the stimulation of the Gut-Associated Lymphoid Tissue (GALT) [22] (Fig.2).
- Structural. The intestinal microbiota seems to be involved in controlling the cellular proliferation of the intestinal epithelium and the immunity cells, creating the mucosal barrier. Specifically, some bacteria keep the integrity of the desmosomes (through the expression of adhesion proteins) and the tight junctions, limiting the intestinal permeability. Furthermore, it prevents the epithelial cells' apoptosis through the secretion of soluble proteins (p40 and p75) and through the regulation of the level of the endocannabinoid, which is responsible for the barrier effect of the mucosa [23] (Fig.2).
- Metabolic. The intestinal bacteria synthesize essential and non-essential amino acids, produce vitamins, and take part in the biotransformation of the bile acids. They are also involved in the metabolic pathways of non-digestible carbohydrates, such as non-starchy polysaccharides, starches, cellulose, and pectins. Through a process of fermentation, they are transformed into short-chain fatty acids (SCFAs), acetate, propionate, butyrate (in a ratio of 60:25:15), and gas (CO and H). Besides representing nourishment for some bacterial species, these molecules are used in cholesterol synthesis, gluconeogenesis, proliferation, growth, and expansion of the intestinal cells, and prevention from obesity [24] (Fig.2).

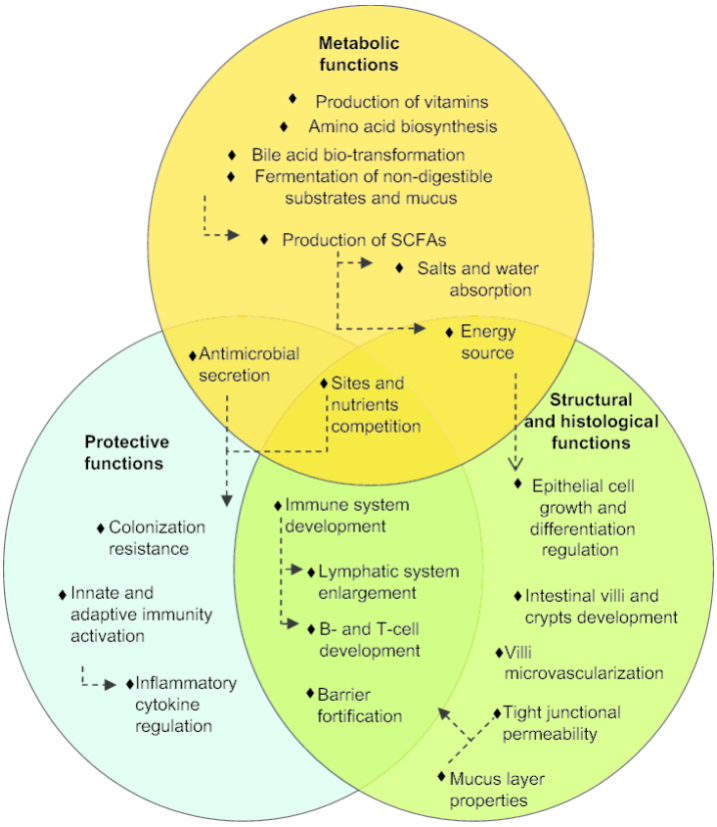


Fig. 2: Main functions of the human gut microbiota [25].

1.2.1 Composition of Human Microbiota

The composition and colonization density of the resident microbial flora is significantly different and complex in the various anatomical sites of the body. It is influenced by many environmental parameters, such as pH, oxygen levels/redox state, availability of nutrients, water activity, and temperature [26]; [27]. The microorganisms which colonize the intestinal tract, concerning their activity and their impact on human's health, can be classified as commensal bacteria (neutral), pathogens (negative entities), and probiotics (positive entities). Everyone holds a personal bacterial fingerprint (a specific profile different from that of other individuals) and a core of at least 57 species common to everyone [28]. The Phyla and their most represented genera are:

- *Firmicutes* (Gram-positive, including Mycoplasma, Bacillus, Clostridium, Faecalibacterium, Ruminococcus, Eubacterium, Staphylococcus, Streptococcus, Lactobacillus, Lactococcus, Enterococcus, Roseburia)
- *Bacteroidetes* (Gram-negative, including Bacteroides, Prevotella and Corynebacterium)

- *Actinobacteria* (Gram-positive, including Bifidobacterium, Collinsella, Propionibacterium)
- *Cyanobacteria* (Gram-negative)
- *Fusobacteria* (Gram-negative)
- *Lentisphaerae* (Gram-negative)
- *Proteobacteria* (Gram-negative, including Escherichia, Klebsiella, Shigella, Salmonella, Citrobacter, Helicobacter)
- *Spirochaeates* (Gram-negative)
- *Verrucomicrobia* (Gram-negative).

The presence in the colon of a small bacterial world subset of 9 Phyla (out of 30 existents in the Bacteria domain) is the result of a strong selective pressure that during evolution has played a role both on the microbial colonizers and on the intestinal niche group. The number and complexity of microbes increase gradually from stomach to colon (Fig. 3): the number of bacterial cells in the gastrointestinal tract shows a continuum in growth in the oral-aboral direction, going from 10^{1-3} bacteria/g in the stomach and duodenum to 10^{4}-10^{7} in the jejunum and ileus until it reaches 10^{12} cells/g of intestinal contents in the cecum and right colon [27]. Inside the stomach, the bacterial count is lower considering that the acid conditions kill almost every bacterium that passes through, acting as a first defensive barrier against external infection. On the other hand, most of the bacteria are in the lower part of the gastrointestinal tract, especially in the large intestine: in the most proximal tract, bile and pancreatic secretions are also toxic and unsuitable for most of the microorganisms' growth. The terminal part of the ileus is an area of transition between the jejunum, which mostly contains facultative anaerobes and the dense population of anaerobes that we can find inside the colon [27]. In the colon, thanks to the very low oxygen concentrations, the anaerobe microorganisms considerably exceed the aerobic bacteria, reaching concentrations of $1x10^{12}$ CFU/ml. At every intestinal level, the microbiota composition changes, even though its diameter, with some bacteria that tend to adhere to the mucous surface, while others prefer the lumen. The motor activity of the terminal ileus and the biomechanical and physiological characteristics of the ileus-colic junction contributes to this bacterial gradient. Certainly, a decisive factor in the deposition of bacteria at the centre of the lumen or on the intestinal surfaces is bowel motility.

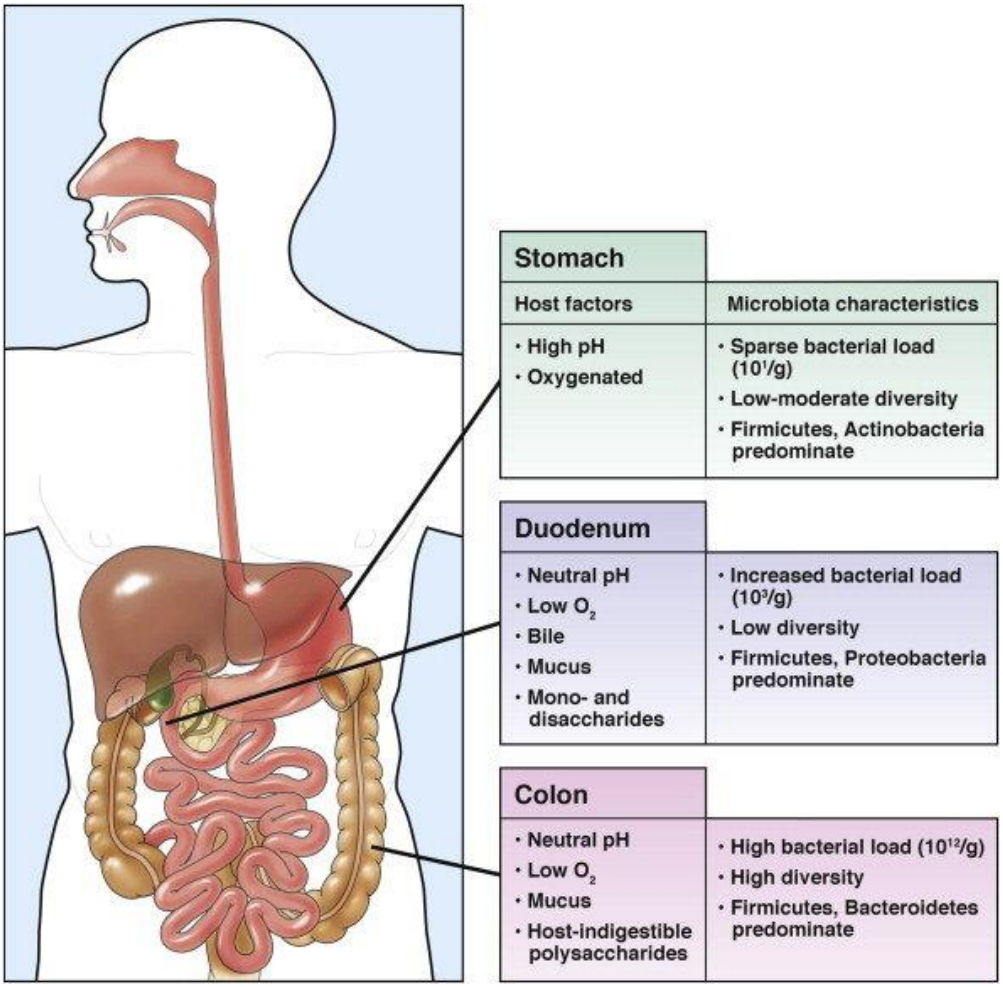


***Fig 3**:* Regional differences in the gastrointestinal tract affect microbial niches [29].

1.2.2 Factors influencing composition and variety of the microbiome

One of the critical elements driving NCDs intergenerational nature, playing a pivotal role in "the first 1000 days", is the gut microbiome, the dynamic organ sensitive to environmental factors that modify its composition over the host's lifespan [30] [31].

The bacterial establishment process is a complex phenomenon that begins in utero. In the last decade, the intrauterine environment was considered sterile. Still, recent evidence supports an intrauterine maternal-to-fetal exchange of microbes, challenging the traditional "sterile womb" that has been acknowledged worldwide for more than a century [12] [31] [32]. Most gut microbes are either harmless or have beneficial properties for the host and protect against invading pathogens; disruption of the normal balance within the gut microbiome (also called dysbiosis) has been associated with NCDs development [31].

Many factors influence the shaping of gut microbiota in this critical window of plasticity, including gestational age, maternal pre-pregnancy Body Mass Index (BMI), weight gain during pregnancy, mode of birth, feeding types, weaning, birth environment, use of antibiotics, besides ethnic/geographical background [31] [33] (Fig. 4).

Among these factors, overweight and obesity are of great concern since the number of pregnant women affected by overweight or obesity has increased in high-income and middle-income countries [34]. These subjects are likely to face several complications such as gestational diabetes, hypertension, and delivery by cesarean section [35]. These undesired effects are, at least partly, modulated by the related changes in gut microbial composition during pregnancy and lactation [36] [37]. Such changes impact maternal and offspring health, altering host metabolic pathways and remodelling the expression of genes regulating them [38].

Another critical factor that influences the composition of the child microbiome is the type of delivery. During labor, the newborn first encounters a considerable quantity of different maternal microbes from the maternal reproductive tract, gut, and skin, in addition to those that came from the environment. Vaginally delivered infants, indeed, are exposed to maternal vaginal and fecal microbiota; hence facultative anaerobic species such as Escherichia coli, Staphylococcus, and Streptococcus colonize the infant's gut and create anaerobic environments in the first few days of life that allow strict anaerobes like Bacteroides and Bifidobacterium spp. to grow [39]. On the contrary, in infants born by caesarian delivery, typical mother-to-infant microbial transmission is disrupted. Human skin and oral bacteria dominate the infant's gut, including Staphylococcus, Streptococcus, Corynebacterium, increasing the incidence of irritable bowel syndrome, autoimmune/allergic diseases, asthma, type I diabetes, and obesity. Rather, vaginally delivered infants have higher bacterial diversity and richness and a higher prevalence of colonization with Bacteroides and Lactobacilli [40].

Changes in the maternal microbiome composition could also be vertically transferred to the newborn, not only at birth but also during lactation, promoting the inoculum of an altered microbial community that may have short- and long-term health consequences [31]. Feeding mode (formula or breastfeeding) seems to have a great impact on the gut microbial composition so that the World Health Organization (WHO) recommends that infants should be exclusively breastfed for the first 6 months of life [41]. Human milk is the best link between infant and mother. It has a complex and dynamic composition that is very different from formula-based products (e.g., nutritional value and composition), such as the presence

of specific bioactive compounds as human milk oligosaccharides (HMOs). HMOs are beneficial to infants as they not only help better development but also strengthen the immune system of the newborn, protect against allergies, and may also offer protection from coeliac disease, obesity, type-2-diabetes, diarrhea, and many other metabolic disorders [42]. Shreds of evidence suggest that HMOs play a significant role by stimulating the growth of Bifidobacteria and Bacteroides, modulating infant's health with a prebiotic effect. It is also demonstrated that human milk contains specific proteins and stimulation factors that enhance the growth of beneficial bacteria in the infant's gut, which further help in the breakdown of complex oligosaccharides present in human milk [42].

Later, with the introduction of solid food, the gut microbiome undergoes an important shift to an adult-type microbiome. As solid foods are included in the diet, the microbiota starts evolving from a simple Bifidobacteria-rich environment to a different one, rich in species such as Bacteroides, able to metabolize starches present in a more complex dietary pattern [38]. Even if there is no clear evidence of a clear association between the timing of food introduction and childhood overweight or obesity, some evidence suggests that its introduction at four months or earlier may increase the risk of childhood overweight [43]. Certainly, the composition, rather than timing of food introduction, has been more clearly related to later obesity risk; in particular, high intakes of energy and sugar and animal protein in infancy could be associated with an increase in body weight and fatness [43].

Further, the family context (biodiversity in the houses, in the neighbouring environment, and in family members with close and continuous contact with the baby), presence of older siblings, and hygienic practices can modify the gut microbiome composition of the newborn during the first weeks of life. A study reports that early-life exposure to older siblings is associated with a lower risk of asthma with a Bifidobacterium-dominant fecal microbiota profile [44]. Another research shows that the presence of older siblings may accelerate the Faecalibacterium gut colonization; this bacterial species is highly abundant in the microbe of healthy individuals, but it is present at reduced levels in individuals with gastrointestinal inflammatory diseases, so its presence is a marker of a healthy gut and is associated with anti-inflammatory properties [45].

Lastly, another essential perinatal factor that could alter an infant’s microbiome is the unnecessary use of antibiotics. Indeed, in developed countries, there is an intense and often inappropriate use of antibiotics, particularly during the first year of life; this excessive exposure is associated with significant differences in the gut microbiome, particularly in reduced abundance and diversity of fecal Bifidobacteria until two years of age [46]. Several

clinical studies have shown a direct correlation between the number of occurrences of the use of antibiotics and the nature of antibiotics used, and the development of asthma in adolescents [47]. These studies suggest that perinatal use of antibiotics can disrupt the normal colonization of the gut microbiome resulting in a loss of immunologic/metabolic. developmental homeostasis and expression of disease later in life [47].

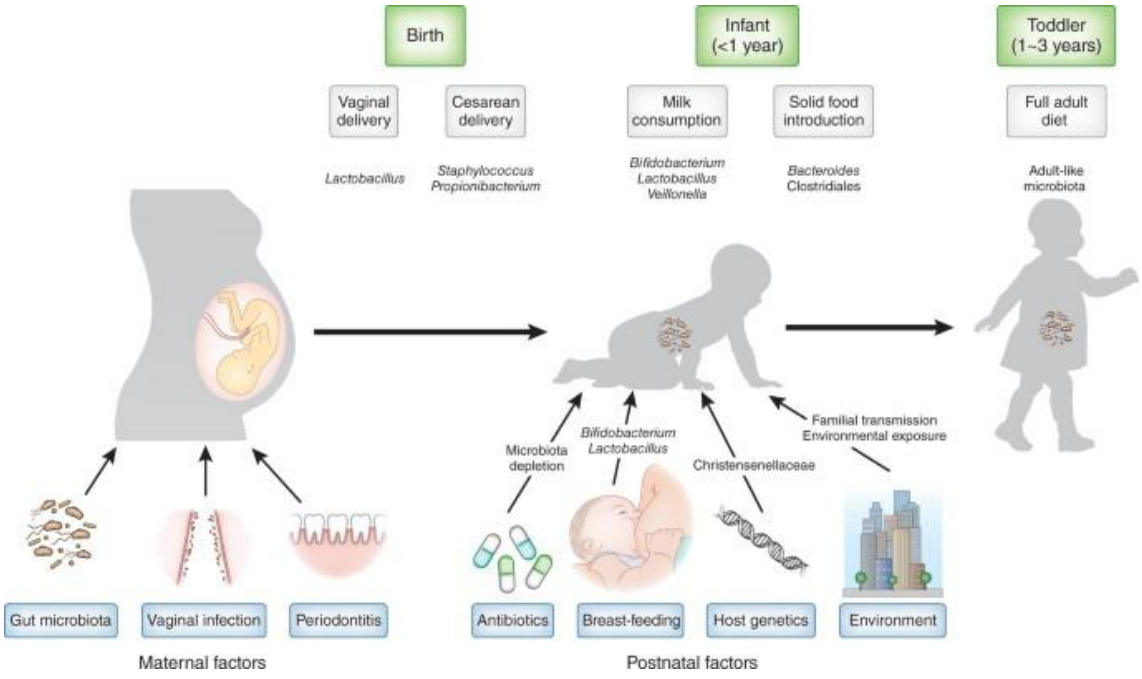


Figure 4. Factors that influence the development and the composition of the gut microbiome [48].

2. AIM OF THE STUDY

The A.MA.MI (Alimentazione Mamma e bambino nei primi Mille giorni) study aims to investigate the intestinal microbiome development of infants (ages 0-11.99 months) and to analyze how prenatal and postnatal factors, including maternal BMI and weight gain during pregnancy, type of feeding, time and type of weaning, and the presence of siblings in the family, could influence the infant gut microbiome composition at one year of age (Fig.5). This study was conducted at the laboratory of Dietetics and Clinical Nutrition at University of Pavia, Italy; it will provide the starting point for a future prospective and observational study assessing the potential association between maternal diet and lifestyle with newborns microbiome composition, their influence on childhood obesity development and the associated increased risk of CVD in adulthood.

2.1 Primary outcome

Investigate intestinal microbiome of the infant at birth (T0) and three follow-ups, one month after birth (T1), six months after birth (T2), and one year after birth (T3) to evaluate the relative abundance of the dominant microbes, to assess the microbial diversity and the inter-individual variation.

2.2 Secondary outcome

Investigate the possible correlation between infant intestinal microbiome and maternal lifestyle (maternal diet, physical activity, weight status gestational exposures), mode of delivery, feeding mode (formula or breastfeeding), family environment exposure, and infant diet.

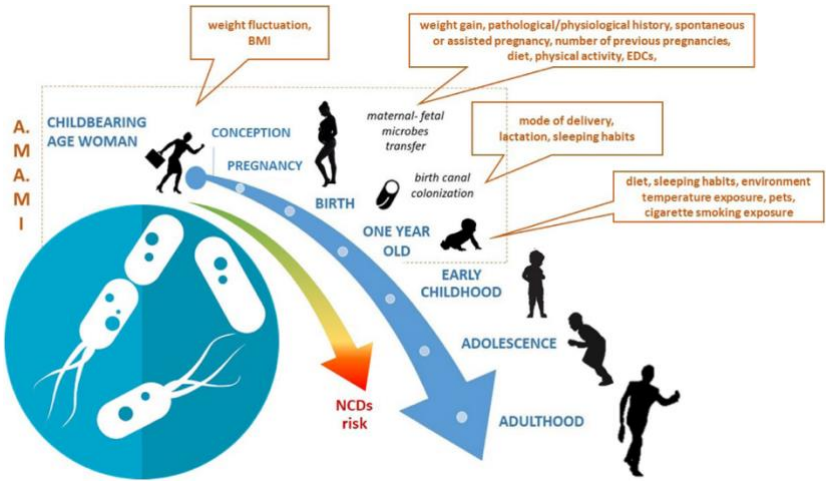


Figure 5. Planning of the A.MA.MI study [31]

3. MATERIALS AND METHODS

3.1 Design of the study

The present study A.MA.MI is a longitudinal prospective observational (ClinicalTrials.gov identifier: NCT04122612) that was conducted on a group of mother-infant pairs (dyads) to identify a possible correlation between maternal habits and infant intestinal microbiome during the first year of life. The study was authorized by the Human Ethics Committee of Fondazione IRCCS Policlinico S. Matteo of Pavia, and it was managed according to the Good Clinical Practice guidelines. The written informed consent of the parents/legal guardian was provided. The Human Ethics Committee of Fondazione IRCCS Policlinico S. Matteo of Pavia approved this procedure [31].

3.2 Participants

In that study, 63 dyads were recruited from the Neonatal Unit of Fondazione IRCCS Policlinico S. Matteo of Pavia, to complete the study with a minimum of 50 mother-infant pairs (mother/newborn), and according to the following inclusion criteria:

- Infants of both sexes born to regular or cesarean delivery
- Gestational age between 37 and 42 weeks
- Italian-speaking parents
- Ability of the parent/guardian to provide informed consent
- Ability of the mother to answer the structured interview/questionnaires.

The exclusion criteria were:

- Infants or mothers with genetic/congenital conditions
- Infants hospitalized in the neonatal intensive care unit directly after birth
- Infants elected for other clinical studies
- Presence of gestational diabetes
- Presence of hyperthyroidism during pregnancy.

During the mother's routine visit before the delivery, the parent/legal guardian was informed about the characteristics of the study and the chance of their active involvement. Then, they were asked to sign the informed consent.

Antibiotic intrapartum prophylaxis was conducted according to the American College of Obstetricians and Gynecologists (ACOG) guidelines 2019 [31] [49].

3.3 Questionnaires and interviews

After the recruitment of the participants, mothers were submitted to some questionnaires to investigate about [31]:

- Pathological/physiological history, type of current pregnancy (spontaneous or assisted pregnancy), number of previous pregnancies, and anthropometric parameters (height, weight pre-and post-pregnancy, Body Mass Index (BMI) pre-and post-pregnancy). Based on pre-gestational data as maternal height and pre-gestational weight was calculated body mass index and women were stratified as Normal Weight (NW - BMI ≤ 24.9 kg/m2) or Overweight/with Obesity (OW/OB - BMI ≥ 25 kg/m2). Instead, gestational weight gain (WG) was defined as body weight increase from pre-pregnancy to delivery and was compared with recommended WG ranges by IOM guidelines for each pregnancy category (NW, 11.5-16 kg; OW, 7-11.5 kg; OB, 5-9 kg) [31] [50].

- Dietary habits were evaluated using a previously validated questionnaire (Appendix 1) divided into two sections [51]: the Food Frequency section (FF) that evaluates the consumption of daily food and beverages such as pasta, bread, cereal products, fruits and vegetables, dairy product, meat and meat derivatives, legumes, fish, eggs and cheese and also alcoholic beverage, desserts and sweet. The Dietary Habit section (DH) examines daily and weekly food habits, frequency of meals, portions, water consumption, soft drinks, and alcoholic beverages. Each section is described by questions with the following categories: always, often, sometimes, never. Each response has attributed a score ranging from 0 to 3. According to the National Dietary Guidelines, the minimum score is assigned to the less healthy choice, and the maximum score is assigned to the healthiest one [31].

- Physical activity habits were assessed using the short version of a validated questionnaire on physical activity and sedentary behavior assessment (International Physical Activity Questionnaire, IPAQ) (Appendix 2) [52]. IPAQ is a tool tested and

developed for the adult population (age range of 15–69 years) to study the level of physical activity. The IPAQ short version (7 items) is structured to provide information about the time spent in vigorous, moderate-intensity activity, walking, and sedentary activity. The total score calculation is determined by the sum of the frequencies (in days) and duration (in minutes) of those activities; each activity is expressed by its energy requirements defined by METs (Metabolic Equivalent of Tasks) to produce a score in MET minutes.

MET values and Formula for computation of Met-minutes:

-Walking MET-minutes/week = 3.3 * walking minutes * walking days
-Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days
-Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days [52]

Thresholds of activities intensities:
Less than 3 MET: light intensity activity
3-6 MET: aerobic physical activity of moderate intensity
Over 6 MET: high intensity aerobic physical activity

The MET values adopted in this formula were obtained from the IPAQ validity and reliability study [52].

At T1 (one month after birth), T2 (six months after birth), and T3 (one year after birth), mothers were interviewed about [31]:

- Medical history of the child, environmental influences (how many people live in the house, presence of pets, temperature, socio-economic level of the family, infant's exposure to passive smoking), type of feeding (formula or breastfeeding), time and characteristics of weaning, infant's sleep duration, use of antibiotics or probiotics and use of supplements were evaluated both in mothers and children.
- Dietary habits using a previously validated questionnaire [51].
- Physical activity habits using a previously validated questionnaire [52].

Mothers were also interviewed at T3 to investigate infants' dietary habits at 1 year of age, using a previously validated questionnaire [53], adapted using the forward-back translation method [31] showed in Appendix 3.

The timeline of all data collection is shown in Fig. 6 [31].

3.4 Maternal and infant's anthropometric parameters

Maternal anthropometric parameters, such as height (cm) and weight (kg), were measured at each time from T0 to T3 using standardized procedures, and BMI (Kg/m2) was then calculated; waist circumference (WC) (cm) was measured from T1 to T3 [31] (Fig.6).
Bodyweight was measured with mothers wearing only their underwear and without shoes using a steelyard scale (precision ±100 g); body height was measured on mothers without shoes using a stadiometer (precision ±1 mm). Waist circumference was obtained using a flexible steel tape measure with participants standing, with crossed arms, placing the hands-on opposite shoulders. The measurement was performed after gently exhaling on the horizontal plane between the lowest portion of the rib cage and the uppermost lateral border of the right ilium [31] [54].
Each child was planned to be visited before discharge from the hospital (T0), at one month (T1), at six months (T2), and at one year after birth (T3) (Fig.6). Infant anthropometric parameters such as weight (kg), length (cm), and head circumference (cm) were also measured (T0-T3) with standardized procedures [31] [54].
Bodyweight was measured with naked infants using a steelyard scale (precision ±100 g). The length was obtained by laying infants on an infantometer with the feet toward the mobile portion of the instrument and the head against the fixed portion, making sure that the head was lying in the Frankfort horizontal plane. If it was impossible to reach both legs stretched in the correct position, the examiner ensured that at least one leg was straight with the foot flexed against the mobile portion. The head circumference was measured using a non-stretch measuring tape which placed around the infant's head with the tape adherent to the frontal bones of the skull slightly above the eyebrows, perpendicular to the long axis of the face, above the ears, and over the occipital prominence at the back of the head. According to standard assessment procedure, the tape was moved up and down over the back of the head to locate the maximum circumference and tightening the insertion tape to fit it perfectly around the head and compress the hair and underlying soft tissue slightly [31] [54].

3.5 Sample collection

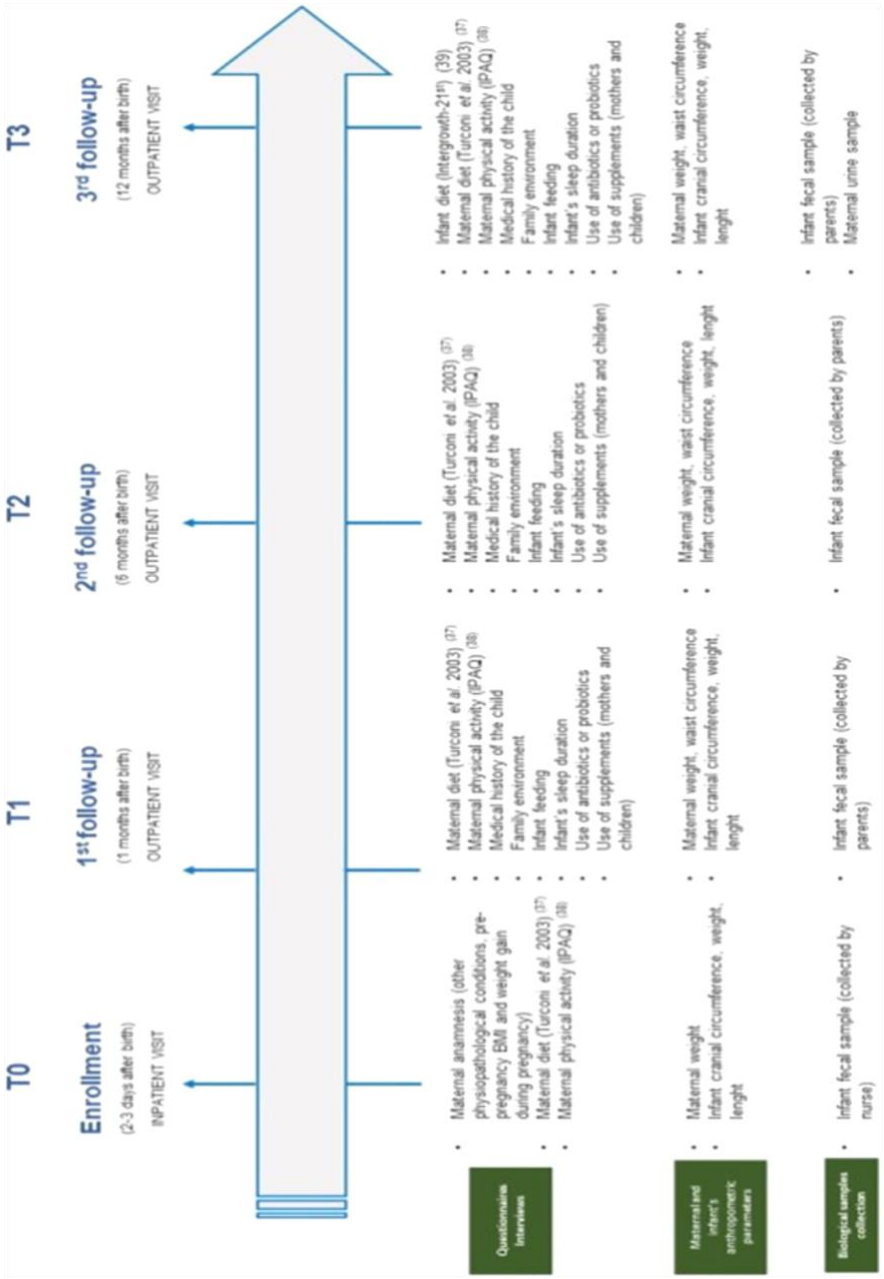
Each infant stool sample (T0 – T3) was collected into two different screw-top containers and labelled with the newborn's unique study ID number (Fig.6). One sample was stored at − 80 °C at Neonatal Intensive Care Unit, IRCCS Policlinico San Matteo Foundation (Pavia, Italy), and the other one was stored at − 80 °C at the Institute of Human Anatomy, Department of Public Health Experimental and Forensic Medicine, School of Medicine, University of Pavia (Pavia, Italy), until analyzed. At T0, a trained nurse collected a stool sample of the newborn, and at the end of the visit, a member of the research team scheduled the second visit with the child's parents/legal guardian (1month from the birth, T1) (Fig.6), and gave them instructions for the collection of the second stool sample independently at home [31].

The infants' parents were contacted by a research team member 1–2 days before the scheduled visit at T1 to check the health of the newborn. They were asked to submit a stool sample in a second moment if:

- the child was currently ill with fever, respiratory illness, or gastrointestinal illness
- the child had a diarrheal illness lasting more than 24 h in the past 7 days
- the child has been treated with antibiotics in the past 7 days.

The stool sample collection at T2 and T3 followed the same procedures [31] (Fig.6).

Fig.6: Timeline and data collection [31].



3.6 16S rRNA Metagenomic Sequencing Library Preparation, Gene Amplicon Sequencing and Analysis

At the end of each recruitment, samples were shipped on dry ice to Genomix 4 Life Srl (C/O Laboratory of Molecular and Genomic Medicine - Campus of Medicine and Surgery, Baronissi, Salerno, Italy, spin-off of the University of Salerno, Fisciano, Italy), where 16S metagenetic analysis was carried out. Samples shipped had only the study ID number, and no clinical or personal information was included. Next-generation sequencing (NGS) analyses, comprising DNA extraction and primary bioinformatics investigation, were conducted by Genomix4life S.R.L. (Baronissi, Salerno, Italy). DNA extraction was performed with Invimag Stool kit (Stratec) utilizing a negative extraction control. The final yield and quality of extracted DNA were determined using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and Qubit Fluorometer 1.0 (Invitrogen Co., Carlsbad, CA). 16S rRNA gene amplification was performed with primers: Forward: 5′-CCTACGGGNGGCWGCAG-3′ and Reverse: 5′-GACTACHVGGGTATCTAATCC-3′ [55], which target the hypervariable V3 and V4 regions of the 16S rRNA gene. Each PCR reaction was assembled according to Metagenomic Sequencing Library Preparation (Illumina, San Diego, CA). A negative control is included in the workflow; it consists of all reagents used during sample processing (16S amplification and library preparation) but does not contain a sample to assess potential contamination. Libraries were quantified using a Qubit fluorometer (Invitrogen Co., Carlsbad, CA) and pooled to an equimolar amount of each index-tagged sample to a final concentration of 4 nM, including the Phix Control Library. Pooled samples were subject to cluster generation and sequenced on the MiSeq platform (Illumina, San Diego, CA) in a 2 × 300 paired-end format [31]. The raw sequence files generated (fast files) underwent quality control analysis with FastQC. The 16S metagenomics analysis performs the taxonomic classification of 16S rRNA targeted amplicon reads after OTU clustering based on the 97% similarity (3% of divergence) [31]. The algorithm is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang et al., 2007 [56] Taxonomic databases used to perform taxonomic classification are RefSeq RDP 16S v3 May 2018 DADA2 32bp. The obtained sequences were uploaded to a public database, and the following extremes refer to the submission to NCBI database (http://www.ncbi.nlm.nih.gov/bioproject/675753, extremes: Submission-ID: SUB8491261; BioProject ID: PRJNA675753) [31].

3.7 Statistical analysis

3.7.1 Power analysis

The A.MA.MI study was initially formulated on a relatively small scale (at least 60 dyads) to achieve the study with at least 50 mother-infant pairs and provide the initial feasibility of the study protocol. The aim is to investigate the microbiome variability in infants and how maternal and infant factors reasonably relate to microbiome development. The information obtained will also be used to establish better the sample size of a more extensive study to confirm the results of the former study. To reduce losses during follow-up, researchers planned to keep participants constantly notified and remind them of all the follow-up activities throughout phone calls [31].

3.7.2 Statistical analysis

Data are summarized using descriptive statistics, such as means and standard deviations or median and interquartile range (IQR) for quantitative variables and relative frequencies for qualitative ones. Principal component analysis (PCA) and Spearman correlation have been used to evaluate differences in the microbial composition over multiple follow-up times; to assess the association of the intestinal microbiome composition with the prenatal and postnatal determinants previously described, t-tests/ANOVA or Mann-Whitney/Kruskal-Wallis has been chosen. Diversity indexes and the relative abundances of different phyla and genera have been compared across groups described by presence/absence or different categories of the prenatal or postpartum specific factor. To obtain the characteristic of excess zeros at lower taxonomic levels and model the confusing abundance of bacteria data, a zero-inflated model has also been performed and results compared. Friedman tests or Repeated-measure ANOVA have been applied, with adjustment for multiple comparisons, to determine the relationship between the intestinal microbiome development and the condition of adverse growth parameters. A posthoc multilevel mixed-effects linear regression test has been performed in the case of group-time interaction. Finally, to define the relationship between intestinal microbiome composition and development and prenatal or neonatal factors, generalized linear models (GLM) have been performed. All the tests are two-tailed; statistically significant levels are set at 0.05. All analyses have been conducted using STATA 15 [31].

At T2, a multivariable association between 16S rRNA gene data abundances at different taxonomic levels in infants' microbiota was conducted using the MaAsLin2 R package

(https://huttenhower.sph.harvard.edu/maaslin/). Meantime, data and group differences were investigated and analyzed by paired or unpaired two-tailed Student's t-test. PCA analysis was utilized to assess the beta diversity occurring within our population to evaluate variations in the microbial composition [T0, vs.T2]. The dudi.pca function within the "ade4" R package (https://cran.r-project.org/web/packages/ade4/) was applied to perform a PCA of data frames. The resulting PCA and dudi class objects were plotted with the "factoextra" R package (https://cran.r-project.org/web/packages/factoextra/index.html).

At T3, data were summed using descriptive statistics (mean, median, ± SD or IQR), as appropriate for quantitative variables and relative frequencies for qualitative ones. Alpha diversity indices were analyzed according to a two-tailed Mann-Whitney's test computing the exact p-values (corrected for multiple comparisons) using GraphPad Prism version 8.0, GraphPad Software, San Diego, California, USA. Multivariable association between 16S rDNA-seq data abundances and variables (metadata) was conducted using the MaAsLin2 R package (https://huttenhower.sph.harvard.edu/maaslin/), which allowed the investigation metadata as fixed or random effects. The principal component analysis (PCA) was applied to assess the beta diversity after subgrouping the cohort based on the time and duration of breastfeeding. The dudi.pca function within the "ade4" R package (https://cran.r-project.org/web/packages/ade4/) was adopted to perform a PCA of data frames. The resulting PCA and dudi class objects were plotted with the "factoextra" R package (https://cran.r-project.org/web/packages/factoextra/index.html).

5. RESULTS

The enrolment started in October 2018 then data and samples at T0, T1, T2 and T3 were collected.

5.1 Dyads general characteristics at recruitment

A total of 63 dyads, attending the Neonatal Unit, IRCCS Policlinico San Matteo, Pavia (Italy) were recruited. General characteristics of the dyads at recruitment are presented in Table 1 in Appendix 3.

Thirty-six women (48.1%) enrolled were at their first pregnancy. The type of conception was spontaneous in most women 96.8%, (n = 60), and only 3.2% (n = 2) underwent assisted reproductive technologies (ART). The delivery mode was spontaneous vaginal delivery in 85.5% of women (n = 53). 58.6% of infants (n = 34) were breast-fed while 41.1% (n = 24) were formula-fed [31].

4.2 Infant microbiome analysis at T0-T1

Of 118 neonatal stool samples, the average reads that passed filter quality were 106,298.81 ± 69,276.42 sequences. Bacterial communities were analyzed, estimating alpha diversity (number of identified species OTUs) and Shannon diversity index [31].

4.2.1 Comparison of newborns vs 1-month-old infants

Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Cyanobacteria, and Verrucomicrobia described more than 98% of 16S rRNA gene sequences in all samples. Phyla with relative abundance < 0.1% were grouped in the "others" category. The relative abundance of Firmicutes was increased at T1 when compared to T0 (T0: 19.71%, T1: 27.52%; $p = 0.047$), associated with a reduction of Proteobacteria (T0: 46.23% vs. T1: 33.56%; $p = 0.008$) and Fusobacteria (T0: 1.65% vs. T1: 0.02; $p = 0.000$) (Fig. 7a) [31]. At T1, at genus and species levels, a distinct drop of genera and species of Fusobacteria was found. Among Firmicutes, Blautia, Lactobacillus species as L. gasseri and L. taiwanensis, Ruminococcus (R. gnavus) and Veillonella (i.e. V. atypica, V. criceti, and V. dispar) revealed a marked increment at T1 ($p < 0.035$) [31]. Contrarily, Streptococcus spp. showed distinct trends among the species; indeed, some species suche as S. infantis, S. intermedius, S. sanguinis and S. tigurinus decreased ($p < 0.009$); S. fryi and S. vestibularis rised 30 days after birth (T1) ($p < 0.046$) [31]. Furthermore, some genera of the Actinobacteria decreased matching T1 vs. T0 ($p < 0.041$). On the other hand, Collinsella (T0: 0.05%, T1: 0.56%; $p = 0.047$) and particularly Bifidobacterium, both genera of Actinobacteria, showed a significant rise (T0: 1.93%, T1: 12.39%; $p = 0.000$) after one month of age [31]. On the opposite, only Trabulsiella (Enterobacteriaceae) and Sutterella(Sutterellaceae), both genera of Proteobacteria, were higher after 30 days compared to t0 ($p < 0.048$). No notable increase was seen among genera of Bacteroidetes, whereas Capnocytophaga, Chryseobacterium, Porphyromonas, Prevotella, and Tannerella were lower at T1 than T0 ($p < 0.011$) [31].

4.2.2 Comparison of delivery mode at T0: vaginal delivery vs cesarean section

At the phylum level, no statistical differences were observed between the meconium samples from those neonates born via cesarean section (CS) vs. those born with vaginal delivery (VD) (Fig. 7b) [31]. The principal differences seen between the two groups were at genus and species levels. Many genera and species of Firmicutes (including Lactobacillaceae, particularly Lactobacillus and Pediococcus, Bacillus, Selenomonas, and Staphylococcus), were higher in neonates born via CS than VD (VD: 0.43%, CS: 9.41%; $p = 0.000$). Moreover, species of Streptococcus (S. anginosus, S. intermedius, S. oralis, and S. tigurinus), Veillonella (V. atypica and V. denticariosi) and Clostridium (i.e. C. histolyticum, C. neonatale, and C. paraputrificum) were discovered to be higher in the CS-group ($p < 0.035$).

Among Actinobacteria and Proteobacteria, the OTUs that exhibited statistical differences ($p < 0.05$) according to the delivery mode were all higher in the CS group [31].

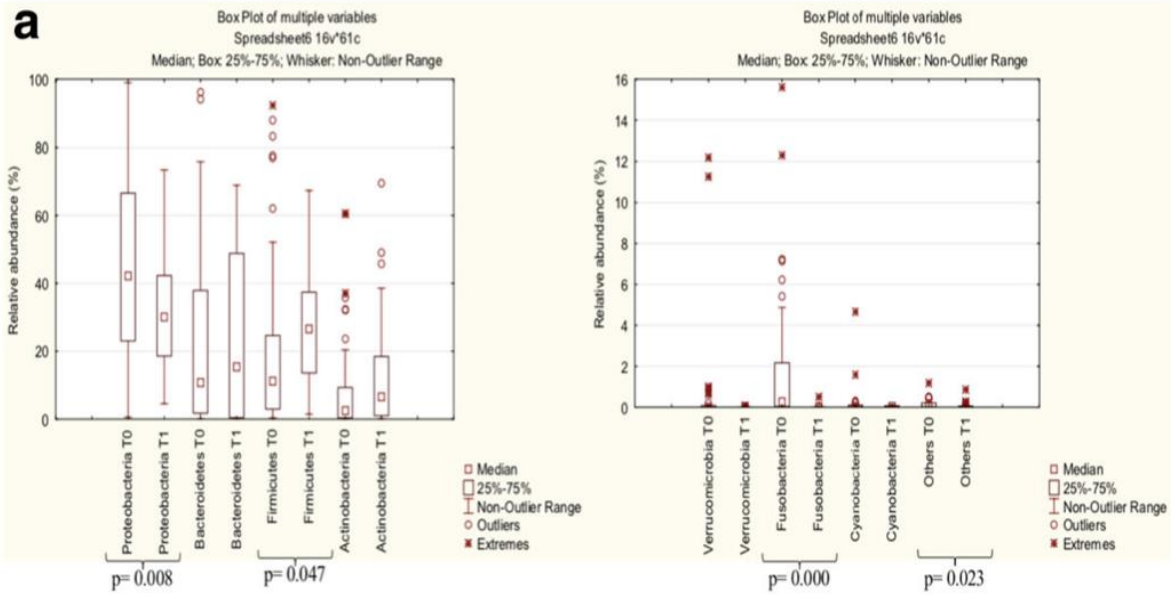
4.2.3 Comparison of adequate maternal pre-pregnancy BMI vs high maternal pre-pregnancy BMI at T0

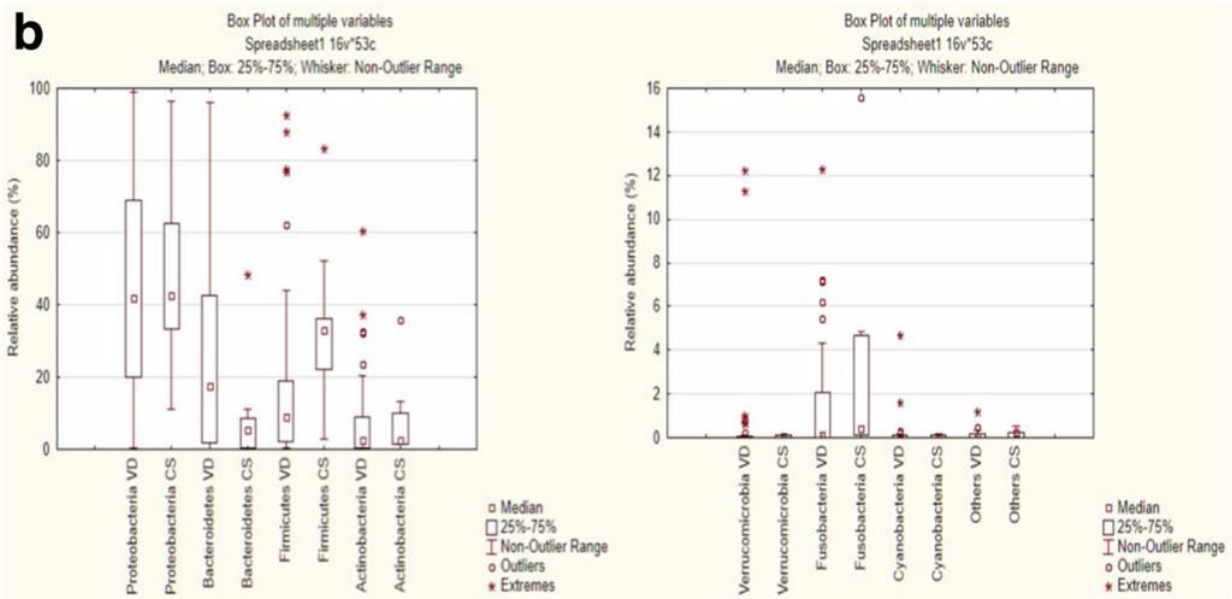
No significant differences were observed at the phylum level (Fig. 7c) [31], comparing samples from neonates born from mothers with pre-pregnancy normal body weight (BMI < 25 Kg/m2) vs. those born from mothers with overweight or obesity (BMI ≥25 Kg/m2) [31]. Otherwise, at the genus level, neonates born from mothers with BMI ≥ 25 Kg/m2 showed higher abundances of Streptococcus ($p = 0.026$), Propionibacterium, Actinomyces (Actinobacteria; $p < 0.04$), and Kingellaand Hylemonella (Proteobacteria; $p < 0.045$). At the species level, Alkaliphilus peptidifermentans was higher in neonates born to mothers with overweight/obesity than those born to mothers with normal weight (0.78 and 0.13%, respectively; $p = 0.016$) [31]; Then, inside Klebsiella, three species (K. variicola, K. granulomatis, and K. pneumoniae) were significantly more abundant in meconium of neonates born to mothers with overweight/obesity than those born to mothers with normal weight ($p < 0.023$) [31].

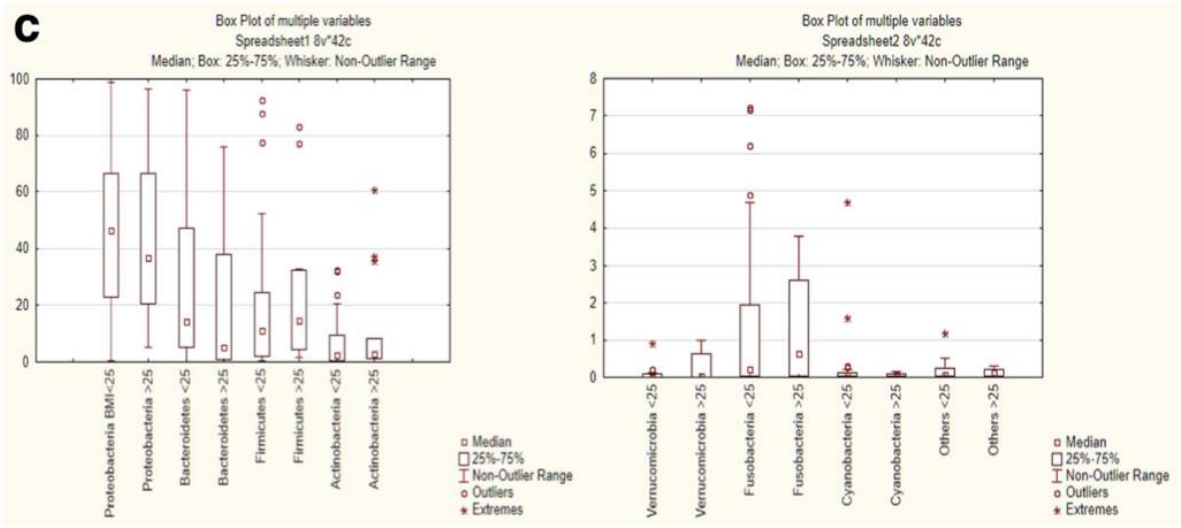
4.2.4 Comparison of breastfeeding vs formula feeding at T1

No statistical differences were found at the phylum level within the T1-fecal samples when comparing formula-fed newborns (FF) with breastfed newborns (BF) (Fig. 7d) [31]. At the species level, two species of Bacteroides (B. rodentium and B. uniformis) and two of Parabacteroides (P. goldsteinii and P. merdae) were seen to be higher in FF than BF ($p < 0.042$) [31]. Likewise, amongst Firmicutes, Butyrivibrio proteoclasticus, Enterococcus casseliflavus and E. gallinarum, Veillonella atypica and V. montpellierensis were higher in FF ($p < 0.05$) [31]. Among Proteobacteria, only Citrobacter werkmanii and Enterobacter aerogenes differed between the two-sampled groups ($p < 0.05$). No statistical differences were found among species of Actinobacteria and minor phyla [31].

Fig.7: Relative abundance (%) of total bacteria (16S rRNA) at the phylum level. Phyla with relative abundance < 0.1% in all samples were grouped in "Others". Box plots showed: a) total bacteria found at T0 (meconium) and T1 (1 month of age); b) total bacteria found in the meconium of neonates born via cesarean section (CS) or vaginal delivery (VD); c) total bacteria found in the meconium of neonates born to normal weight (BMI < 25 Kg/m^2) or mothers with ove rweight or obesity (BMI ≥25 Kg/m^2) and d) total bacteria fond in T1 samples of neonates fed with formula (FF) or breastfeeding (BF). (*p = p-value) [31].



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Box Plot of multiple variables
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Median; Box: 25%-75%; Whisker: Non-Outlier Range
Proteobacteria BMI<25
Proteobacteria >25
Bacteroidetes <25
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Firmicutes <25
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Actinobacteria <25
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Outliers
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Box Plot of multiple variables
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Median; Box: 25%-75%; Whisker: Non-Outlier Range
Verrucomicrobia <25
Verrucomicrobia >25
Fusobacteria <25
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Cyanobacteria <25
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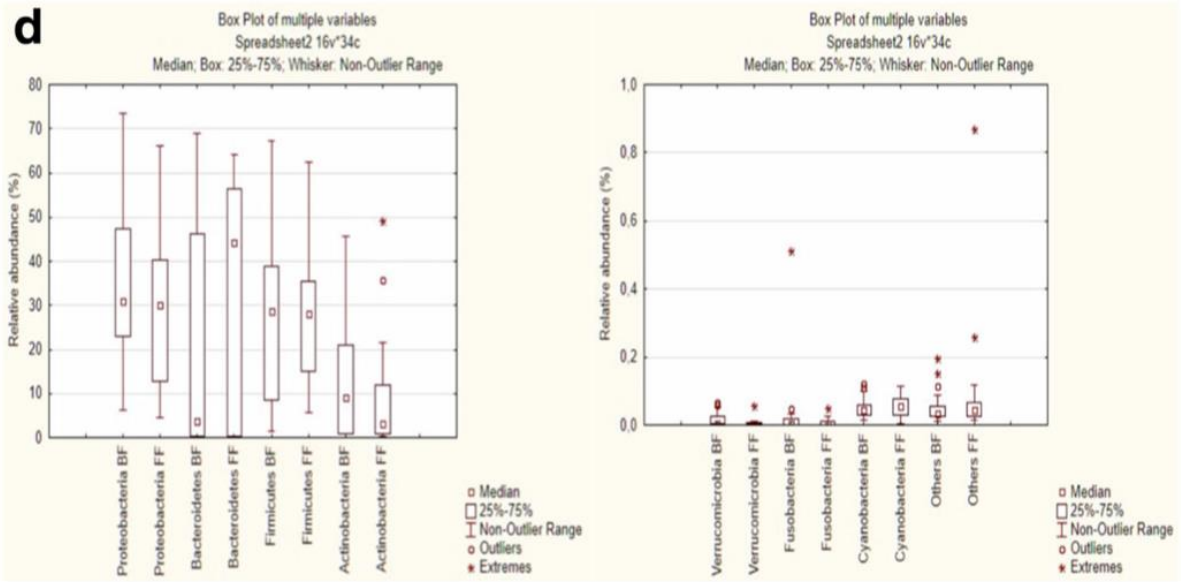
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Box Plot of multiple variables
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Median; Box: 25%-75%; Whisker: Non-Outlier Range
Relative abundance (%)
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Proteobacteria FF
Bacteroidetes BF
Bacteroidetes FF
Firmicutes BF
Firmicutes FF
Actinobacteria BF
Actinobacteria FF
Median
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Outliers
Extremes
Box Plot of multiple variables
Spreadsheet2 16v*34c
Median; Box: 25%-75%; Whisker: Non-Outlier Range
Relative abundance (%)
Verrucomicrobia BF
Verrucomicrobia FF
Fusobacteria BF
Fusobacteria FF
Cyanobacteria BF
Cyanobacteria FF
Others BF
Others FF
Median
25%-75%
Non-Outlier Range
Outliers
Extremes

4.3 Infant microbiome analysis at T2

4.3.1 Dyads general characteristics at T2

At T2, a total of 10 dyads dropped out. General characteristics of the dyads are presented in Table 2 in Appendix 4.

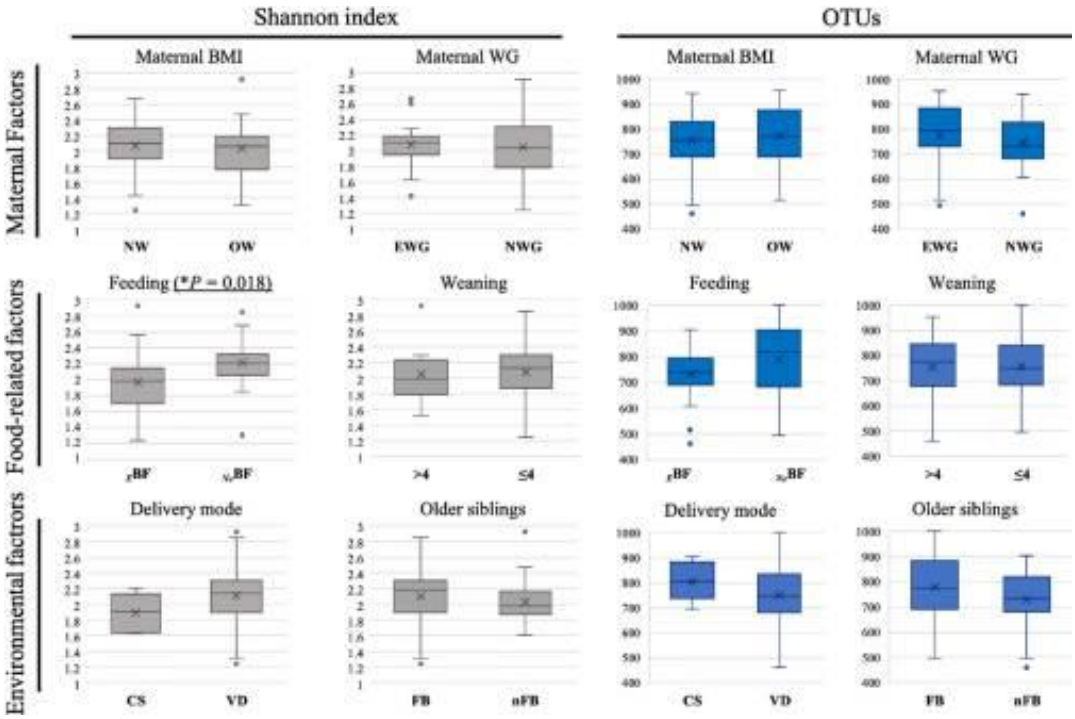
4.3.2. Microbiome analysis at T2

To investigate which factors impact the early microbiota colonization of infants in the first six months of life, samples were grouped based on

- prenatal maternal factors [pre-pregnancy body mass index (BMI) and gestational weight gain (WG)]
- perinatal factor (type of delivery)
- postnatal factor (diet-related factors such as type of feeding and weaning and presence/absence of older siblings in the household) [38].

Alpha diversity, assessed using the Shannon index and the number of operational taxonomic units (OTUs), was determined according to the different factors mentioned above (Fig. 8) [31]. We observed that exclusively breastfed babies ($_{E}$BF), when compared to not exclusively breastfed ones ($_{Ne}$BF), showed lower Shannon index values ($P = 0.018$). Any other of the studied factors determined significant differences in alpha diversity [38].

Fig.8: Box plots of the alpha diversity (Shannon index and number of operational taxonomic units (OTUs) identified) among T2 fecal samples (infants at 6 months of age) grouped according to different factors influencing early microbiota composition. Maternal factors: maternal pre-pregnancy body mass index (BMI) and gestational weight gain (WG). NW [n.37]: normal pre-pregnancy BMI (BMI < 25 kg/m2), OW [n.12]: excessive pre-pregnancy BMI (BMI ≥ 25 kg/m2); EWG [n.20]: excessive gestational WG, NWG [n.29]: optimal gestational WG). Food-related factors: Feeding (EBF [n.31]: exclusive breast-feeding, NeBF [n.21]: not exclusively breast fed, mixed fed or exclusively formula fed) and Weaning (≤4 [n.41]: before 4 months of age, >4 [n.9]: after 4 months of age). Environmental factors: Delivery mode (CS [n.7]: cesarean section, VD [n.46]: vaginal delivery) and presence of Older siblings (FB [n.30]: first-born, nFB [n.22]: not first-born). *P = p-value [38].



4.3.3 Factors Affecting Early Microbial Colonization of Infant Gut Microbiota

Maternal pre-pregnancy BMI within suggested rates (BMI < 25 kg/m2) or higher (BMI ≥ 25 kg/m2) and gestational WG, optimal or excessive according to IOM reference values [49], were used to assess if maternal weight could influence an infant's gut microbiota structure till six months of age. At the phylum level, we didn't find significant differences both for BMI and gestational WG (Figs 9i, ii) [38]. Furthermore, no significant difference was observed at the deeper taxonomic levels, particularly family and genus.

When considering delivery mode (VD vs. CS), an increased amount of Bacteroidetes in VD was detected (P = 0.021; Table 3, Fig 9iii) [38]. Despite this result at the phylum level, no other statistical difference at deeper levels was detected [38].

Assessing the type of feeding, EBF or NeBF (mixed or exclusively formula-fed), no differences were found at the phylum level (Fig.9iv) [38]]. In particular, the family of Ruminococcaceae strongly correlated with the NeBF group (P < 0.0001, qvalue = 0.011; Table 3) [38].

Moreover, Ruminococcaceae subclusters were more abundant in FF, especially Flavonifractor and the Clostridium cluster IV of Firmicutes. Also, other genera of Firmicutes mainly characterized the microbiota of FF infants, specifically Faecalicoccus (and other taxa of Erysipelotrichaceae Incertae Sedis), Romboutsia, and Oribacterium (P < 0.005). On the other hand, Staphylococcus was higher in EBF than in NeBF (P= 0.003; Table 3) [38].

Concerning the introduction of solid food in the diet, we observed that the weaning before 4 months of age (≤4), at least at two months of age (≤4), was not sufficient to define significant shifts in the microbiota composition comparing to infants weaned after the 4th month of age (Fig. 9v) [38].

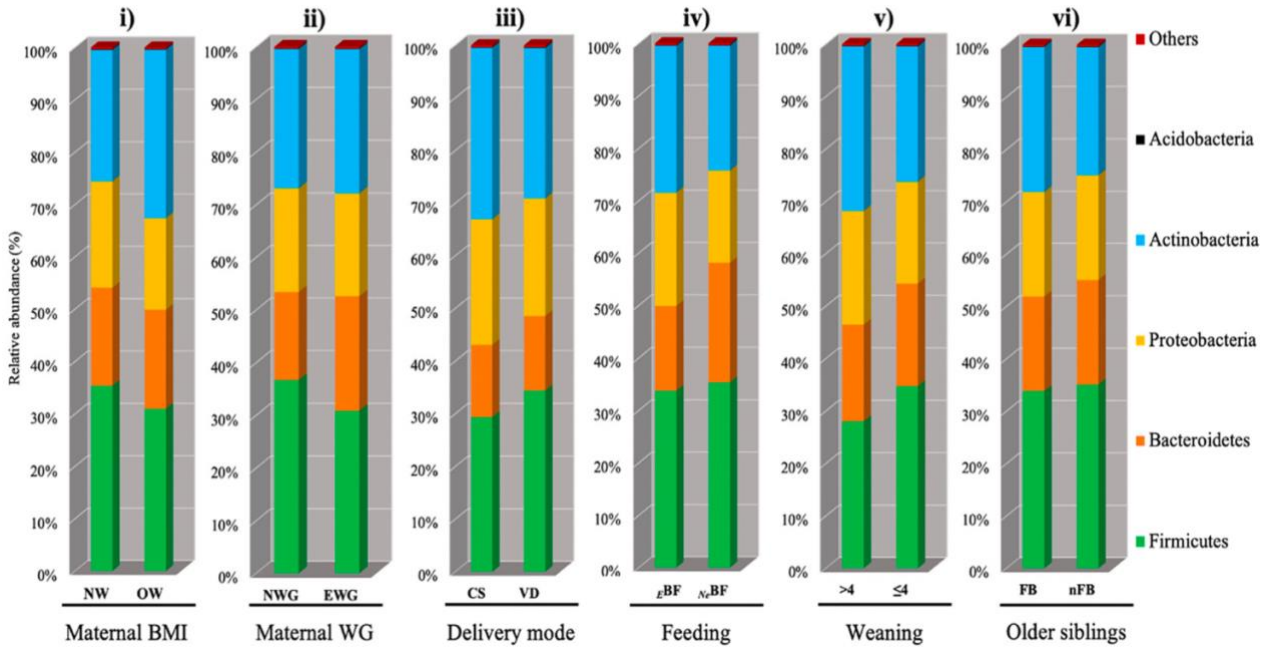
Among family factors, the presence of older siblings in the household did not reveal differences at the phylum level (Fig. 9vi) [38]. Despite this, firstborn babies (FBs) showed a higher abundance of Clostridiaceae, Clostridiales Incertae Sedis XIII, and Peptostreptococcaceae (P<0.003) when compared to infants with older siblings (nFBs) [38]. These results were not confirmed when p values were adjusted for multiple comparison. (Table 3).

Analyzing metadata (maternal BMI, gestational WG, type of delivery, feeding, weaning, and the presence of older siblings) as fixed effects in a regression model to evaluate the significant multivariable association among all infants, MaAsLin2 software using family and genus relative abundances was run. Considering that prenatal factors (both maternal BMI and gestational WG) did not show any significant difference, we developed a statistical analysis with fixed metadata only for perinatal and postnatal factors (mode of delivery, type of feeding, weaning, and the presence of older siblings).

Within families, Ruminococcaeae was confirmed to positively correlate with NeBF babies (Fig.10) [38]. Further, five families were negatively associated with VD infants: Cellulomonadaceae, Corynebacteriaceae, Actinomycetaceae, Streptomycetaceae, and Micromonosporaceae (Fig.10) [38]; Colwelliaceae was negatively associated with babies weaned before 4 months of age, while Staphylococcaceae and Clostridiales Incertae Sedis XIII were negatively related with nFB gut microbiota (Fig.10) [38]. However, all the results did not confirm the significance for adjusted p-values ($P < 0.05$; qval > 0.05) [38].

Concerning genera, in NeBF samples (Fig.11) [38], Flavonifractor showed a similar trend to Ruminococcaceae. Furthermore, three genera were negatively associated with VD infants (Corynebacterium, Propionibacterium, and Streptacidiphilus), while Staphylococcus was negatively associated with nFB. As reported in family results, also at the genus level, significance was not verified for adjusted p-values ($P < 0.05$; qval > 0.05) [38].

Fig.9: Influence of different factors on early microbiota composition at the phylum level (16S rRNA gene amplicon sequencing) in T2 fecal samples (infants at 6 months of age). Starting from the left: i) and ii) Maternal factors: maternal pre-pregnancy body mass index (BMI) and gestational weight gain (WG), respectively. NW [n.37]: normal pre-pregnancy BMI (BMI < 25 kg/m^2), OW [n.12]: excessive pre-pregnancy BMI (BMI ≥ 25 kg/m^2); EWG [n.20]: excessive gestational WG, NWG [n.29]: optimal gestational WG). iii) Delivery mode (CS [n.7]: caesarean section, VD [n.46]: vaginal delivery). iv) Feeding (EBF [n.31]: exclusive breast-feeding, NeBF [n.21]: not exclusively breast fed, mixed fed or exclusively formula fed). v) Weaning (≤4 [n.41]: before 4 months of age, >4 [n.9: after 4 months of age). vi) Presence of older siblings (FB [n.30]: first-born, nFB [n.22]: not first-born) [38].



Tab.3: Statistically different phyla, families, and genera (16S rRNA gene amplicon sequencing) found in fecal samples of infants at 6 months of age (T2) [38].

Taxon	metadata	feature	value	coef	Stderr	N	pval	qval	[factor] IQR (median)	[factor] IQR (median)
									[CS]	**[VD]**
Phylum	**Delivery**	Bacteroidetes	VD	0.85	0.36	53	0.021	0.209	0.38–0.87 (0.45)	0.44–37.1 (14.86)
									(FB)	**(nFB)**
Family	**Firstborn**	*Clostridiales Incertae Sedis XIII*	nFB	−0.12	0.03	53	0.001	0.108	0.01–0.01 (0.01)	0.00–0.01 (0.01)
	Firstborn	*Clostridiaceae*	nFB	−0.50	0.16	53	0.003	0.130	0.31–4.06 (0.81)	0.19–0.55 (0.23)
	Firstborn	*Peptostreptococcaceae*	nFB	−0.62	0.19	53	0.002	0.130	0.04–0.88 (0.32)	0.02–0.08 (0.04)
	Firstborn	*Planctomycetaceae*	nFB	0.10	0.03	53	0.003	0.130	*ND**	0.00–0.01 (0.00)
									[$_E$BF]	**[$_{Ne}$BF]**
	Feeding	*Ruminococcaceae*	**$_{Ne}$BF**	0.63	0.15	53	<0.0001	0.011	0.12–0.22 (0.15)	0.19–2.19 (1.41)
Genus	**Delivery**	*Propionibacterium*	VD	−0.22	0.05	53	<0.001	0.031	**[CS]**	**[VD]**
									0.00–0.01 (0.00)	*ND**
	Delivery	*Thiomicrospira*	VD	−0.15	0.04	53	<0.001	0.031	0.00–0.01 (0.01)	*ND**
	Delivery	*Streptacidiphilus*	VD	−0.23	0.07	53	0.002	0.210	0.00–0.02 (0.01)	0.00–0.01 (0.00)
									[$_E$BF]	**[$_{Ne}$BF]**
	Feeding	*Flavonifractor*	**$_{Ne}$BF**	0.99	0.25	53	<0.001	0.112	0.00–0.01 (0.00)	0.01–1.25 (0.06)
	Feeding	*Erysipelotrichaceae Incertae Sedis*	**$_{Ne}$BF**	0.79	0.25	53	0.003	0.218	0.01–0.01 (0.01)	0.01-1.24 (0.09)
	Feeding	*Romboutsia*	**$_{Ne}$BF**	0.59	0.18	53	0.002	0.218	0.00–0.01 (0.00)	0.00–0.22 (0.02)
	Feeding	*Staphylococcus*	**$_{Ne}$BF**	−0.51	0.17	53	0.003	0.218	0.01–0.15 (0.02)	0.00–0.01 (0.01)
	Feeding	*Faecalicoccus*	**$_{Ne}$BF**	0.21	0.06	53	0.002	0.218	*ND**	0.00–0.01 (0.00)
	Feeding	*Sulfurimonas*	**$_{Ne}$BF**	0.15	0.05	53	0.003	0.218	0.00–0.01 (0.00)	0.01–0.01 (0.01)
	Feeding	*Clostridium_IV*	**$_{Ne}$BF**	0.47	0.16	53	0.005	0.232	0.01–0.04 (0.01)	0.02–0.11 (0.06)
	Feeding	*Oribacterium*	**$_{Ne}$BF**	0.22	0.08	53	0.005	0.232	0.01–0.02 (0.01)	0.02–0.03 (0.02)

*VD, vaginal delivery; CS, caesarean section; $_E$BF, exclusive breastfed; $_{Ne}$BF, combined or exclusive formula-feeding; FB, first-born; nFB, not first-born; IQR, interquartile range (25th–75th percentile). *ND: not detected within all samples belonging to the considered group.*

Fig.10: MaAsLin2 significant results and associations between postnatal factors (delivery, feeding, weaning, and presence of older siblings) and gut microbiota composition at the family level of infants at six months of age (T2). Based on normalized obtained significant results, the color scale-bar showed a positive relationship (red) and a negative one (blue) between taxa and factors, ranging from the highest positive normalization (+3) to the lowest one (−3) [38].

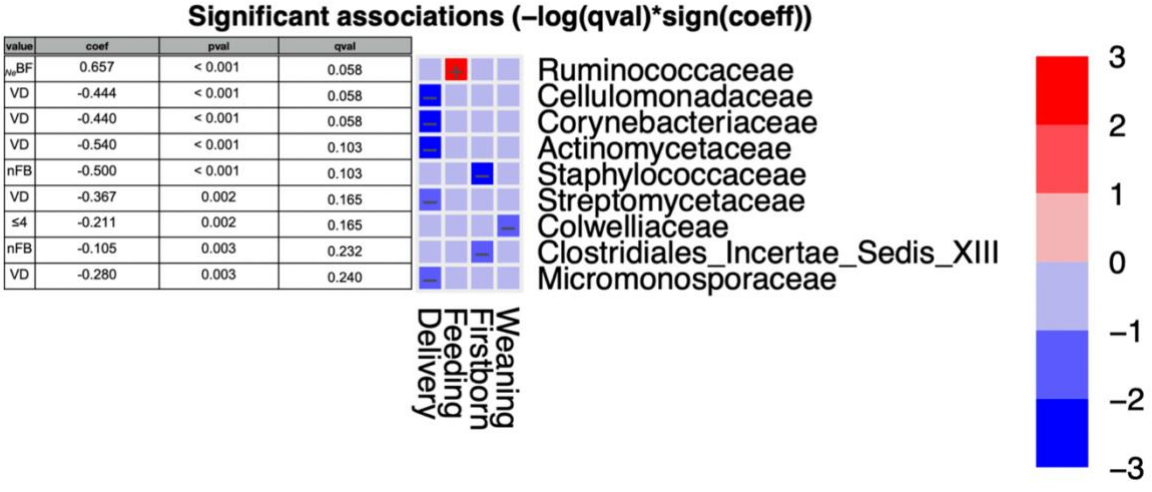
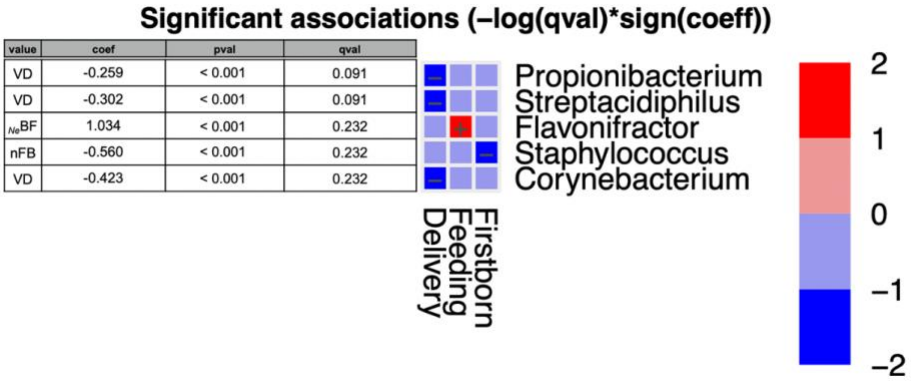


Fig.11: MaAsLin2 significant results and associations between postnatal factors (delivery, feeding, weaning, and presence of older siblings) and gut microbiota composition at the genus level of infants at six months of age (T2). Based on normalized obtained significant results, the colour scale-bar showed a positive relationship (red) and a negative one (blue) between taxa and factors, ranging from the highest positive normalization (+2) to the lowest one (−2) [38].



4.3.4 Multivariate Analyses

To compare infant microbiota composition from birth to six months of age, we conducted a multivariate analysis (PCA) between T2 and baseline T0 samples. We examined pre-pregnancy BMI (Fig.12A) [38], gestational WG (Fig.12B) [38], and delivery mode (Fig.12 C), using genera with a median relative abundance greater than 0.1%, at least in one of the sampled times, as variables [38]. In all of the three studied conditions, as shown by the PCA group ellipses, time of sampling seems to impact more on sample stratification than on related metadata (BMI, WG, and delivery). Among the variables, Fusobacterium, Leptotrichia, Mycobacterium, Serratia, and Rothia had a deeper impact on T0-sample spatial distribution, whereas the genera Bifidobacterium, Siccibacter, Photorabdus, Veillonella, and Vibriomainly characterized infants' gut microbiota at T2 [38].

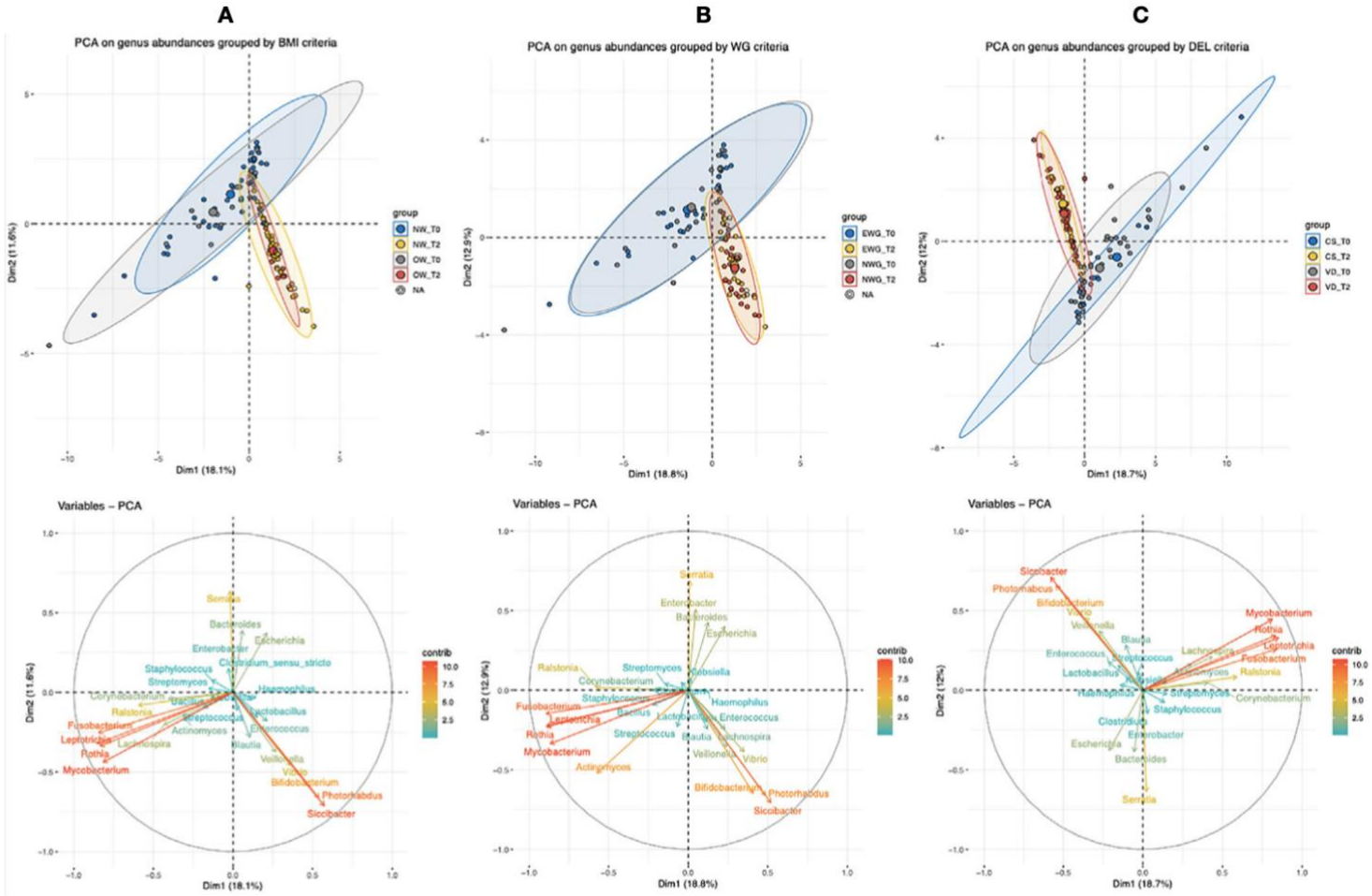
Considering both T0 groups (NW and OW), the presence of four samples (three NW and one OW), that seem to be outliers, determined a not complete overlapping of both T0 clouds. On the other side, *Bifidobacterium*, *Siccibacter*, and *Photorabdus* relative abundances contributed to shifting a subset of NW samples at T2, therefore, determining elongation of the NW cloud (Fig.12A) [38].

We also observed that at both times T0 and T2 the ellipses of NWG and EWG overlapped, and therefore, no PCA differences were associated with weight gain (Fig.12 B) [38].

Considering the type of delivery, CS and VD samples exhibited a more heterogeneous microbiota at T0, whereas at T2 the two delivery conditions allowed the construction of reduced and almost completely overlapped clouds (Fig.12C) [38].

Therefore, a multivariate analysis was also run only on T2 fecal sample-set (infants at 6 months of age) performing a PCA and using bacterial genera (16S rRNA gene amplicon sequences) with a median value of relative abundance greater than 0.1% and therefore contributing to describe at least 50% of the whole population, resulting in 17 bacterial genera (FigS1b) [38]. The two PCA principal components (Dim1 and Dim2; FigS1a) [38] described 52% of the total variance. The cos2 graduated scale values computed with R factoextra package described the quality of the sample. The best PCA sample quality is determined by high cos2 relative values. Observing the homogeneity of the sample in the PCA score plot where only a few linear distances marked some samples as outliers, it has been decided to perform another and more sensitive multivariate analysis [38]. To understand how the aforementioned factors (maternal and diet-related ones, as well as environmental ones) affected infants' early microbial colonization, the samples were clustered. By using the same filtered (median > 0.1%) set of genera, (whose vectors have been plotted in PCA biplot pf samples and variables, FigS1b) [38], discriminant analysis of the principal components (DAPC) was considered. Hence, a DAPC without superimposing any *a priori* condition was performed. As a result of the best fit cluster number identification, the "find.clusters" R function provided that four was the best-supported cluster number for the sample set. This information was used to run the DAPC. In the DAPC scatter plot (FigS2) [38] only cluster "4" was poorly populated (three samples), while the other three clusters (1, 2, and 3) included at least nine samples and were all placed into different quarters of the axes [38].

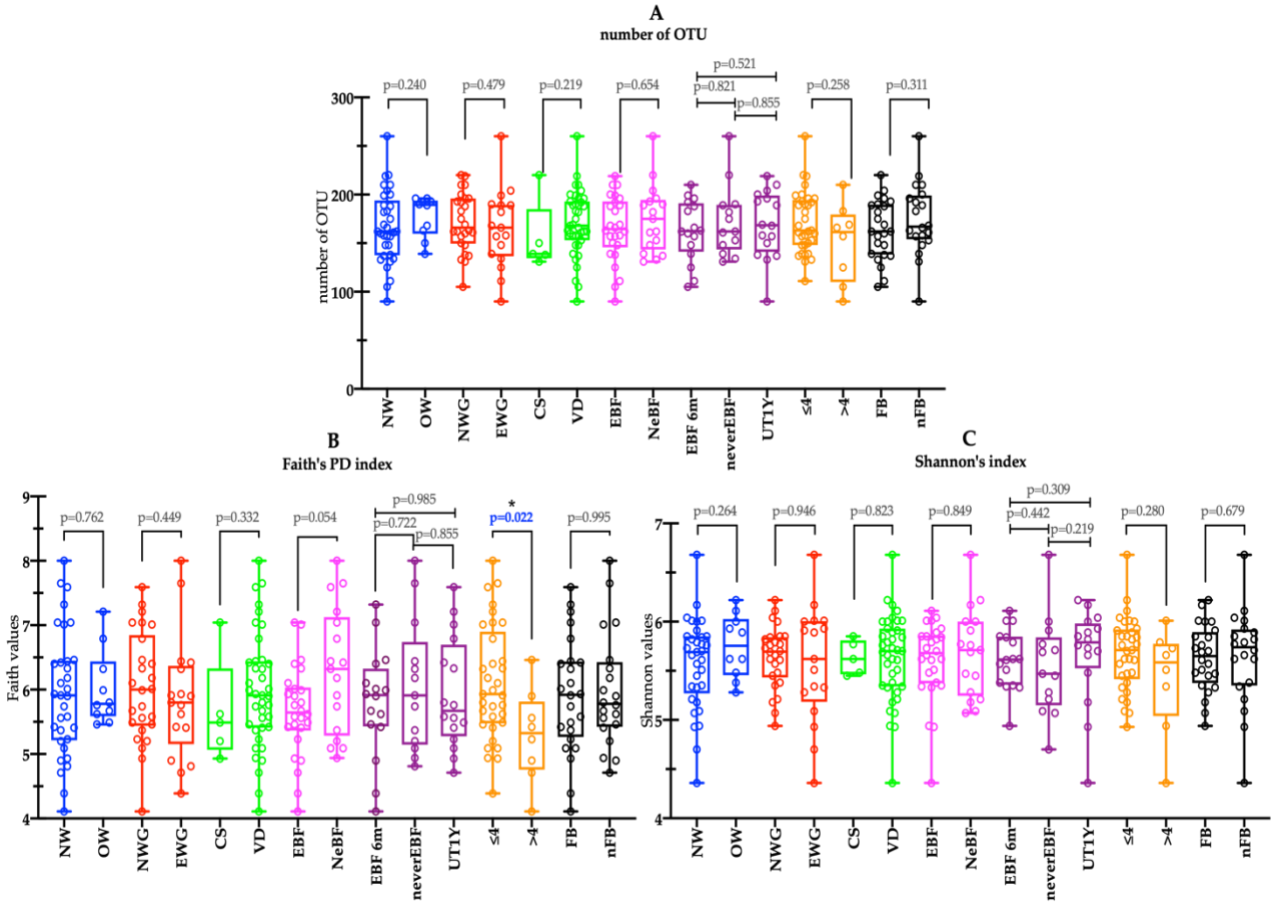
Fig. 12: Principal component analysis (PCA) of bacterial genera with a median abundance >0.1% (16S rRNA gene amplicon sequencing) of infants' T0 (meconium) and T2 (six months of age). Infants were grouped based on the relative metadata: (A) maternal pre-pregnancy BMI (NW, normal BMI, or OW, mothers with overweight/obesity), (B) maternal gestational weight gain (NWG, normal WG, or EWG, excessive WG), and (C) type of delivery (VD, vaginally delivered, or CS, by caesarean section) [38].



4.4 Microbiome analysis at T3

The amplicon 16S rRNA sequencing analysis was conducted on the 45 fecal samples of infants one year after birth (T3). Samples were grouped according to different metadata to investigate how and which prenatal and postnatal factors can influence the early microbiota colonization during the first year of life. Specifically, pre-pregnancy BMI and gestational WG have been collected as prenatal variables, while the type of delivery was accepted as a perinatal determinant. Type of feeding, time of weaning, and the presence/absence of older siblings in the household have been managed as postnatal factors. The number of assigned operational taxonomic units (OTU), Faith's phylogenetic diversity (Faith's PD) index, and Shannon's index have been applied to define the alpha diversity of our fecal samples. In particular, the number of OTU (Fig. 13A) showed no significant differences. Evaluating the Faith's PD index (Fig. 13B), a close significance was observed for the type of milk-feeding; at one year after birth, infants exclusively breastfed for the first six months of life presented lower but not significantly (exact pval = 0.054) indices than non-exclusively breastfed peers. According to Faith's PD index, the only significant difference was observed in infants weaned preterm (before 4 months of age, ≤4) when matched to ones weaned after the 4th month (exact pval = 0.022). Despite this, the same association did not show significance according to Shannon's index (Fig. 13C).

Figure 13. Alpha diversity. The number of operational taxonomic units (OTU), Faith's phylogenetic diversity index, and Shannon's diversity index (respectively A, B, and C panel) were used to describe the alpha diversity in fecal samples of infants at 1 year of age grouped according to different pre-, peri-, and postnatal variables (metadata). In detail, used metadata were: i) maternal pre-pregnancy BMI (blue boxplots) within recommended values (NW: BMI < 25 kg/m^2) or higher (OW: BMI ≥ 25 kg/m^2); ii) gestational WG (red boxplots), if optimal (NWG) or excessive (EWG) according to IOM reference values; iii) type of delivery (green boxplots), if caesarean section (CS) or vaginally delivered infants (VD); iv) type of feeding (pink boxplots), according to an exclusively breastfed for the first 6 months of age (EBF) or not (including both mixed and exclusively formula-fed, NeBF); v) milk-feed up to the year of age (purple boxplots), based on infant that took at least one breastfeed up to the year of age (UT1Y), infants exclusively breastfed till 6 months of age (EBF 6m), and infants that never were exclusively breastfed (neverEBF); vi) time of weaning (orange boxplots), if solid foods have been given before (≤4) the 4th month of age or after (>4); vii) absence/presence of older siblings in the household (FB and nFB, respectively; black boxplots).



Reporting (*), it means a significant difference (computed as exact p-value <0.05) according to the two-tailed Mann-Whitney's test.

4.4.1Factors affecting early microbial colonization of infant gut microbiota

According to the maternal pre-pregnancy BMI, no differences have been found (Tab. 4); meanwhile, according to the maternal gestational WG, slight differences have been found in Actinobacteria and Firmicutes abundances. Although both phyla did not show a significant corrected p-value (qval > 0.05), the MaAsLin2 analysis showed as Firmicutes were more related to NWG (pval = 0.034). At the same time, Actinobacteria seems to be more associated with an EWG (pval = 0.021). No other differences have been noted at the deeper taxonomic levels.

Different delivery modes showed differences only at the family level, with a tendency of Pasteurellaceae higher in infants born with CS (CS; pval = 0.011), even if this was not verified for adjusted p-values (qval > 0.05).

According to the type of feeding, EBF or not NeBF during the first six months of life, Acidaminococcaceae appeared to be more associated with NeBF (pval = 0.019, qval > 0.05). In

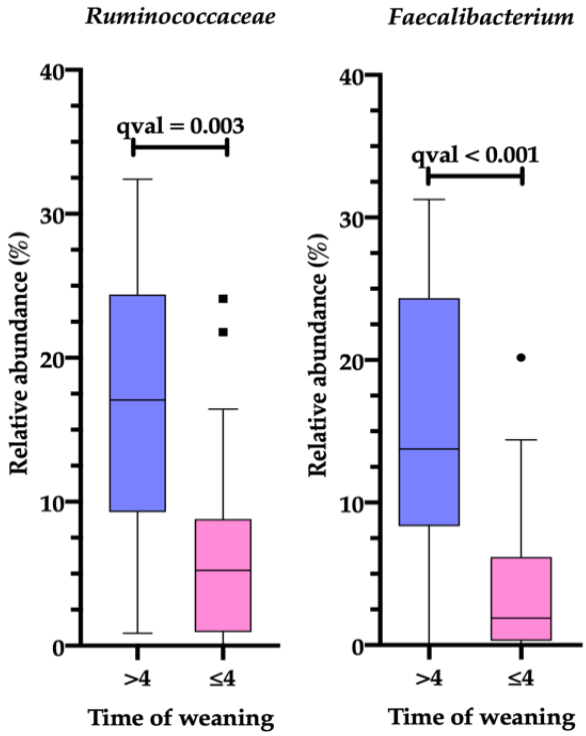
contrast, Lactobacillaceae mainly described the EBF microbiota (pval = 0.02, qval > 0.05). Concerning the feeding, a more extensive investigation was carried out. Infants were further shared in three groups: those that took at least one breastfeeding up to the year of age (ut1Y), infants that never have been breastfed (neverEBF), and infants that were breastfed up to six months of age (EBF6m). Interestingly, neverEBF infants exhibited a microbiota lower in Firmicutes and largely colonized by Bacteroidetes than both the other groups (pval ≤ 0.028, qval > 0.05). Further, analysis showed a negative association between neverEBF and Bacteroidaceae (and Bacteroides at genus level; pval = 0.005, qval > 0.05). Lactobacillaceae and Enterobacteriaceae followed the Firmicutes result showed a negative association with neverEBF infants (pval = 0.049 and 0.031, respectively). However, the above-reported differences were not confirmed for adjusted p-values (qval > 0.05).

Weaning was also evaluated to study the impact of diet-related factors. Infants were split in weaned preterm (≤4 months of age) or not (>4 months); we did not find differences at the phylum level. At deeper taxonomic levels, instead, those weaned after the 4th month of age showed a significantly increased abundance of Ruminococcaceae (pval = 0.0001) and Faecalibacterium (pval < 0.0001); the significance was also confirmed for adjusted p-values (qval < 0.001; Fig. 14). At the same time, the GI microbiota of preterm weaned (≤4) showed a tendency to be more colonized by Veillonellaceae (pval = 0.015), even if this was not significant for adjusted p-values (qval > 0.05).

Table 4. Significant different microbial taxa (phylum, family, and genus level) were found in fecal samples of infants one year after birth (T3). Differences have been evaluated according to different metadata, specifically: i) maternal pre-pregnancy BMI, ii) maternal gestational weight gain (WG), iii) type of delivery, iv) type of feeding for the first 6 months of life, v) milk-feed till the year of age, vi) introduction of solid foods (weaning), and vii) presence/absence of older siblings in the household. Into the column named "referring group" was reported the variable (optimal/excessive gestational weight gain, NWG and EWG, respectively; cesarean section-delivered, CS; exclusively and no-exclusively breastfed till 6 months after birth, $_{E}$BF and NeBF, respectively; never exclusively breastfed, never$_{E}$BF, weaned before/later than 4th month of age, ≤4/>4, respectively) to which coefficient (coeff) and standard error (stderr) referred.

Metadata	Taxon	Referring group	coeff	stderr	pval	qval
Maternal BMI						
	no significant differences					
Gestational WG						
	Actinobacteria	EWG	6.55	2.99	0.034	0.102
	Firmicutes	NWG	9.35	3.92	0.022	0.102
Type of Delivery						
	Pasteurellaceae	CS	3.09	1.15	0.011	0.242
Feeding followed in the first 6 months of life						
	Acidaminococcaceae	NeBF	2.48	1.01	0.019	0.232
	Lactobacillaceae	$_{E}$BF	3.01	1.25	0.020	0.232
Milk-feed till the year of age						
	Bacteroidetes	never$_{E}$BF	15.12	6.46	0.024	0.171
	Firmicutes	never$_{E}$BF	-10.84	4.78	0.028	0.171
	Bacteroidaceae	never$_{E}$BF	18.26	6.21	0.005	0.244
	Lactobacillaceae	never$_{E}$BF	-3.20	1.57	0.049	0.586
	Enterobacteriaceae	never$_{E}$BF	-4.34	1.94	0.031	0.586
	Bacteroides	never$_{E}$BF	18.26	6.21	0.005	0.371
Weaning						
	Ruminococcaceae	>4	10.73	2.54	0.0001	0.003
	Veillonellaceae	≤4	5.54	2.20	0.015	0.178
	Faecalibacterium	>4	10.96	2.22	0.00001	0.0004
Older siblings						
	no significant differences					

Figure 14. Relative abundance (16S rRNA gene sequencing) of *Ruminococcaceae* and *Faecalibacterium* in fecal samples of infants one years old (T3) grouped according to the time of weaning, early-weaned (≤4) or weaned after the 4th month of age (> 4).



Therefore, to assess the significant multivariable association between our samples and their relative metadata, in the first moment, we ran the MaAsLin2 R-package using pre and perinatal factors. However, we did not show significant results (Tab. 5). The same investigating approach was adopted using all postnatal factors. We found that all the three variables analyzed (feeding, weaning, and the presence of older siblings) showed at least a tendency according to a significance reached only for pval and not for qval. Firstborn babies showed an increased abundance of phyla included in the group named "Others" and in Proteobacteria (pval < 0.05, qval > 0.05). At the family level, the presence of older siblings promoted the abundance of Streptococcaceae (pval < 0.016). At the same time, the EBF harboured Veillonellaceae, Lactobacillaceae, and Enterobacteriaceae (pval ≤ 0.04), with a decrease in the abundance of Bacteroidaceae (pval = 0.035). However, all the results mentioned above, and the high abundance of Veillonellaceae in preterm weaned infants (pval = 0.002), were not significant for adjusted p-values. Otherwise, Faecalibacterium and Ruminococcaceae were significantly associated with infants weaned later than the 4th month of age (pval < 0.001, qval ≤ 0.03). The latter was also found when all collected metadata (pre-, peri-, and post-) have been evaluated together, confirming the strict association between Faecalibacterium and infants weaned since the 4th month of age (pval < 0.001, qval = 0.046).

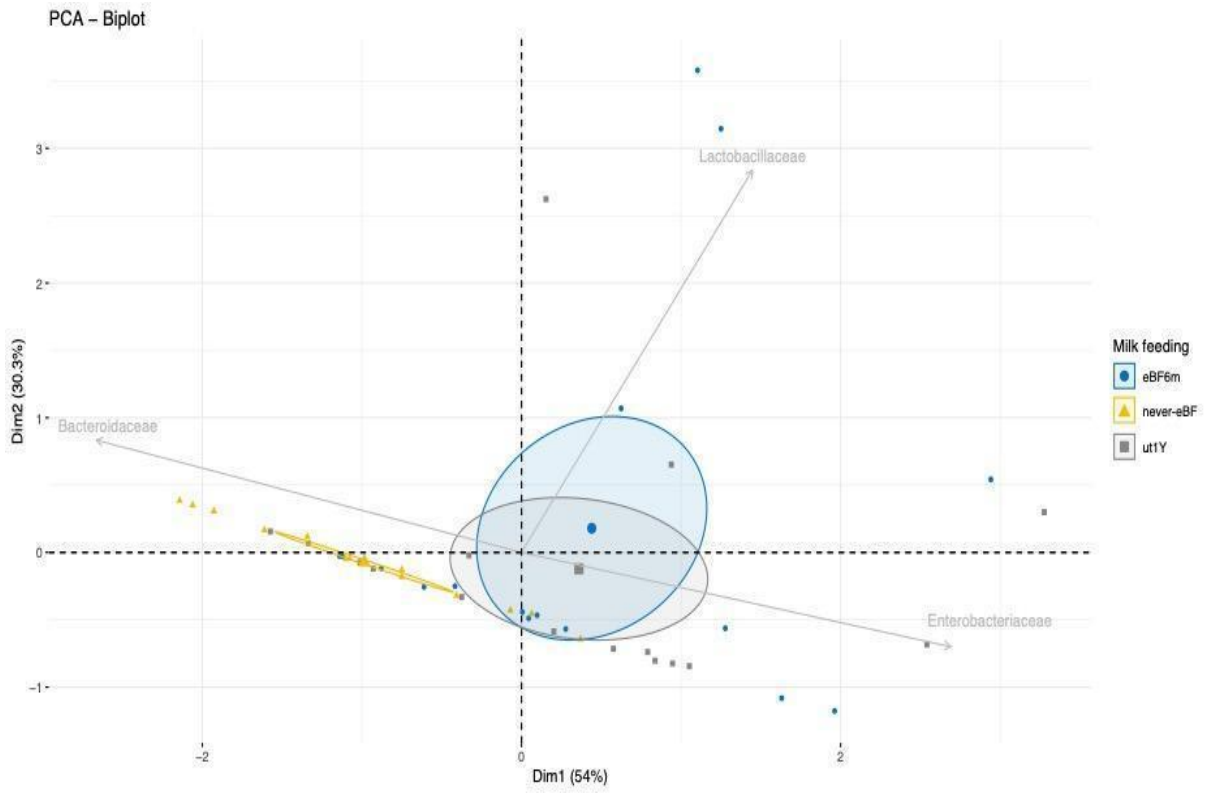
Table 5. Significant associations, according to MaAsLin2 R-package, were found between gut microbial taxa (phyla, families, and genera) of infants one-year-old (T3) and the relative metadata. In the first instance, significant associations have been searched according to pre- and perinatal variables (maternal pre-pregnancy BMI, maternal gestational weight gain (WG), and type of delivery). Subsequently, the significant association have been searched according to only to postnatal variables (type of feeding for the first 6 months of life, introduction of solid foods (weaning), and presence/absence of older siblings in the household) and, lastly, using all collected metadata (pre-, peri-, and postnatal variables). Into the column named "referring group" was reported the variable (infants with/without older siblings, nFB and FB, respectively; no-exclusively and exclusively breastfed, NeBF and $_{E}$BF, respectively; weaned before/after the 4th month of age, ≤4 and >4, respectively) to which coefficient (coeff) and standard error (stderr) referred.

Metadata	Taxon	Referring group	coeff	stderr	pval	qval
Pre- and perinatal variables (maternal pre-pregnancy BMI, maternal gestational weight gain, and type of delivery)						
	no significant differences					
Postnatal variables (milk feeding, weaning, and presence of older siblings)						
	Others (phyla < 0.1%)	FB	0.16	0.07	0.030	0.239
	Proteobacteria	FB	6.20	3.06	0.049	0.239
	Ruminococcaceae	>4	10.21	2.67	0.0004	0.030
	Veillonellaceae	≤4	6.82	2.09	0.002	0.077
	Streptococcaceae	nFB	2.12	0.85	0.016	0.281
	Veillonellaceae	$_{E}$BF	5.66	1.92	0.005	0.122
	Lactobacillaceae	$_{E}$BF	2.89	1.36	0.039	0.391
	Enterobacteriaceae	$_{E}$BF	3.69	1.73	0.040	0.391
	Bacteroidaceae	NeBF	12.68	5.81	0.035	0.391
	Faecalibacterium	>4	10.57	2.34	0.00005	0.006
All variables (maternal pre-pregnancy BMI and gestational weight gain, type of delivery, type of milk feeding, weaning, and presence of older siblings)						
	Faecalibacterium	>4	9.99	2.45	0.0002	0.047

4.4.2 Principal component analysis (PCA) of breast-milk consumption till the first year of life

Due to the collected results regarding the feeding, we further investigated how bacterial families could discriminate samples according to the duration of breastfeeding. With this goal, samples were sub-grouped in exclusively breastfed till six months of age (EBF6m), never exclusively breastfed (neverEBF), and infants taken at least one breastfed up to 1 year of age (ut1Y). Consequently, a principal component analysis (PCA) has been conducted (Fig.15). Assessing the beta diversity, the PCA reported more than 84% of the variance (accounting for PC1 and PC2) and showed distinct clustering of never-EBF with a positive relationship with Bacteroidaceae. Notably, the abundance of Lactobacillaceae was negatively associated with them. The size of clouds measured the highest probability to find samples belonging to the related group (EBF6m, neverEBF, or ut1Y). Hence, it seems that no differences existed between ut1Y and EBF6m. This relationship was mainly determined by positive scores of both Lactobacillaceae and Enterobacteriaceae.

Figure 15. Principal component analysis (PCA) of infants one years old (T3) sub-grouped according to the duration of breast-feeding, if exclusively breast-fed up to six months of age ($_{E}$BF6m), never exclusively breast-fed (never-$_{E}$BF), and infants fed with breast milk at least once/day up to 1 year of age (ut1Y), based on the abundance of bacterial families showing p-values < 0.05 (i.e., *Bacteroidaceae*, *Lactobacillaceae*, and *Enterobacteriaceae*). The size of blue, yellow, and grey ellipses (including $_{E}$BF6m, never-$_{E}$BF, and ut1Y samples, respectively) are weighted based on the highest probability to find a related sample into the PCA-plane.



5. Discussion

Early life events have multiple and long-lasting consequences that can reach adulthood; the same can be stated for the acquisition and development of microbiota during the first years of life. Gut microbiota alterations during early infancy have been proved to influence the risk of obesity during childhood and diabetes [57], non-alcoholic fatty liver disease (NAFLD) [58], allergic diseases and asthma, and allergic disease [59].

Gut microbiota construction can be influenced by a wide range of perinatal and postnatal factors, such as the mode of delivery (cesarean section and vaginal delivery), feeding (breastfeeding/formula/mixed feeding), and cessation of breastfeeding) [31] [60] [61], but also the family environment [59].

Our study aims to investigate the intestinal microbiome development of infants (ages 0-12 months) and to analyze how prenatal and postnatal factors could influence this development and construction in the first year of life.

Furthermore, according to previous studies [62] [63] [59], our data reveals that the infant gut microbiome experiences dynamic changes during the first 12 months of life with newborns showing a low microbial diversity and microbiota structure, which progressively increases during the time.

In particular, our results show that the meconium at birth (T0) is characterized by a more significant abundance of Proteobacteria, particularly those species relating to the genus *Escherichia* [31]. Previous investigations explained that the facultative anaerobes increase in the primitive gut, leading to a relatively aerobic intestinal environment [64] [62]. In this process, facultative anaerobes, as some Enterobacteriaceae, find the optimal conditions for growth [62]; Firmicutes, Bacteroidetes, and Actinobacteria start to increase later during infancy [65] [66]. Similarly, this intestinal microbial evolution was also observed in our study, with the relative abundance of Firmicutes significantly increased at one month of life (T1), and Proteobacteria and Fusobacteria significantly decreased [31].

About prenatal factors (maternal pre-pregnancy BMI and gestational WG), data revealed that those children born from mothers with overweight/obesity become at risk to develop obesity during life [67], even if determinants behind this relationship are not fully known.

Our study examined the microbial composition of newborns from mothers with pre-pregnancy BMI < 25 Kg/m2 or ≥ 25 Kg/m2. Previous investigations showed an increased and significant variation in gut microbiome composition at different stages of life in those children born to women who were affected by overweight or obesity during pregnancy [36]

[68] [69]. Collado et al. [36] stated that mothers with overweight had higher levels of *Bacteroides* genus in the third trimester of pregnancy; these higher levels may be vertically transmitted to the newborn during VD. In line, other studies reported that, among VD neonates, the relative abundance of gram-negative *Bacteroides spp*. is more associated with infants born to mothers affected by overweight or obesity than those born to normal-weight mothers [70]. Contrarily, it was proved that maternal pre-pregnancy BMI was negatively associated with fecal levels of *Bacteroides* when the infants were one month of age [36].

In contrast with these previous findings [36] [68] [69], no significant differences in the relative abundance of *Bacteroides spp*. were detected [31]. Concerning Firmicutes levels and the higher ratio Firmicutes/Bacteroidetes often commented in subjects affected by overweight or obesity [71], we observed an over-representation of *Streptococcus* in babies born to mothers whit a pre-pregnancy BMI ≥ 25 Kg/m2 [31], in contrast with Kameron Y. Sugino et al. [72]. Certainly, more researches are required to explain the specific changes in offspring microbiome composition related to maternal weight. The main differences between the two groups (pre-pregnancy BMI < 25 Kg/m2 and pre-pregnancy BMI ≥ 25 Kg/m2) were associated with the relative abundance of specific genera and species. In particular, ¾ of the statistically different species referred to the *Klebsiella* genus was higher in neonates born to mothers with BMI ≥ 25 Kg/m2 [31]. *Klebsiella* continues to be one of the main causes of nosocomial infections since it frequently develops multidrug resistance [73] [31].

When analyzing fecal samples at 6 and 12 months of age (T2-T3), we did not find differences according to maternal factors (pre-pregnancy BMI and gestational WG) [38]. Additionally, in line with previous studies, we detected only partial and not significant differences in Firmicutes and Bacteroidetes abundances [38] [74] [75].

The mode of delivery is thought to be a crucial and dominant factor in gut microbiota development. Birth by CS has been linked with different adverse consequences on immune development that could drive to allergies, infection, and inflammatory disorders [61] [76] [77] [78]. Analysis within CS birth's impact on microbiota and health consequences debates that results may be affected by intrapartum antibiotics [78]. Moreover, the diminished success of breastfeeding after CS sums alterations in healthy microbiota development [78].

The gut microbiome composition of babies born with VD is recognized to be predominantly affected by the maternal vaginal and gut microbiome; on the contrary, the gut microbiome of infants born with CS is essentially characterized by the microbes belonging to the surrounding environment (e.g., hospital environment) and mother’s skin [61]. Many studies

have recognized different intestinal microbial profiles between VD and CS babies, with lower diversity and richness in infants born by CS compared to those born vaginally [61].
In our sample, those neonates born to CS presented a higher relative abundance of facultative aerobes such as *Staphylococcus,* *Clostridium*, *Corynebacterium*, and *Streptococcus,* presumably acquired from maternal skin and hospital environment [31].
Our findings concerning bacterial taxonomic analyses at birth (T0), 1 month (T1) of infants' age [31], emphasized pre-and perinatal variables (maternal weight and type of delivery) as the main affecting factors. Differently, at six months (T2) and one year of age (T3), infants presented only a few differences when pre-and perinatal factors have been considered [38]. Indeed, after grouping samples according to maternal pregestational BMI, WG during pregnancy, and mode of delivery, no differences were found [38]. Therefore, it is possible to emphasize the most significant influence of "more recent" postnatal factors able to overcome the influence of "older" ones. Herein, the main differences were found between postnatal variables [38].
Feeding type is one of the main factors triggering early microbial shaping, and differences in the gut microbial composition between BF and FF infants are well documented [71]. Overall, the primary key factor able to improve and boost up the immune system is exclusive breastfeeding. The World Health Organization (WHO), indeed, suggests and recommends exclusively breastfeeding infants for at least the first six months of life [79].
In the present study, EBF samples reported lower Shannon Index values when compared to NeBF one [38]. Higher values of alpha diversity in adults' microbiota are related to health status, ecosystem resistance, and resilience [80]; nevertheless, in infants, it has been observed that is the formula-feeding define a high value of alpha diversity [81] [82]. Breastfed infants, on the contrary, are mainly defined by *Bifidobacterium* species; therefore, their presence markedly influences and reduces the alpha diversity index [81] [38].
Bifidobacterium species are recognized as one of the most important classes of probiotics, prevailing on other taxa, particularly able to metabolize Human Milk Oligosaccharides (HMOs) [83]. In the present study, we did not observe a high abundance of *Bifidobacteriaceae* (or relative subtaxa) in EBF infants in the first six months of life [38]; this is probably since our NeBF group also included infants fed with mixed feeding (formula and breast milk) [38]. This condition could have subdued the clustering of our samples. Nevertheless, despite this potential overlapping, we noted that our NeBF group was essentially defined by high abundances of *Ruminococcaceae*, in particular *Flavonifractor* and taxa ascribed to the *Clostridium cluster IV* [38]. This is in line with former studies [84]

[82] [85] [86], in which *Lachnospiraceae* or *Ruminococcacae* were recorded to be higher in the absence of specific competition with *Bifidobacteriaceae*, replacing them as butyrogenic bacteria in NeBF infants gut environment [87] [88].

The presence of *Flavonifractor* in NeBF babies has been previously described [89] [90]. This genus is associated with a high level of cytokines [91] and food allergies [92]. Recently, Bui et al. reported an enhancement of the microbial pathways for Nε-fructosyllysine degradation in formula-fed infant's stools; fecal microbiota of EBF was not able to develop on Nε-fructosyllysine medium [88]. The authors also observed that in the EBF gut microbiota, there was a reduction of the Intestinimonas-Flavonifractor-Pseudoflavonifractor group, largely defining FF's stools. The transfer of the Nε-fructosyllysine/lysine pathway genes seems to be vertical, from mothers to offspring, but the selective outcome of Nε-fructosyllysine/lysine-fermenting microorganisms dominated in formula-fed infants, suggests that the type of milk might affect their outgrowth [93]. We also found a positive association between *Erysipelotrichaceae Incertae Sedis* and NeBF [38], previously reported to be linked to asthma and allergy development [94] and presumably acting in the butyrate metabolism [95].

Additionally, at one year of age, we examined differences associated with a long-term milk-feeding; we found a clear difference in *Bacteroidaceae, Lactobacillaceae,* and *Enterobacteriaceae* abundances between infants who were never fed with human milk (neverEBF) than those exclusively breastfed up to 6 months of age (EBF6m) and those who had taken at least one breastfed up to 1 year (ut1Y). NeverEBF samples positively correlated with *Bacteroidaceae* and negatively with *Lactobacillaceae*. Rather, no differences were observed between EBF6m and ut1Y, highlighting a long-term contribution of human milk to drive the early microbiota establishment. Although splitting samples into three groups, these three bacterial families came to light, mainly describing EBF, *Bacteroidaceae* negatively, whereas *Lactobacillaceae* and *Enterobacteriaceae* positively. As explained before, this seems to be a long-term contribution to human milk. The presence of low abundances of *Lactobacillaceae* and *Enterobacteriaceae*, due to not exclusively breastfeeding, supported an increase of *Bacteroidaceae*. Due to a large number of species of Bacteroides able to colonize the human gut [96] and acknowledging their ability to quickly adapt to variations in nutrient availability [97], it does not surprise a heightened abundance of Bacteroidaceae according to a progressive suspension of milk-based feeding and progression in weaning [98]. Moreover, the absence of competitors could determine the overgrowth of *Bacteroidaceae*. Not all *Bacteroidaceae's* species are opportunistic

pathogens, but a substantial number are a lipopolysaccharide (LPS) producers [99]; due to the strict association of LPS with histone acetylation and methylation processes, some *Bacteroidaceae* sub-taxa could contribute to the onset of NCDs later in life [100]. This is an additional point of view strengthening the power of breastfeeding, which acts as a major driver of gut microbiota health since it harbours helpful probiotics and indirectly avoids the overgrowth of potentially pathogenic microbes.

Concerning weaning, previous research has shown that the introduction of solid foods and its timing could play a consistent part in the development of childhood overweight and obesity, with an enhanced risk when the weaning starts before 4 months of age [101] [102]. However, this risk has been observed in babies aged at least 2 years. This study did not find any statistically significant difference resulting from an early introduction of solid foods on younger ones [38]. That result could arise from the different types of feeding (exclusive breast-, mixed-, or exclusive formula feeding) in the first six months of life, determining overlap in the microbiota composition [38]. However, from our point of view, early weaning does not define any advantage in terms of GI microbiota maturation at six months of age [38]. Therefore, taking into account the literature cited before, breastfeeding continues to be the gold standard for optimal nutrition in the first six months of age, shaping microbiota interests.

At one year of life (T3), we found a higher abundance of Ruminococcaceae (at the family level) and *Faecalibacterium* (at genus level). High abundances of *Faecalibacterium*, as well as *Ruminococcaceae*, were linked to infants that started to introduce solid foods from 4 months of age. Undoubtedly, the notable increase in alpha diversity and appearance in *Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae* species, reflects the enhanced complexity of the infant's diet, in which fibers from fruits and vegetables and cereals, as well as non -milk protein sources (e.g., meats, fish, eggs, dairy products, and legumes) [98]. Dietary fibers and complex carbohydrates are preferred energy sources for microbes, and the main catabolic end products are short-chain fatty acids (SCFAs) acetate, butyrate, and propionate [103]. Whereas the acetate (together with lactate, formate, and succinate) is produced in high quantities in early infancy (e.g., by *Bifidobacterium, Lactobacillus*, and *Enterobacteriaceae spp*.), butyrate and propionate concentrations are initially very low but increase with infant age [98] [104]. Also, protein fermentation products, such as branched-chain fatty acids (BCFAs), are undetectable during breastfeeding but follow a similar pattern of increase with age [98]. These changes in SCFAs and BCFAs correspond with the introduction of solid foods and the end of breastfeeding [98] [104].

Faecalibacterium was previously described as one of the main early gut colonizers due to its involvement in lactose metabolism [105], reduced in the gut microbiota of children with allergic asthma [106], and investigated for its role in preventing diabetes onset [107]. The impact of weaning on *Faecalibacterium* abundance is still unclear; probably, it could correlate to a longer milk-feeding that harboured a gut microbial community more stable than infants who started to reduce milk-feeding earlier. On the contrary, the gut microbiota of early-weaned infants could have undergone a marked perturbation, which affected the survival of *Faecalibacterium*. In our set of children, due to the low number of enrolled subjects, it is hard to assess differences occurring in infants weaned before or later then 4th month. Undoubtedly, in line with our results, breastfeeding remains the gold standard of nutrition at least for the first six months of life, but additional evidence about the combined effect of breastfeeding and different times of introduction of solid food introduction might be able to increase the knowledge under a multi-factorial point of view.

In addition to microorganisms acquired from the maternal and diet sources described before, infants are exposed to a great mixture of other microbes deriving from the surrounding environment, including siblings, pets, and home surfaces. Exposure to new microorganisms consequently increases with increasing age [108]; meanwhile, the impact of such exposure decreases as the child grows and available ecological niches in the gut get conquered [108], The presence of older siblings affects the composition of the infant's microbiota [109] [38], with the microbial richness increasing with increasing numbers of older siblings [109]. Furthermore, starting from the "*Hygiene Hypotheses*" [110], Strachan observed that the presence of older siblings in the household decreased the risk of developing allergies in infants [111]. In the present study, babies with an older sibling at six months of age displayed lower Peptostreptococcaceae, Clostridiaceae, and *Clostridiales Incertae Sedis XIII* [38]; other data recorded an early maturation of the microbial colonization mediated by the "adult" associated genus Faecalibacterium [45]. Therefore, we suggest that older siblings in the household expose infants to "other and new" environmental bacterial patterns and indirectly reduce the abundance of the neonatal microbial colonizer [38]. Thus, the presence of older siblings shows to determine an early shift towards an "adult" microbiota profile. When analyzing infant's microbiome at one year of age (T3), we did not find significant differences, confirming that exposition to new microorganisms increases with age and that the impact of such exposure decreases as the child grows.

6. CONCLUSION

According to the most recent literature, the first year of life is a critical window of plasticity for healthy growth. Different and several prenatal and postnatal determinants can affect gut microbiota development, directly contributing to infants' gut microbiota maturation and determining potential disease outbreaks later in life.

In particular, in this study, according to the literature, we showed how the dynamic microbial community grows and develops rapidly from birth and during the first year of life.

Our conclusions concerning bacterial taxonomic analyses at birth (T0), after 1 month after birth (T1) emphasized pre-and perinatal variables (maternal weight and type of delivery) as the main affecting factors. Differently, at 6 months (T2) and one year of age (T3), infants presented only a few differences when pre-and perinatal factors have been considered. Hence, it is possible to highlight the most significant influence of "more recent" 'postnatal factors (e.g., feeding and weaning), being able to overcome the influence of "older" ones.

Dietary factors appear as the major players. This study confirmed the observation that exclusively breastfeeding remains the gold standard of nutrition at least in the first six months of life. At the same time, the study demonstrates the long-term contribution till the year of life. Nonetheless, additional evidence is needed to evaluate the interplay between milk-feeding and weaning.

Among the strengths of the A.MA.MI study, it is possible to recognize the adopted multifactorial approach based on collecting various babies' and maternal variables, that allowed us to provide a more profound structure into the field of early gut microbial colonizers. Furthermore, data analysis required a multidisciplinary team, and we were able to provide these expertises, including microbiologists, nutritionists, pediatrics, bioinformatics, and biostatistics. However, as previously reported, the small number of enrolled subjects represented the most significant limitation that needs to be considered together with the lack of groups having the same number of samples. Overall, our findings add to evidence of the complex and multifactorial interplay of different maternal, neonatal and environmental factors on GI microbiota composition in the first stage of life. Indeed, this study suggests the need for future studies to assess different factors working as co-variables during the first steps of babies' life.

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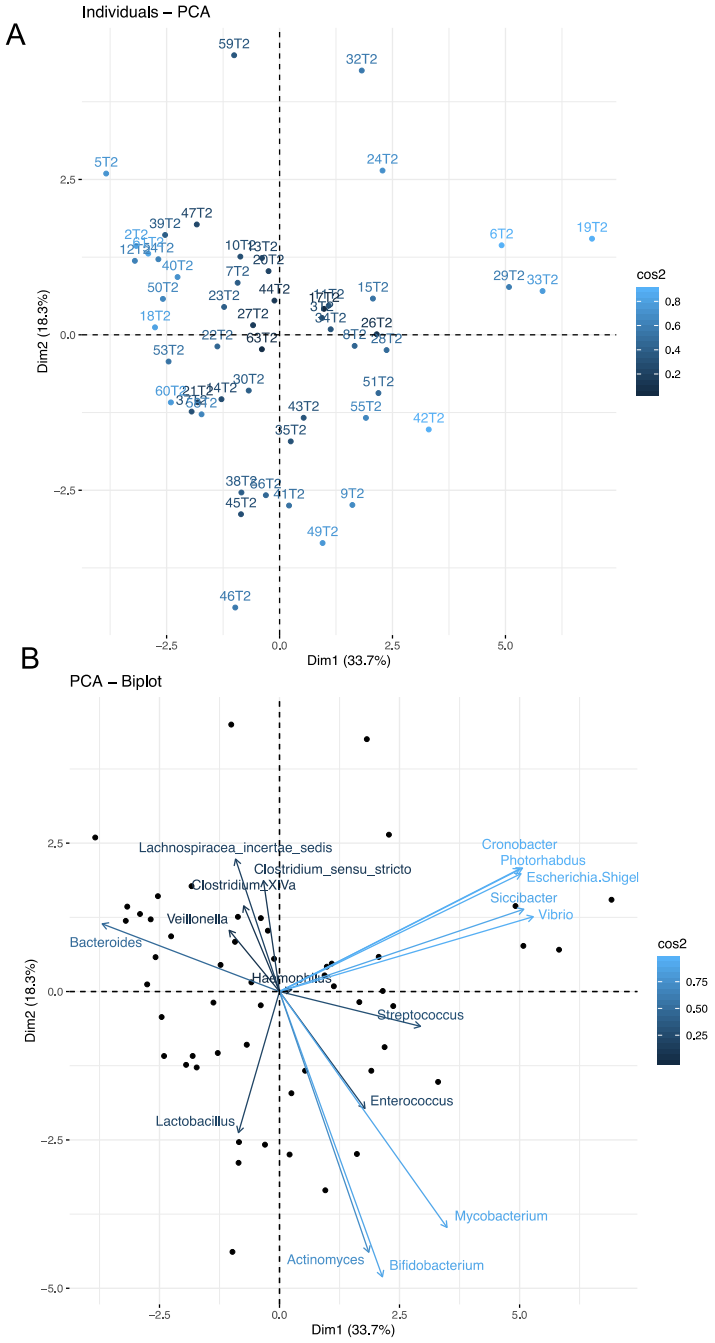
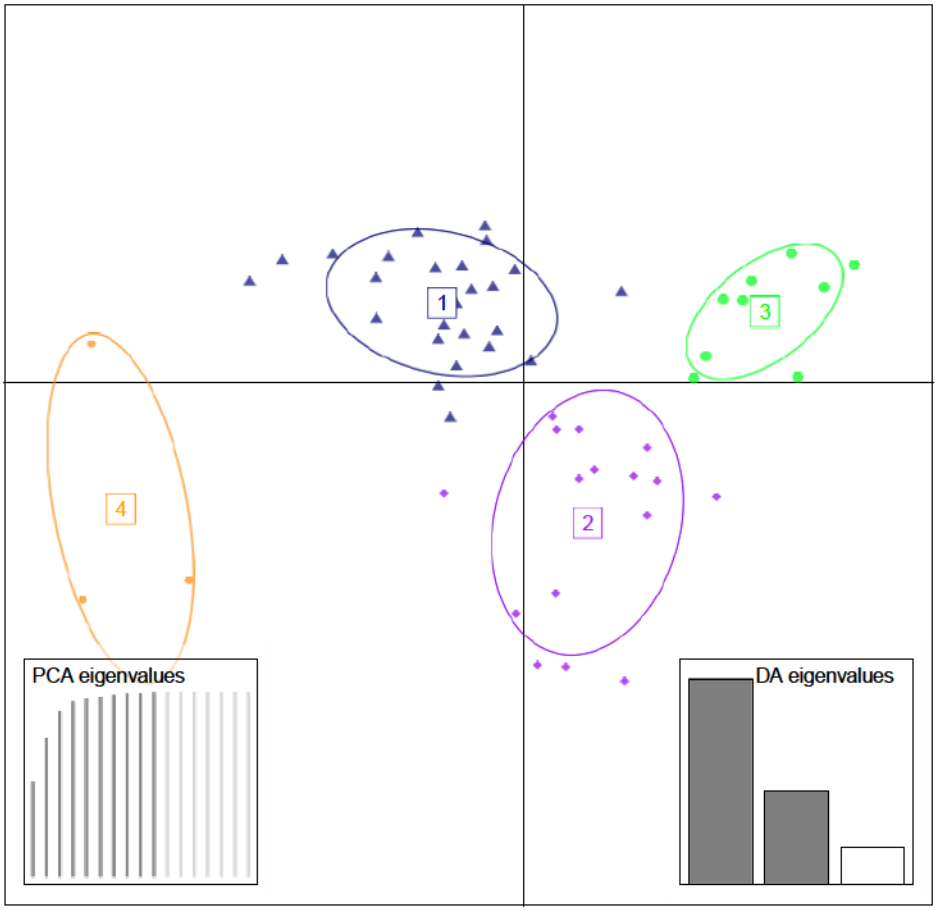
SUPPLEMEMTARY FIGURES

Supplementary Figure 1 (Fig.S1). Principal components analysis (PCA). The PCA was performed using genera with a median relative abundance (16S rRNA metagenetics) >0.1%. Panel A shows PCA of all T2 fecal samples (infants at six months of age). Panel B biplot of individuals and variables calculated by using factoextra R package. The factoextra cos2 calculated PCA values shows the importance of a principal component for a given observation (vector of original variables). Components with a large value of cos2 contribute to a relatively large portion of the total distance.



Supplementary Figure 2 (Fig.S2). Discriminant analysis of principal components (DAPC) plus cluster assignment. The DAPC was performed using genera with a median relative abundance (16S rRNA metagenetics) >0.1% and without superimposing any sample membership. In DAPC scoreplot each of the T2- fecal samples (gut microbiota of infants at six months of age) of the four clusters (1, 2, 3, and 4) has a different point shape and color. The table on the right reports DAPC sample assignment to each cluster.



Sample name	Cluster
2T2, 7T2, 10T2, 12T2, 13T2, 14T2, 18T2, 20T2, 21T2, 22T2, 23T2, 27T2, 30T2, 37T2, 39T2, 40T2, 44T2, 47T2, 50T2, 53T2, 54T2, 58T2, 60T2, 61T2, 63T2	1
3T2, 6T2, 8T2, 11T2, 15T2, 17T2, 19T2, 24T2, 26T2, 28T2, 29T2, 33T2, 34T2, 42T2, 51T2, 55T2	2
9T2, 35T2, 38T2, 41T2, 43T2, 45T2, 46T2, 49T2, 56T2	3
5T2, 32T2, 59T2	4

APPENDIX 1: *Food Questionnaire (Turconi et al.)* [51]

ISTRUZIONI PER LA COMPILAZIONE DEL QUESTIONARIO

Il questionario deve essere compilato in ogni sua parte, rispondendo ad ogni domanda con una sola risposta. Non lasciare nessuna domanda senza risposta.

Si garantisce che i dati rilevati verranno utilizzati a solo scopo di ricerca nel rispetto della legge sulla *"privacy"* (tutela delle persone e di altri soggetti rispetto al trattamento dei dati personali secondo la legge 675/1996).

SEZIONE A. FREQUENZE DI CONSUMO DI ALIMENTI
Le domande si riferiscono alle sue abitudini alimentari attuali
Quando più alimenti compaiono insieme, è sufficiente consumarne anche uno solo per rispondere affermativamente alla domanda

A. 1 Quante volte a settimana consuma **latte/cappuccino/caffèlatte/ yogurt ?**
- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 2 Quante *porzioni* al giorno?
- □ 1
- □ 2
- □ 3
- □ 4

A. 3 Quante volte a settimana consuma **pasta/riso/pane/patate/pizza**?
- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 4 Quante *porzioni* al giorno ?
- □ 1-2
- □ 3-4
- □ 5-6
- □ più di 6

A. 5 Quante volte a settimana consuma **frutta/verdura**?
- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 6 Quante *porzioni* al giorno ?
- □ 1-2
- □ 3-4
- □ 5-6
- □ più di 6

A. 7 Quante volte a settimana consuma **carne**?
- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 8 Quante volte a settimana consuma **pesce**?
- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 9 Quante volte a settimana consuma **uova**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 10 Quante volte a settimana consuma **formaggio**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 11 Quante volte a settimana consuma **salumi/insaccati**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 12 Quante volte a settimana consuma **piatti a base di legumi**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 13 Quante volte a settimana consuma **dolciumi in genere**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 14 Quante volte a settimana consuma **patatine fritte**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 15 Quante volte a settimana consuma **vino/birra** ?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 16 Quanti *bicchieri* al giorno?

- □ 0
- □ 1
- □ 2
- □ 3

A. 17 Quante volte a settimana consuma **aperitivi/cocktail alcolici/superalcolici**?

- □ tutti i giorni
- □ quasi tutti i giorni (4-5)
- □ talvolta (2-3)
- □ mai o quasi mai (0-1)

A. 18 Quanti *bicchieri* al giorno?

- □ 0
- □ 1
- □ 2
- □ 3

SEZIONE B. ABITUDINI ALIMENTARI
Le risposte si devono riferire a quella che è la situazione "più abituale"

B. 1 Fa la prima colazione ?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 2 Quale bevanda consuma a colazione ?
- □ latte/yogurt/caffèlatte/cappuccino
- □ succo di frutta/spremuta
- □ tè/caffè
- □ cioccolata

B. 3 La sua colazione è a base principalmente di:
- □ biscotti/fette biscottate/merendine/ cereali da prima colazione/pane
- □ frutta
- □ salumi, formaggi, uova
- □ pizza/focaccia/toast

B. 4 Consuma almeno due porzioni di frutta al giorno ?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 5 Consuma almeno due porzioni di verdura al giorno ?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 6 Consuma almeno un bicchiere di latte o un vasetto di yogurt al giorno?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 7 Consuma almeno 1-1,5 litri di acqua al giorno?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 8 I suoi pasti si concludono abitualmente con un dessert o un dolce?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 9 Ai suoi pasti, oltre all'acqua, beve abitualmente vino o birra ?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 10 Consuma abitualmente tutti i giorni: colazione, pranzo e cena ?
- □ sempre
- □ spesso
- □ raramente
- □ mai

B. 11 La sua alimentazione giornaliera :

- □ varia ogni giorno
- □ varia solo qualche volta alla settimana
- □ varia solo nel fine settimana
- □ varia raramente in quanto è molto monotona

B. 12 La sua alimentazione giornaliera è a base principalmente di :

- □ alimenti ad alto contenuto proteico (carne, pesce, uova, formaggi, legumi secchi)
- □ alimenti ad alto contenuto di grassi (salumi, focacce, patatine fritte, dolci a base di panna e crema)
- □ alimenti ad alto contenuto di carboidrati (pane, pasta, riso, patate, biscotti, fette biscottate, crostate)
- □ alimenti vari

B. 13 I suoi spuntini consistono principalmente in :

- □ frutta/spremute/succhi di frutta/frullati/yogurt
- □ fette biscottate/biscotti/crackers/pane/grissini
- □ patatine fritte/pop corn/noccioline
- □ caramelle/cioccolatini/gelati/dolci/bibite

B. 14 Le bevande che consuma più spesso fuori pasto sono :

- □ acqua
- □ bibite zuccherine (cola, aranciata, tè freddo, gassosa, acqua tonica, ecc.)
- □ birra/vino
- □ succhi di frutta/spremute/frullati

APPENDIX 2: *Ipaq Questionnaire (Craig et al.)* [52]

QUESTIONARIO INTERNAZIONALE SULL'ATTIVITA' FISICA

Siamo interessati a conoscere i tipi di attività fisica che le persone fanno come parte della vita quotidiana. Le domande riguarderanno il tempo che lei ha trascorso in attività fisiche negli **ultimi sette giorni**. Cortesemente, risponda ad ogni domanda anche se non si considera essere una persona attiva. Pensi, per favore, alle attività svolte al lavoro, come parte del lavoro svolto in casa ed in giardino, per spostarsi da un luogo all'altro e nel suo tempo libero come divertimento, esercizio fisico o sport.

Pensi a tutte le attività **vigorose**, energiche che ha svolto negli **ultimi sette giorni.** Le attività fisiche **vigorose** sono quelle che richiedono uno sforzo fisico duro e che la fanno respirare con un ritmo molto più frequente rispetto al normale. Pensi *soltanto* a quelle attività fisiche che lei ha svolto per almeno 10 minuti consecutivamente.

1. Durante gli **ultimi sette giorni**, in quanti giorni lei ha svolto attività fisica vigorosa come sollevare oggetti pesanti, zappare, fare aerobica, o pedalare in bicicletta ad una certa velocità?

_____ **giorni per settimana**

☐ Nessuna attività fisica vigorosa ----------→ **Vada alla domanda 3**

2. Quanto tempo in totale di solito trascorre in attività fisiche **vigorose** in uno di quei giorni?

_____ **ore per giorno**

_____ **minuti per giorno**

☐ Non sa / non è sicuro/a

Pensi a tutte quelle attività **moderate** che lei ha svolto negli **ultimi sette giorni**. Le attività moderate sono quelle che richiedono uno sforzo fisico moderato e che la fanno respirare con un ritmo un po' più frequente rispetto al normale. Pensi soltanto a quelle attività fisiche che lei ha svolto per almeno 10 minuti consecutivamente.

3. Durante gli **ultimi sette giorni,** quanti giorni lei ha svolto attività fisica **moderata** come portare pesi leggeri, andare in bicicletta ad un ritmo regolare oppure giocare il doppio a tennis? Non includa il camminare.

_____ **giorni per settimana**

☐ Nessuna attività fisica moderata ----------→ **Vada alla domanda 5**

4. Quanto tempo lei di solito dedica alle attività fisiche **moderate** in uno di quei giorni?

_____ **ore per giorno**

_____ **minuti per giorno**

☐ Non sa / non è sicuro/a

Pensi al tempo da lei trascorso **camminando** negli **ultimi sette giorni**. Includa il tempo trascorso sia al lavoro sia a casa, nello spostarsi da un luogo ad un altro e qualsiasi altro cammino che lei ha fatto solo per divertimento, sport, esercizio fisico o per passatempo.

5. Durante gli **ultimi sette giorni,** in quanti giorni lei ha **camminato** per almeno 10 minuti di continuo?

_____ **giorni per settimana**

☐ Nessuno ----------→ **Vada alla domanda 7**

6. Di solito quanto tempo ha trascorso, in uno di quei giorni, **camminando?**

_____ **ore per giorno**

_____ **minuti per giorno**

☐ Non sa / non è sicuro

L'ultima domanda riguarda il tempo trascorso stando **seduto** dal lunedì al venerdì negli **ultimi sette giorni**. Includa il tempo in cui rimane seduto al lavoro, in casa, nello svolgere un corso di formazione, durante il suo tempo libero. Questo può includere il tempo trascorso seduto alla scrivania, nel far visita ad amici, leggendo, o seduto/a o sdraiato/a per guardare la televisione.

7. Durante gli **ultimi sette giorni**, in un giorno della settimana, quanto tempo ha trascorso stando seduto?

_____ **ore per giorno**

_____ **minuti per giorno**

☐ **N**on sa / non è sicuro

Qui termina il questionario, grazie per la collaborazione.

APPENDIX 3: *Intergrowth21 Questionnaire* [53]

Questionario di Frequenza Alimentare (FFQ)

Studio di Follow-up postnatale – Visita ad 1 anno

(Tradotto da: https://www.intergrowth21.org.uk/protocol.aspx?lang=1/ https://www.medscinet.net/Intergrowth/patientinfodocs/PIFS1%20-%20IFU%20-%20final.pdf)

Codice mamma-bambino —

Data di nascita G G M M A A

Data della visita G G M M A A

Per favore, risponda a tutte le domande si/no mettendo una 'x' in corrispondenza della casella

Sezione 1. Alimentazione di suo/a figlio/a nel primo anno di vita

1. Alle dimissioni dall'ospedale, suo/a figlio/a:

   - era esclusivamente allattato al seno? ☐ Se si, vada alla D2

   oppure - ha rivevuto un allattamento misto? ☐ Se si, vada alla D2

   oppure - era esclusivamente con latte artificiale? ☐ Se si, vada alla D6

2. Durante il primo anno di vita, ha mai dato a suo/a figlio/a latte tirato dal seno? ☐si ☐no

3. Se sta ancora allattando suo/a figlio/a, quanti pasti al giorno (incluso il latte tirato dal seno)? __
   N.pasti/giorno

4. Che età aveva suo/a figlio/a quando ha smesso di allattarlo esclusivamente al seno? M M Mesi S Settimane

5. Che età aveva suo/a figlio/a quando ha iniziato a dargli il latte in formula? M M Mesi S Settimane

6. Che tipo di latte in formula ha dato a suo/a figlio/a (barra il maggior numero possibile)?

Formula standard	Formula idrolizzata	Formula ad alto contenuto calorico	Formula a base di soia	Altri tipi di latte in formula

7. A che età ha iniziato a dare a suo figlio/a altri tipi di latte? M M mesi S settimane

8. Se si, che tipo/i di latte?

   Vaccino Screm. ☐ Vaccino parz. Screm. ☐ Vaccino Intero ☐ Latte di soia ☐ Altri latti veg ☐ Altro ☐

9. Che età avevo suo/a figlio/a quando sono stati introdotti I primi cibi solidi? M M mesi S settimane

10. In generale, dove è preparata la maggior parte del cibo di suo/a figlio/a?

    a casa ☐ famiglia/amici ☐ ristorante ☐ negozio ☐

11. Chi dà da mangiare principalmete a suo/a figlio/a?

    Madre ☐ Padre ☐ Nonna/o ☐ baby-sitter ☐ altro ☐

12. Suo/a figlio/a segue una dieta speciale?

    Vegetariana ☐ Senza glutine ☐ basso contenuto ☐ basso contenuto ☐ Altro ☐ Nessuna ☐

Studio di Follow-up postnatale – Visita ad 1 anno

Questionario di Frequenza Alimentare

Codice Diade mamma/figlio_____ **Data della vista**_____

Per favore indichi con una 'X' nella tabella quanto spesso suo/a figlio/a ha consumato questi cibi negli ultimi 28 giorni

Sezione 2: Questionario Frequenza Alimentare

Negli ultimi 28 giorni, quanto spesso il/la bambino/a ha consumato…?		MAI	Volte/mese	Volte/settimana		Volte/giorno		Non applicabile
			1-3 volte	1-3 volte	> 3 volte	1-3 volte	> 3 volte	
13.	Latte materno							
14.	Formula/latte soia/altro latte							
15.	Latte animale							
16.	Succhi/spremute/centrifugati di Frutta/verdure							
17.	Bevande zuccherate							
18.	Bevenade con dolcificanti (ipocalorici)							
19.	Acqua							
20.	Zuppa (brodo di carne/pesce/verdure)							
21.	Latticini							
22.	Cereali cotti (es porridge)							
23.	Cereali da colazione							
24.	Frutta e verdura ricca in Vit A(es: carote, spinaci albicocche, ecc)							
25.	Altra frutta							
26.	Altra verdura							
27.	Cereali in chicco (es.riso)							
28.	Legumi (es fagioli lenticchie)							
29.	Tuberi (es patate)							
30.	Pasta							
31.	Pane / crackers							
32.	Biscotti / snacks dolci							
33.	Patatine / snacks salati							
34.	Dolci / caramelle							
35.	Uova							
36.	Carne rossa / interiora (es:. Manzo/fegato)							
36/A	Prosciutto cotto/maiale							
37.	Pesce							
38.	Pollame-carne Bianca (es: pollo, tacchino, coniglio, agnello)							
39.	Cibo Take-away food (es. pizza…)							
40.	Burro							
41.	Margarina/oli vegetali							
42.	Olio di Oliva/Extraverg.							

NOME E FIRMA DEL RICERCATORE_____

APPENDIX 4

Table 1. *General characteristics of the dyads at recruitment. Data are presented as median and InterQuartile Range (IQR)* [31].

Dyad	***Parameters***	***Median***	***IQR***
Mothers *(n=63)*	Age (years)	33.0	29.0-37.0
	Pre-pregnancy Weight (kg)	60.0	53.0-65.5
	Pre-pregnancy height (cm)	164.0	160.0-170.0
	Pre-pregnancy BMI (Kg/m^2) 65% (n=42); normal weigh 26,3% (=16); overweight/obesity 8,2% (n=5); underweight	22.0	19.5-25.0
	Weight gain during pregnancy (kg) 54,1% (n=33) adeguate weight gain during pregnancy *	12.5	10.0-15.0
Infants *(n=63;31 M/32 F)*	Gestational age at birth (weeks)	40.0	39.0-41.0
	Birth weight (kg)	3.3	3.1-3.5
	Lenght (cm)	50.0	50.0-52.0
	Cranial circumference (cm)	34.0	33.5-35.0

* according to the Institute of Medicine (US), guidelines [Institute of Medicine, US; Weight gain during pregnancy: re-examining the guidelines. Washington, DC. National Academies Press; National Academy of Sciences]

APPENDIX 5

Table 2: *General characteristics of the dyads are presented as Average, Median and Standard Deviation (SD) [38]*

Dyad	***Parameters***	***Average***	***Median***	***SD***
Mothers *(n=53)*	Age (years)	33	34	5
	Pre-pregnancy Weight (kg)	61.98	60	0.71
	Pre-pregnancy height (cm)	164.5	164	4.95
	Pre-pregnancy BMI (Kg/m^2)	22.76	33	1.41
	Weight gain during pregnancy (kg)	12.67	12.5	0.71
	54,1% (n=33) adeguate weight gain during pregnancy *			
Infants *(n=53; M:28; F:25)*	Gestational age at birth (weeks)	39.4	40	0
	Birth weight (kg)	3.12	3.05	0.46
	Weight (kg) at T2	7.29	7.33	0.46
	Length at T2 (cm)	66.65	66	0.71
	Cranial circumference at T2 (cm)	42.91	43	1.41

[a] according to the Institute of Medicine (US), guidelines [Institute of Medicine, US; Weight gain during pregnancy: re-examining the guidelines. Washington, DC. National Academies Press; National Academy of Sciences]

APPENDIX 6

Table 3. *General characteristics of the dyads at T3 presented as Average, Median and Standard Deviation (SD)*

Dyads T3	***Parameters***	***Average***	***Median***	***SD***
Mother *(n=45)*	Age (years)	33	34	6
	Pre-pregnancy BMI (Kg/m^2)	22.5	22	3.6
	Weight gain during pregnancy (kg) adeguate weight gain during pregnancy *	12.3	12.5	4.3
Infant *(n=45; M=26; F=19)*	Gestational age at birth(weeks)	40	40	1.1
	Weight (Kg)	9.5	9.5	0.86
	Lenght (cm)	75.2	75.5	2.5
	Cranial Circumference (cm)	46.1	46	1.6

[a] according to the Institute of Medicine (US), guidelines [Institute of Medicine, US; Weight gain during pregnancy: re-examining the guidelines. Washington, DC. National Academies Press; National Academy of Sciences]