NUTRITION DURING THE FIRST 1000 DAYS AND FETAL PROGRAMMING

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NUTRITION DURING THE FIRST 1000 DAYS AND FETAL PROGRAMMING

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Factors Associated With Weight, Length, and BMI Change in Adolescents' Offspring in Their First Year of Life

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Background: Young maternal age is associated with negative outcomes at birth and with offspring's growth. In low- and middle-income countries, adolescents' offspring growth little has been studied.

Objective: To determine the association of maternal sociodemographic characteristics with weight, length, and BMI change in adolescents' offspring in their first year of life.

Methods: This is a one-year follow-up study that included adolescent mothers and their offspring from 2010 to 2017. The infant anthropometric variables were performed at birth, 3, 6, and 12 months. Maternal health, pregnancy, and social variables were evaluated as well as birth outcomes. Crude, percentage, Z score, and percentile changes of weight, length, and BMI were evaluated from birth to 1-year-old. Statistical analyses were adjusted by maternal chronological age, socioeconomic status, breastfeeding duration, the timing of introduction of complementary feeding, among other variables.

Results: We examined 186 dyads (mother-infant). The median maternal age was 15.5 years, and the mean pre-pregnancy BMI was 20. The mean gestational age was 39.1 weeks for infants, birth weight was 3,039 g, and length at birth was 49.5-cm. Maternal chronological age, the timing of introduction of complementary feeding, socioeconomic status, and maternal occupation were associated with offspring's weight gain at 12 months. Length gain was associated with exclusive breastfeeding. Socioeconomic status and occupation were associated with offspring's BMI change. When performing adjusted multivariable analyses, weight and length at birth were associated weight and BMI at 12 months.

Conclusions: Weight at birth may negatively predict infant's weight and BMI changes at 12 months, while length at birth may positively predict the changes. Maternal

chronological age, socioeconomic level, occupation, and the timing of the introduction of complementary feeding were associated with the weight change. Only exclusive breastfeeding was associated with length Z-score change in adolescents' offspring in their first 12-months of life.

Keywords: body weight, child growth, infant, pregnancy during adolescence, breastfeeding, Mexico

INTRODUCTION

Adolescent pregnancy is associated with an increased risk of eclampsia, puerperal endometritis, systemic infections, maternal mortality, and preterm delivery compared to pregnant adults (≥20 years old) (1). Most of the research related to adolescent pregnancy and their offspring outcomes comes from high-income countries (2), even though teen pregnancy has higher rates in low- and middle-income countries (3). Pregnant adolescents may be at risk of being physically immature due to delayed completion of growth, especially when they have a pre-pregnancy body mass index (BMI) of underweight (4, 5).

The reasons for the adverse maternal and neonatal outcomes in adolescents are not clear yet, but it seems that inadequate/absent prenatal care is one of the main risk factors (6). Evidence shows that adverse perinatal outcomes in adolescents can be lowered with adequate prenatal care (7). In this sense, the lack of prenatal care in adolescent mothers has been related to a high risk of low birth weight and higher risk of perinatal mortality (8). Regarding neonatal outcomes, their anthropometric measurements can be influenced by several factors, like maternal age, pre-pregnancy weight, excessive gestational weight gain, and offspring's gender and gestational age (9–11).

Social and cultural factors are also associated with perinatal outcomes in pregnant adolescents. Many pregnant adolescents drop out of school, leading to a cycle of unemployment and poverty for life. These structural challenges resonate to promote the care of adolescent mothers. For example, the adolescents who go back to school often stop breastfeeding their children, contributing to the low rates of exclusive breastfeeding (8% at 6 months) (12). Therefore, it is predicted that adolescent mothers will have more difficulties in caring for their children, such as in breastfeeding practice and vaccines for children (2).

For the adolescents' offspring, the risk of perinatal morbidity and mortality, low birth weight, small for gestational age, and severe neonatal conditions is increased (13). Also, being an adolescent mother is associated with less knowledge about parenting and infant growth; for these reasons, adolescents may become less confident in their parental abilities, as well as being immature in their behavior and expressing more problematic parenting beliefs, and therefore, less able to meet the needs of their children (14, 15). On the other hand, as adolescents are still growing, there is a nutrient competition between the mother and the fetus (16).

Abbreviations: BMI, Body Mass Index; ENSANUT, National Health and Nutrition Survey; INPer, Instituto Nacional de Perinatología; p-BMI, Pre-pregnancy Body Mass Index; SD, Standard Deviation; WHO, World Health Organization.

Currently, very few longitudinal studies have investigated the after-birth growth of adolescents' offspring. Therefore, the purpose of the study was to determine the association of maternal sociodemographic characteristics with weight, length, and BMI change in adolescents' offspring in their first year of life.

MATERIALS AND METHODS

Study Design and Subjects

We conducted a prospective cohort study at Mexico's Instituto Nacional de Perinatología (INPer) from 2010 to 2017. INPer is a public tertiary care institution that treats high-risk pregnancies of women who do not have formal employment or health insurance. The participants were 186 dyads of pregnant adolescents and their offspring. The children were born in the toco-surgical unit of INPer, and we followed them from birth to their first year of life. Sampling was non-probabilistic, and women who met the selection criteria were included consecutively. The participants were pregnant adolescents from 10 to 19 years old, primiparous, with a singleton pregnancy, with <20 weeks of gestation, and without chronic, metabolic, or genetic diseases. All participants signed informed assent, and their respective parents or guardians signed informed consent. We eliminated the participants that had gestational diabetes or hypertensive disorders during pregnancy. Also, dyads were eliminated if the offspring had a disease that affected growth, such as hypothyroidism, diabetes, heart diseases, neoplasms, or 21-trisomy. A structured questionnaire was used to gather sociodemographic data, like age, marital status, level of education, occupation, socioeconomic level, pre-pregnancy weight, history of diseases, and smoking and alcohol drinking habits.

Procedures

Data Gathering

Trained health workers obtained the mothers' and their offspring's data using the questionnaire, clinical records, and performing interviews during the postnatal nutritional visits. We calculated the mother's chronological age at the moment of the delivery from the baseline data. We obtained the date of birth, sex, gestational age, birth weight, and length at birth directly from clinical records. We calculated gestational age at baseline evaluation or from the clinical record according to the last menstrual period. Neonatal outcomes were defined as follows: low birth weight <2,500 g, and small for gestational age as birth weight below the 10th percentile for specific gestational age and sex, according to the World Health Organization (WHO) criteria using AnthroPlus-WHO® (17).

Follow-Up Visits

Trained health professionals performed anthropometric measurements of the offspring at 3, 6, and 12 months. Birth weight was determined in grams on a pediatric digital scale Tanita 1583 (CMS Weighing Equipment Ltd., London, UK) with an accuracy of 0.01 kg. Length at birth was measured in centimeters with an infanto-meter Harpenden with an accuracy of 0.1 cm (CMS Weighing Equipment Ltd., London, UK). Length-for-age and weight-for-length were converted into sex-specific and gestational-age-adjusted Z-scores, respectively. Growth retardation was defined as a Z-score of length-for-age and weight-for-length and BMI below −2 SD, respectively (18).

Breastfeeding practice was prospectively assessed every 3 months during follow-up visits and was associate with growth variables: weight, length, and BMI. According to the definitions provided by the WHO (19), we evaluated any type of breastfeeding practice as the duration in months.

The socioeconomic level was considered a potential confounder; it was assessed with six dimensions of well-being within the household: Human Capital, Practical infrastructure, Connectivity and entertainment, Health infrastructure, Planning and future, and Basic infrastructure and space (20).

Ethical Considerations

The Institutional Review Board and Ethics Committees from INPer approved the study with registration number 212250-49451. All data gathering was confidential. All participants received nutritional counseling to help them to improve their eating habits.

Statistical Analysis

Frequencies and percentages were obtained for categorical variables. We calculated measures of central tendency and dispersion for numerical variables. We used the Kolmogorov-Smirnoff test to assess the distribution of numerical variables. Crude, percentage, Z score, and percentile changes of weight, length, and BMI were evaluated from birth to 1-year-old. Sociodemographic and clinical variables and breastfeeding duration were classified into two categories; then, weight, length, and BMI changes were compared between categories using a *t*-Student or a *U* Mann–Whitney test. Several univariate linear models were carried out to identify the variables that explained the weight, length, and BMI changes. These models were adjusted for possible confounding variables such as breastfeeding, maternal age, gestational age, educational lag, educational level, type of delivery, among others. All data were analyzed using the 21st version SPSS for Windows (IBM® Corp, Armonk, NY, USA). Statistical significance was declared at $p < 0.05$.

RESULTS

A total of 186 dyads (adolescent mothers and their children) participated in the study. All pregnancies were at term. Mean maternal age was 15.5 years and the median pre-pregnancy BMI was 20. Eighteen percent of the adolescents had a job or was a student; for educational level 27.4% had elementary school and 72.6% were at junior high, 36.6% of participants were ≤15 years, and 72.1% were from very low, low, and low-median socioeconomic levels.

TABLE 1 | General characteristics of adolescent mothers and their offspring, $n = 186$ dyads.

	Mean ± SD	Range
Adolescent mother characteristics		
Age (years)	15.5 ± 1	12–17
Age at menarche (years)	11.5 ± 1	9–14
Height (cm)	155.5 ± 4	148–165.8
Pre-pregnancy BMI (kg/m^2)	20	19–22
Gestational weight gain (kg) [a]	12.4 (9.6–16.7)	−16–37
Educational lag (years) [a]	2 (0-2)	0–6
	Frequency (%)	
Sociodemographic characteristics		
Occupation [b]		
• Housewife	153 (82)	
• Had a job/student	33 (18)	
Socioeconomic level [b]		
• Very poor	8 (4.3)	
• Very low	23 (12.4)	
• Low	103 (55.4)	
• Middle-low	38 (20.4)	
• Middle	14 (7.5)	
Marital status [b]		
• Married/cohabiting	92 (49)	
• Single	94 (51)	
Education (years)	9 (8–9)	5–12
Type of delivery [b]		
• Vaginal	85 (46)	
• Cesarean section	101 (54)	
Offspring's feeding characteristics		
Timing of the introduction of complementary feeding (months) [a]	5 (4–6)	3–8
Exclusive breastfeeding (months) [a]	3 (1–7)	0–12
Offspring's characteristics at birth		
Gestational age (weeks)	39.1 ± 1	37–41.6
Sex [b]		
• Boy	71 (38)	
• Girl	115 (62)	
Anthropometric measurements		
• Weight (g)	3,039 ± 382	2,175–3,820
• Z-score weight	−0.5 ± 0.8	−2.8–1.18
• Percentile weight	34 ± 24	0.2–88.1
• Length (cm)	49.5 ± 1.5	46–53
• Z-score length	0.04 ± 0.8	−2.76–1.91
• Percentile length	51.1 ± 26	0.3–97
• BMI	12.4 ± 1	9.2–15.9
• Z-score BMI	−0.88 ± 1.2	−4–1.88
• Percentile BMI	29.6 ± 27	0–97

SD, Standard Deviation; Range, Minimum–Maximum value.
[a] *Median (25 percentile-75 percentile).*
[b] *Frequency (percentage).*

The offspring' characteristics were as follows: 46% of the children were born by vaginal delivery, and 62% were female. The mean gestational age was 39.1 weeks of gestation, mean birth weight 3,039 g, and mean length at birth was 49.5 cm (**Table 1**).

Boys and girls had similar birth weights (3,019 ± 344 vs. 3,071 ± 436 g, respectively; p = 0.366). In contrast, the length was greater among the boys than the girls were (49.7 vs. 49.2 cm, respectively; $p = 0.028$). There were no statistical differences in birth weight between mothers ≤15 years than mothers of 16 to 19 years (3,010 ± 401 vs. 3,055 ± 371, $p = 0.435$); neither did the length at birth (49.4 vs. 49.5 cm, $p = 0.603$). Low birth weight prevalence was 6.5% (12 out of 186), the timing of the introduction of complementary feeding was ≤5 months in 125 (67.2%) infants, and only 35.5% were exclusively breastfed for at least 6 months.

After 12 months of follow-up, there was only one case of low weight for age (0.5%). In contrast, 17 children (9.1%) were already in the percentiles of overweight or obesity. The rest of the children had an appropriate weight for their age. The offspring's mean weight at 12 months was lower in mothers ≤15 years compared to ≥16 years (9,307 *vs.* 9,739 g, $p = 0.003$). Infants who initiate complementary feeding before 6

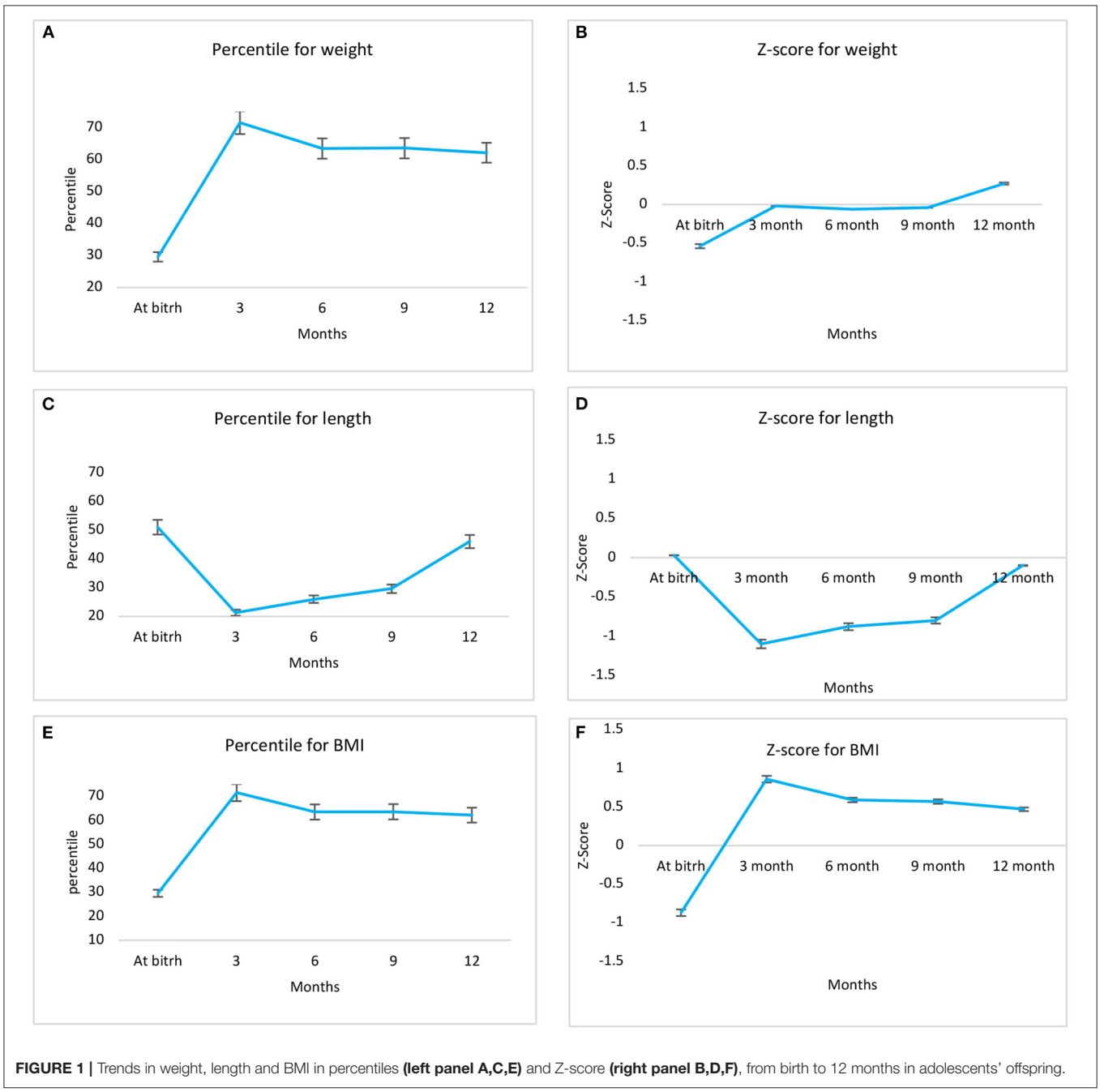

FIGURE 1 | Trends in weight, length and BMI in percentiles **(left panel A,C,E)** and Z-score **(right panel B,D,F)**, from birth to 12 months in adolescents' offspring.

months of age weighted less at 12 months of age than infants who initiated complementary feeding after 6 months of age (9,423 vs. 9,906, $p = 0.001$).

Figure 1 shows length and BMI trends (percentile and Z score) from birth to 12 months old.

Table 2 shows that adolescent's offspring from a low socioeconomic level had a mean weight change at 12 months of age lower than the children of mothers from a middle socioeconomic level. When comparing the offspring's weight at 12 months of age by mother's occupation, children from homemakers had higher weight than children of adolescent mothers who had a job or were students. Weight change during the first 12 months of life in adolescent mothers' offspring was different according to the mother's age, the timing of the introduction of complementary feeding, and the socioeconomic level.

Regarding the length measurements, the change in length Z-score during the first 12 months of life in adolescent mothers' offspring differs only by the type of breastfeeding (0.050 ± 1 vs -0.27 ± 1, $p = 0.040$), the rest of sociodemographic variables did not have any statistical significance (**Table 3**).

Table 4 shows that the change in the BMI percentile during the first 12 months of life in adolescent mothers' offspring was statistically different by socioeconomic level and the mother's occupation during pregnancy.

Only 35.5% of the children were exclusively breastfed for >6 months, and 67.2% began complementary feeding before 5 months of age. Weight, length, and BMI change at 12 months of the adolescent mothers' offspring were not statistically different according to the type of breastfeeding or the timing of the introduction of complementary feeding.

Tables 5, **6** show that weight and length at birth were the covariates that predicted the weight and BMI change at 12 months and maternal age, mother's occupation, and socioeconomic level, although with less power. Birth weight inversely predicted the anthropometric changes at 12 months according to all the performed models.

TABLE 2 | Weight change (delta) from birth to 12-months-old, according to maternal sociodemographic characteristics.

	Grams	Percentage	Z-score	Percentile
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Weight				
	6,544 ± 924	319 ± 42	0.82 ± 1	23.5 ± 31
Maternal age				
≤15 years ($n = 68$)	6,300 ± 879*	313 ± 45	0.69 ± 1	19 ± 33
≥16 years ($n = 118$)	6,684 ± 924	322 ± 41	0.89 ± 0.9	26 ± 29
Timing of the introduction of complementary feeding				
≤ 5 months ($n = 125$)	6,418 ± 912*	318 ± 45	0.77 ± 1	21.5 ± 32
≥ 6 months ($n = 61$)	6,802 ± 902	321 ± 38	0.90 ± 0.9	27.6 ± 28
Breastfeeding at 6 months old				
Exclusive ($n = 66$)	6,560 ± 902	317 ± 40	0.78 ± 1	24.5 ± 32
Non-exclusive ($n = 120$)	6,531 ± 942	320 ± 44	0.83 ± 1	22.8 ± 30
Education level				
Elementary school ($n = 51$)	6,559 ± 861	320 ± 40	0.88 ± 0.9	25.7 ± 25
High school ($n = 135$)	6,538 ± 948	319 ± 44	0.79 ± 1	22.7 ± 33
Educational lag				
No ($n = 54$)	6,364 ± 993	315 ± 45	0.74 ± 1	19 ± 35
Yes ($n = 132$)	6,617 ± 888	321 ± 42	0.85 ± 1	25 ± 29
Socioeconomic level				
Low ($n = 134$)	6,436 ± 906*	315 ± 42*	0.71 ± 1*	20.6 ± 30*
Middle ($n = 52$)	6,820 ± 922	330 ± 43	1.08 ± 1	31.0 ± 33
Mother's occupation during pregnancy				
Home wife ($n = 153$)	6,564 ± 949*	320 ± 42	0.85 ± 1*	24.5 (31)**
Student ($n = 33$)	6,456 ± 817	314 ± 43	0.68 ± 1	19.4 (33)
Mother's occupation at 4 months postpartum				
Home wife ($n = 164$)	6,592 ± 935*	320 ± 45	0.87 ± 1*	25.5 (31)
Student ($n = 22$)	6,185 ± 764	307 ± 42	0.43 ± 1	8.9 (32)
Marital status				
Single ($n = 94$)	6,423 ± 931	316 ± 44	0.72 ± 1	26.8 (33)
Married ($n = 92$)	6,667 ± 906	322 ± 40	0.91 ± 1	26.2 (28)

SD, Standard Deviation.
**p <0.050 **p ≤0.001.*

DISCUSSION

Birth weight is a health indicator of the offspring; it reflects the quality of nutrition during pregnancy, predicts immediate survival, and is essential to assess subsequent growth. In our study, the mean birth weight was 3,039 ± 382 g, which is very similar to that reported in children of adolescent mothers in Turkey, where the weight of the newborns was 2,934 g for mothers ≤15 years and 3,021 g for the children of mothers from 16 to 19 years (21). In contrast, the mean birth weight reported for Mexican children of adult mothers in Mexico City and the metropolitan area is 3,200 ± 373 g (22, 23). According to the previous data, the offspring in our study weighted 200 g less than these reports (24, 25). The offspring's anthropometric measurements can be influenced by several factors, including pre-pregnancy weight, excessive gestational weight gain, parity, gender, and gestational age (10).

Differences in pre-pregnancy body composition and gestational weight gain may have contributed to birth weight (10, 26). Studies show that adolescents tend to have low birth weight offspring, which suggests that the pregnant adolescent should receive prenatal care to look after the offspring's weight. Therefore, health care professionals should offer special attention to younger adolescents, who are the most likely to have a higher risk of adverse pregnancy outcomes. In addition, the children of adolescent mothers in developing countries are at a disadvantage at birth and during breastfeeding. In this sense, strategies should strengthen measures to prevent adolescent motherhood and to help to improve the nutrition and education of their children.

Also, offspring of mothers <15 years old have a higher risk of low birth weight. This finding is consistent with evidence

TABLE 3 | Change of length (delta) according to sociodemographic characteristics.

	Centimeters	Percentage	Z-Score	Percentile
	Median (p25–p75)	**Mean (SD)**	**Mean (SD)**	**Median p25–p75**
Length (cm)	24 (22.5–26)	149 (6)	−0.14 (1)	−5 (32)
	Median (p25–p75)	**Median (p25–p75)**	**Median (p25–p75)**	**Median p25–p75**
Maternal age				
≤15 years ($n = 68$)	23.5 (22–26)	148 (144-152)	−0.32 (−1.28-0.48)	−8.2 (−37–12)
≥16 years ($n = 118$)	24.5 (23-26)	149 (145-153)	−0.14 (−0.85-0.70)	−3.6 (−24–17)
	Median (p25–p75)	**Mean (SD)**	**Mean (SD)**	**Mean ± SD**
Timing of the introduction of complementary feeding				
≤5 months ($n = 125$)	24.5 (22–26)	148 (6)	−0.21 (1.3)	−5 (31)
≥6 months ($n = 61$)	24.0 (22–26)	149 (6)	−0.02 (1)	−4 (36)
Breastfeeding at 6 months				
Exclusive ($n = 66$)	24.5 (23–26)	150 (6)	0.05 (1)*	0.4 (35)
Non-exclusive ($n = 120$)	24.0 (22–26)	148 (6)	−0.27 (1)	−8.4 (30)
Education level				
Elementary school ($n = 48$)	24.0 (22–26)	149 (6)	−0.08 (1)	−3.0 (34)
High school ($n = 138$)	24.0 (22–26)	149 (6)	−0.17 (1)	−5.7 (32)
Educational lag				
No ($n = 54$)	23.7 (22–26)	148 (7)	−0.30 (1)	−7.8 (35)
Yes ($n = 132$)	24.3 (22.5–26)	149 (6)	−0.01 (1)	−3.8 (32)
Socioeconomic level				
Low ($n = 134$)	24.0 (22.5–26)	149.1 (6)	−0.16 (1)	−4.9 (32)
Middle ($n = 52$)	24.5 (22.5–26)	149.4 (6)	−0.10 (1)	−5.2 (33)
Mother's occupation during pregnancy				
Home wife ($n = 153$)	24.5 (22–24.5)	149 (6)	−0.12 (1)	−4.2 (33)
Student ($n = 33$)	23.5 (23–25.7)	149 (5)	−0.24 (1)	−8.6 (26)
Mother's occupation at 4 months postpartum				
Home wife ($n = 164$)	24.0 (22.5–26.0)	149 (6)	−0.14 (1)	−4.6 (29)
Student ($n = 22$)	23.7 (22.7–26.2)	149 (6)	−0.20 (1)	−8.4 (29)
Marital status				
Single ($n = 94$)	24.0 (22–26)	148 (7)	−0.19 (1)	−5.4 (33)
Married ($n = 92$)	24.5 (22.5–26)	149 (6)	−0.10 (1)	−4.5 (32)

*$p < 0.040$.

showing that adolescent pregnancy is detrimental to the newborn (27–30). However, the low birth weight rate observed in our study is 6.5%, a rate that is lower than the established by the WHO (10% maximum) (31, 32). According to the WHO, in 2000, the prevalence of low birth weight in developed countries was close to 7%, while in South America, it was 9.6%. In 2015, this rate in Latin America and the Caribbean was 8.7% (33). The low rate of low birth weight that we obtained in our study could be attributed mainly to the quality and number of prenatal care visits (34, 35) received at INPer.

One of the main findings of this study was that birth weight and length at birth may predict the weight gain and BMI changes at 12 months old in the offspring of adolescent mothers. Nevertheless, in all statistical models performed, birth weight was the predictor variable on the anthropometric parameters' changes at 12 months old. Regarding the weight and length change during the first 12 months of life, both remained within the parameters of expected gain. However, the length change was slightly lower, although always within the parameters recommended by the WHO (17). Our results agree with that published by Chen et al. (36), who demonstrated that children from adolescent mothers had statistically significant lower weight ($p < 0.001$) and length ($p < 0.001$), although the speed and slope of growth were similar over time when compared with growth standards, and with data from the children of adult mothers. Wu et al. (37) also reported no significant statistical differences between children of adolescent mothers and children of adult mothers regarding children's growth and development

TABLE 4 | Change of BMI (delta) according to maternal sociodemographic characteristics.

	BMI	Percentage	Z-Score	Percentile
	Median (p25–p75)	**Median (p25–p75)**	**Median (p25–p75)**	**Median p25–p75**
BMI	4.6 (3.7-5.9)	137 (127-151)	1.1 (0.36-2.1)	31.5 (127-151)
Maternal age				
≤15 years ($n = 68$)	4.4 (3.6-5.8)	135 (127-152)	0.9 (0.3-2.3)	26 (5.5-56)
≥16 years ($n = 118$)	4.8 (3.7-5.9)	138 (128-151)	1.2 (0.4-1.2)	34 (10-56)
Timing of the introduction of complementary feeding				
≤5 months ($n = 125$)	1.6 (3.6-6.1)	137 (127-153)	1.0 (0.3-2.3)	29.8 (7.8-57.7)
≥6 months ($n = 61$)	4.8 (3.8-5.6)	138 (129-148)	1.3 (0.5-1.9)	34.8 (14-52.9)
Breastfeeding at six months				
Exclusive ($n = 66$)	4.1 (3.5–5.4)	134 (126–147)	0.8 (0.3–1.8)	24.8 (5.4–54.6)
Non-exclusive ($n = 120$)	4.9 (3.8–6.0)	139 (128–155)	1.3 (0.5–2.3)	34.4 (11–56.6)
Education level				
Elementary school (<8 y) ($n = 48$)	4.4 (3.8–5.6)	136 (128–147)	1.0 (0.3–1.8)	33.8 (128–147)
High school (>9 y) ($n = 15$)	4.8 (3.6–5.9)	138 (127–154)	1.1 (0.4–2.2)	31.4 (9.3–55.3)
Educational lag				
No ($n = 54$)	4.7 (3.6–6.0)	138 (127–152)	1.4 (0.4–2.3)	32.2 (11.3–57.1)
Yes ($n = 132$)	4.6 (3.7–5.8)	137 (127–150)	1.0 (0.3–2.0)	29.5 (8.4–55.1)
Socioeconomic level				
Low ($n = 134$)	4.4 (3.6–5.6)*	135 (127–148)	0.98 (0.3–1.8)	28.2 (6.2–52)*
Middle ($n = 52$)	5.2 (3.9–6.8)	141 (132–156)	1.5 (0.6–2.5)	39.6 (11.2–74)
Mother's occupation during pregnancy				
Homewife ($n = 153$)	4.7 (3.8–6.0)	137 (128–151)	1.1 (0.45–2.3)	36.6 (11.3–57.1)*
Student ($n = 33$)	4.2 (3.2–5.3)	133 (123–148)	0.7 (0.1–1.7)	18.6 (2.5–34.6)
Mother's occupation at 4 months postpartum				
Homewife ($n = 164$)	4.7 (3.7–5.9)	138 (128–151)	1.1 (0.4–2.2)	33.2 (10–56)
Student ($n = 22$)	4.1 (3.2–5.3)	132 (123–148)	0.7 (0.1–1.7)	19 (2.5–54)
Marital status				
Single ($n = 94$)	4.6 (3.7–5.8)	137 (128–150)	1.1 (0.3–1.9)	31.6 (5.6–55.1)
Married ($n = 92$)	4.7 (3.7–6.0)	137 (127–153)	1.0 (0.4–2.3)	31.4 (11.1–56.0)

*$p < 0.050$.

parameters. These promising results are probably due to factors such as higher maternal education, as well as more favorable living conditions, as demonstrated by Luster et al. (38), in their study with children of teenage mothers and adult mothers from Michigan, USA.

Regarding exclusive breastfeeding, its frequency in our study group was 35.5%, which contrasts with that reported by Wambach and Cole (39), who discuss that adolescent mothers breastfeed for a shorter time than adult mothers. On the other hand, according to the National Health and Nutrition Survey 2012 (ENSANUT-2012) (40), in Mexico, the prevalence of exclusive breastfeeding in the general population was 18.5% in rural areas and 14.5% in urban areas. These figures are lower than what we observed in our study group. An explanation for these contrasting numbers is that INPer is a "child- and woman-friendly hospital," where one of its primary purposes is to promote exclusive breastfeeding for at least 6 months.

Although the prevalence of exclusive breastfeeding was higher than that reported in urban areas of Mexico, it was not associated with weight gain in the adolescents' offspring at 12 months age, which is consistent with previous studies that show that offspring who are exclusively breastfed have a slower growth rate than that reported in infants who are fed with formula milk (41, 42).

Other research also has shown that the duration of breastfeeding is associated with slower growth during the first 12 months of the infant's life, which may contribute to the protective effects of breastfeeding against overweight and obesity at younger ages (43–46).

Our study highlighted that the timing of the introduction of complementary feeding was not associated with weight, length, and BMI changes of the children adolescent mothers at 12 months old. These findings are consistent with those reported by Dewey et al., in a systematic review of the efficacy and effectiveness of complementary feeding interventions in developing countries (47) as well as in the study by Woo et al., which included children of adolescent mothers from Mexico, the United States and China (48). In addition to the studies carried out by Briefel et al. (49), and by Chen et al. (36), in a cohort of adolescent mothers from Taiwan, in which the researchers discussed that this behavior might

TABLE 5 | Univariate general linear model to determine the predictor variables of weight change parameters.

Covariates	Beta	Confidence interval 95%	*p*-value	Eta square partial	R^2 adjusted
Weight change in z-score					0.386
Weight at birth	−0.002	−0.002–0.001	0.000	0.362	
Length at birth	0.135	0.048, 0.221	0.002	0.050	
Mother's occupation	−0.99	−0.608, 0.011	0.059	0.020	
Maternal age	0.299	0.055, 0.543	0.017	0.031	
Weight change in percentage (%)					0.555
Weight at birth	−0.090	−0.103, −0.178	0.000	0.525	
Length at birth	4.739	1.672, 7.806	0.003	0.049	
Socioeconomic level	3.422	−3.313, 0.245	0.154	0.011	
Mother's occupation	−9.331	−20.17, 1.613	0.094	0.015	
Maternal age	10.686	1.950, 19.422	0.017	0.031	
Weight change in percentile					0.323
Weight at birth	−0.048	−0.059,−0.036	0.000	0.274	
Length at birth	0.539	−0774, 6.304	0.012	0.034	
Mother's occupation	−16.606	−26.51, −6.703	0.001	0.057	
Maternal age	8.948	1.139, 16.139	0.025	0.027	
Weight change, crude (g)					0.112
Weight at birth	−0.529	−0.913, −0.145	0.007	0.039	
Length at birth	150.992	57.63, 244.35	0.002	0.054	
Mother's occupation	−338.79	−671.65, −5.94	0.046	0.022	
Socioeconomic level	90.146	−55.33, 235.6	0.223	0.008	
Maternal age	347.571	81.62, 613.51	0.011	0.036	

TABLE 6 | Univariate general linear model to determine the predictor variables of BMI change parameters.

Covariates	Beta	Confidence interval 95%	*p*-value	Eta square partial	R^2 adjusted
BMI change in z-score					0.543
Weight at birth	−0.003	−0.004, −0.003	0.000	0.536	
Length at birth	0.471	0.357, 0.585	0.000	0.269	
Mother's occupation	−0.369	−0.776, 0.038	0.075	0.017	
Socioeconomic level	0.130	−0.046, 0.305	0.146	0.012	
BMI change in percentage (%)					0.561
Weight at birth	−0.046	−0.052, −0.040	0.000	0.557	
Length at birth	6.307	4.852, 7.762	0.000	0.288	
Socioeconomic level	1.852	−0.386, 4.090	0.104	0.015	
Mother's occupation	−3.656	−8.845, 1.533	0.166	0.011	
BMI change in percentile					0.369
Weight at birth	−0.067	−0.081, −0.054	0.000	0.351	
Length at birth	9.020	5.763, 12.278	0.000	0.142	
Mother's occupation	−13.98	−25.60, −2.36	0.019	0.030	
Socioeconomic level	3.522	−1.489, 8.489	0.167	0.011	
BMI change, crude (kg/m^2)					0.408
Weight at birth	−0.004	−0.005, −0.003	0.000	0.398	
Length at birth	0.550	0.380, 0.721	0.000	0.184	
Mother's occupation	−0.520	−1.127, 0.087	0.093	0.016	
Socioeconomic level	0.211	−0.051, 0.473	0.113	0.014	

be due to the adverse socio-economic context in which adolescent mothers live, where the quality and quantity of food is compromised. However, one of the limitations of our study was the lack of information available on this topic. Also, the follow-up was only for 12 months to prove the impact of the timing of the introduction

of complementary feeding and the practice of exclusive breastfeeding on the change in weight and length in the children of adolescent mothers.

Our finding that adolescent mothers who are engaged in household activities had offspring with a higher weight (9,648 ± 978) at 12 months old than offspring of adolescents who had a job or were students (9,648 ± 978 vs. 9,273 ± 903, respectively; $p = 0.044$). This finding adds up to the understanding that the care of the infant by its mother translates into better growth (36, 50). Therefore, others factors like socioeconomic level, mother's occupation, and the timing of the introduction of complementary feeding might be associated with weight, length, and BMI change in children of mothers of any age, not only in adolescents.

Strengths and Limitations

The strength of the study is that the potential confounders were collected prospectively. Also, the longitudinal study design permits the assessment of causal relationships.

One of the study's limitations is that we do not have data regarding diet or nutritional supplementation use during pregnancy and other aspects of the offspring's diet (in addition to breastfeeding) and paternal factors, so it is not known if we can rule out residual confounders.

Conclusions

Weight at birth negatively predicted offspring weight and BMI changes at 12 months, while length at birth positively predicted the changes. Only exclusive breastfeeding was associated with length Z-score change in adolescents' offspring in their first 12-months of life.

The offspring of adolescent mothers had lower weight and height, despite the fact that the speed and slope of the growth patterns were similar over time with the growth standards established by the WHO.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The Institutional Review Board and Ethics Committees from INPer approved the study with registration number 212250-49451. The participant's legal guardian/next of kin provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS, HM-R, and GC-B: Conceptualization. RS, HM-R, GC-B, and MH-T: Data curation and Supervision. RS, HM-R, GC-B, MH-T, MB, and ML-V: Formal analysis. RS: Funding acquisition and project administration. RS, HM-R, GC-B, MH-T, MB, ML-V, and JD: Investigation. RS, HM-R, GC-B, MH-T, MB, ML-V, GG-L, JD, and CM-G: Methodology, writing – original draft and writing – review editing. RS, HM-R, and GC-B: resources and validation. RS, HM-R, GC-B, MH-T, and ML-V: Visualization. All authors contributed to the article and approved the submitted version.

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Comparison of Three Gestational Weight Gain Guidelines Under Use in Latin America

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Presently, three guidelines are used in Latin America to assess adequacy of maternal body mass index (BMI) during pregnancy: (1) the chart proposed by the Institute of Medicine of the United States (IOM), (2) the Rosso-Mardones Chart (RM), and (3) a modified RM chart proposed by Atalah et al. (AEA). The aim of the present review was to explore available information on the sensitivity, specificity, and both positive (PPV) and negative predictive values (NPV) of these charts to detect women at risk of delivering babies with the following signs of abnormal fetal growth: (a) length at birth (BL) <50 cm; (b) birth weight (BW) <3,000 g; and (c) BW ≥ 4,000 or 4,250 g. Data from studies conducted in large samples of Chilean and Uruguayan women indicate that the RM chart has the greatest sensitivity to identify at risk cases. However, predictive values were similar for the three charts. Thus, the use of the RM chart should be preferred. The main limitation for using the IOM weight gain recommendations in Latin American women stems from the fact that their average height is approximately 20 cm lower than US women.

Keywords: guidelines, Latin America, gestational, weight, gain

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INTRODUCTION

Over the last three decades, in most Western countries the proportion of overweight and obese women of reproductive age [body mass index (BMI) 25.0 kg/m² or more] underwent a substantial increase (1). In Chile the proportion of obese women (BMI 30.0 kg/m² or more) was 11% in 1988 and 37% in 2017 (2, 3). A similar trend has been reported in the USA (4, 5). One of the factors contributing to this obesity epidemic would be an excessive weight gain during successive pregnancies (4), an observation that highlights the importance of monitoring this aspect during pregnancy.

Body weight gain in a gravida reflects both maternal physiological adaptations and growth of the fetus, placenta, and accumulation of amniotic fluid. The main maternal adaptations include blood volume expansion and body fat accumulation (6).

Women who are either overweight or underweight at conception are at risk of maternal-fetal complications (1, 6, 7). In obese women pregnancy complications include hypertension, gestational diabetes, dystopian childbirth, and fetal macrosomia. In women with low weight/height the main complication is fetal growth retardation. The risk of these complications increases if obese women gain an excessive amount of weight and thin women gain little weight during their pregnancies.

In both situations the offspring have a higher incidence of metabolic syndrome later in life. Consequently, assessment of maternal weight/height adequacy in early pregnancy and monitoring weight gain during pregnancy are considered key aspects of maternal and child health care (1, 6–8).

Despite consensus regarding the importance of maternal gestational weight gain an agreement has yet to be reached concerning its quantitative aspects. Consequently, a universally used instrument (chart) of desirable weight gain for a given maternal weight/height at conception is lacking (9). Currently, most countries in the Northern Hemisphere use the guidelines of the United States Institute of Medicine (IOM) while most Latin American countries use the Rosso-Mardones instrument (RM) or its modification authored by Atalah et al. (AEA) (9–12). The main objective of this mini review was to compare the accuracy of these instruments to identify pregnancies at risk of fetal growth alterations in Latin American women.

GUIDELINES FOR ANTHROPOMETRIC ASSESSMENT OF MATERNAL NUTRITIONAL STATUS

Available guidelines for adequacy of maternal weight/height use the Quetelet Index, also known as BMI (8) and individual targets for weight gains during pregnancy.

United States Institute of Medicine Guidelines

These guidelines were developed in 1990, but the cut-off points for appropriate BMI were modified in 2009 following World Health Organization (WHO) recommendations for adult non-pregnant women (8, 10, 13). Those BMI cut-offs are calculated pre-conceptionally by asking the women about their usual weight and also measuring their height. The various categories of maternal nutritional status are as follows: (a) Low weight: BMI < 18.5 kg/m^2; (b) Normal: BMI 18.5–24.9 kg/m^2; (c) Overweight: BMI 25–29 kg/m^2; and (d) Obese: BMI $\geq$30 kg/m^2 (8, 10). For these categories, the IOM recommends the following weight gains during pregnancy: (a) Women with "low weight" should gain 12.5–18.0 kg. (b) Women with "normal" BMI should gain 11.5–16 kg. (c) Overweight women should gain 7.0–11.5 kg. (d) Obese women should gain 5.0–9.0 kg. The 2009 IOM guidelines considered for the first time the outcomes of both mother and child during and after delivery and the trade-offs between them (4); the recommended weight gain ranges were those most consistently associated with good outcomes, including reduced post-partum weight retention.

WHO's weight/height ratio categories are based on the relationship between BMI and mortality or life expectancy in adult non-pregnant women. The lower risk of mortality is associated with a BMI between 18.5 and 24.9 kg/m^2 (8). Thus, *strictu senso* they do not represent "normalcy" in a pregnancy situation. Institute of Medicine's recommendations are based on measurements made in a racially mixed general US population. This is certainly advantageous for a worldwide use, but average height of US women is 176 cm (10, 13). Thus, it is significantly higher than women living in the Southern Hemisphere. For example, average height of adult women in Ecuador is 152 cm and in Chile 156 cm (14, 15). This aspect is relevant for two reasons. Firstly, because maternal height is directly and significantly associated with the offspring birth weight (BW) (1, 6, 7, 16). Additionally, because desirable gestational weight gain might differ greatly according with maternal height. For example, a mother who is 140 cm tall and has a "normal" BMI at the beginning of her pregnancy must gain only 10.8 kg to reach term with a normal BMI of 27.6 kg/m^2. However, a woman who measures 180 cm and has a "normal" BMI when she becomes pregnant, to reach at term a normal BMI of 27.6 kg/m^2 must gain 18.1 kg. These cases, who are not uncommon, illustrate the importance of establishing weight gains proportional to maternal height.

The IOM guidelines have proved their usefulness in developed countries. For example, a recent systematic review and meta-analysis of more than 1 million pregnant women, showed that gestational weight gain greater than or less than guideline recommendations, compared with weight gain within recommended levels, was associated with higher risk of adverse maternal and infant outcomes (17). From 23 selected studies, 18 were retrospective, and five were prospective. Ten were from the United States, eight were from Asia (four from China, two from Korea, and one each from Taiwan and Japan), and five were from Europe (one each from Norway, Belgium, Italy, Denmark, and Sweden). Sample sizes ranged from 1,034 to 570,672 women. However, in a large German population of overweight and obese mothers those who gained weight within the IOM recommendations had a lower incidence of pre-eclampsia and fewer non-elective cesarean deliveries, but higher risk for gestational diabetes, small-for-gestational-age birth, pre-term delivery, and perinatal mortality (18). Thus, IOM recommendations would be adequate for underweight and normal weight mothers, but different guidelines or thresholds might be more appropriate for overweight and obese ones (1).

Rosso-Mardones Guidelines

These guidelines were based on a study conducted on the early 1980s in 1,745 healthy Chilean women who had uneventful pregnancies and full-term deliveries. This group was representative of a Chilean general population. Average height of the study subjects was 154 cm. Data was used to establish recommendations for the entire range of BMIs beginning in the 10th week of gestation (11) **(Figure 1).** The recommendations were aimed at an outcome of a baby with a "normal" or "desirable BW," defined as the average BW of the babies born at term, delivered by healthy women, with normal weight/height at the beginning of pregnancy and weight increases considered appropriate for their heights.

The area of "normality" of BMI at the end of pregnancy was defined as one that favors the occurrence of "desirable BWs" (11). We deemed this BMI the maternal "critical body mass" for normal Chilean women, since it would allow optimal fetal growth, as determined by genetic and epigenetic factors. The diagnosis of "low weight" and maternal "overweight" corresponds to mothers whose BMI is below and above this

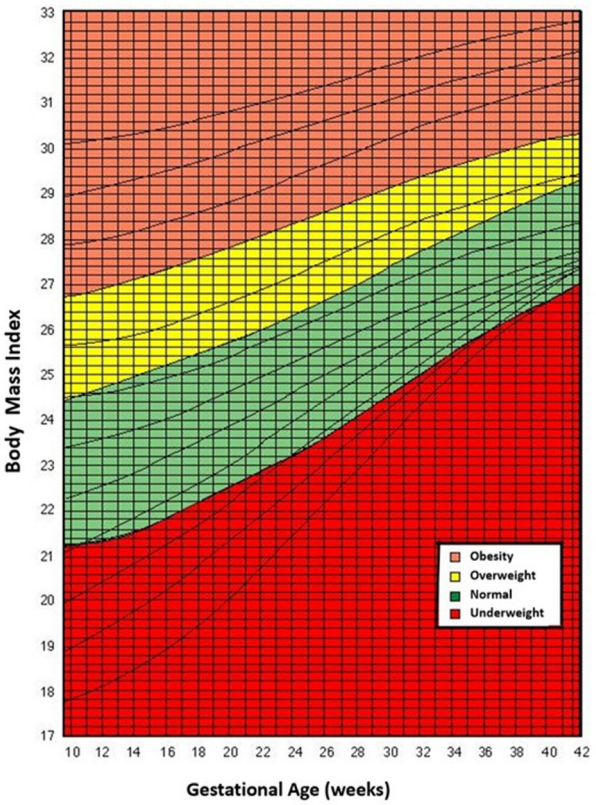

FIGURE 1 | Rosso-Mardones chart.

TABLE 1 | Body mass index (BMI) kg/m^2 cut-off points of the RM chart and the AEA chart for the nutritional classification of women at the beginning and at the end of pregnancy (11, 12).

BMI kg/m^2 cut-offs points	RM chart	AEA chart
Week 10		
Underweight	<21.15	<20.2
Normal	21.15–24.49	20.2–25.2
Overweight	24.50–26.73	25.3–30.2
Obese	>26.73	>30.2
Week 40		
Underweight	<26.55	<25.0
Normal	26.55–28.90	25.0–29
Overweight	28.91–30.03	29.1–33.1
Obese	>30.03	>33.1

"critical body mass." Body mass index cut-offs values of the RM chart are presented in **Table 1** at the beginning and at the end of pregnancy.

Atalah et al. Guidelines

The Chilean Ministry of Health used the RM Chart from 1986 to 2004. In 2005 it was replaced by a modified version proposed by Atalah et al. (12). **Table 1** presents BMI cut-offs of this chart in comparison with the RM chart; there is a clear increment of the normal area in the AEA chart at the beginning and at the end of pregnancy. In contrast with the RM chart, the AEA chart is based on the categories of BMI defined by the IOM. Consequently, the AEA chart classifies as "normal" a percentage of women that according to the RM chart should have been classified as either "underweight" or "overweight." Consequently, from 2005 on, the use of AEA Guidelines meant a marked apparent decrease in the number of pregnant women with "low weight" and "overweight" registered by the Chilean Ministry of Health. Accordingly, these women did not receive the nutritional counseling and support aimed at risk women.

Diagnostic Ability of the Three Instruments

The consequences of underestimation and overestimation of pregnancies at nutritional risk by the various guidelines previously analyzed have been investigated by comparing the sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of the IOM, RM, and AEA guidelines, in pregnant populations of Chile and Uruguay (19–21). In this case the "disease" was defined as the presence of inadequate fetal growth: (a) length at birth (BL) <50 cm; (b) BW <3,000 g; and (c) BW ≥4,000 or 4,250 g.

Sensitivity indicates the proportion of correctly diagnosed cases with the disease and specificity the proportion of healthy cases correctly diagnosed. The PPV indicates the probability that the patient has the disease and the NPV allows knowing the probability that the patient does not have the disease.

The first of these studies was carried out using data obtained in 11,465 healthy Chilean women with singleton pregnancies and gestational age of delivery 39–41 weeks (19). A total sample of 27,613 women with anthropometric and health information was recruited in that study and a subsample of 11,466 healthy pregnant women was selected from the total sample as a control to ascertain the effect of maternal nutritional status on the newborns growth excluding the effect of other factors. The adequacy of the maternal BMI at the beginning of pregnancy was diagnosed by applying the cut-off points of the AEA and RM charts. The comparison of the RM and AEA charts was based on the proportion of children with inadequate fetal growth (according to the indicators described above) in the categories of mothers with low weight and obesity detected by these charts. The RM chart showed higher sensitivity values for diagnosing mothers at risk than the AEA chart, although the predictive values were similar (**Table 2**). Despite this similarity in the proportions of PPV and NPV, the PPV differs in the number of subjects diagnosed with impaired fetal growth investigated by the RM chart, since it reaches almost double those investigated by the other chart, a situation that is repeated in the two studies discussed below (20, 21).

The second comparative study of the RM and AEA charts was carried out in Uruguayan women (20). Data from 23,832 healthy pregnant women, with single deliveries and gestational age of delivery between 39 and 41 weeks were used. The adequacy of

TABLE 2 | Sensitivity, specificity, and positive and negative predictive values for RM and AEA charts corresponding to each target event* (BL < 50 cm; BW < 3,000g and BW > 4,250 g) in the total sample ($n = 27,613$) (19).

Target event	Chart	Sensitivity	Specificity	PPV	NPV
BL < 50 cm	RM	0.17	0.87	0.54	0.54
	AEA	0.10	0.93	0.56	0.53
BW < 3,000 g	RM	0.19	0.86	0.28	0.79
	AEA	0.12	0.92	0.29	0.79
BW > 4,250 g	RM	0.73	0.51	0.05	0.98
	AEA	0.65	0.59	0.05	0.98

BL, birth length; BW, birth weight; PPV: positive predictive value, NPV: negative predictive value.

BMI in early pregnancy was classified using the AEA and RM charts to define nutritional status at the beginning of pregnancy. When comparing the sensitivity, specificity, and PPV and NPV of both patterns to detect women at risk of inadequate fetal growth, the RM chart again showed higher sensitivity values and predictive values similar to the AEA chart.

The third study in this series consisted of a comparison of the IOM and RM charts in the Uruguayan pregnant population of the previous study, using a design, criteria, and definitions similar to those previously described (21). The RM curve showed significantly higher sensitivity values than the IOM criterion. The predictive values of both charts were also similar.

The three studies presented showed lower specificity values for the RM chart. Since in the three studies the more sensitive RM chart had a much higher number of at risk BW and BL cases in the underweight and obese categories, it is preferable to sacrifice the higher specificity of the AEA or the IOM charts and use the RM chart.

DISCUSSION

A growing body of evidence suggests that both high and low gestational weight gains are independently associated with an increased risk of child obesity (22). Multiple randomized controlled trials have been conducted evaluating the efficacy of lifestyle interventions on gestational weight gain, and while those interventions may alter gestational weight gain, they have not been associated with improvement in perinatal outcomes (23). The revised comparisons of the three guidelines permitted to assess improved perinatal outcomes when using the RM chart. Although the comparisons did not use an experimental design, the compared groups had exactly the same control variables; the comparison of identical cohorts is similar to a randomized controlled trial. Those results reveal the public health importance of using the RM chart in the populations proposed.

Possible additional interventions that might improve the effect of gestational weight gain on perinatal outcomes have been the following: (A) Weight loss during pregnancy because it has been associated with decreased risks of macrosomia and cesarean section; however, given an association with low BW, it is not currently recommended (23). (B) Research supports the need to achieve a healthy weight pre-conceptionally. In some studies, pre-pregnancy BMI is strongly related to health outcomes in mother and offspring, with even stronger effects of pre-pregnancy BMI than of gestational weight gain on key outcomes (24).

As indicated by the results and conclusions of a recent seminar on maternal nutrition (25), this is an area of evolving studies. There are numerous gaps of knowledge and unresolved scientific debates on the nutritional requirements of the pregnant woman and, to complicate matters further, ostensible cultural changes are underway, expressed in aspects such as the age of the first pregnancy, the massive incorporation of women into the workforce, a greater interest in nutrition and "healthy" foods, etc. All of these developments open new possibilities and pose unprecedent challenges for the nutritional care of pregnant women. Significantly, perhaps reflecting the fact that, for many specialists, the issue of nutritional assessment of pregnant women has been resolved, this issue was not included in the previously alluded seminar (25).

However, the available evidence indicates, that maternal nutrition is an area that requires urgent attention and a "fresh look" at the strength of the scientific support for public policies. The IOM Guidelines appears to be suitable for the U.S. population and for other regions where pregnant women have a similar average height. However, they could be improved by introducing specific weight recommendations for pregnant women whose heights considerably deviates from average. Therefore, its use in populations with average heights of pregnant women <160 cm does not seem advisable.

As the Chilean experience shows, seemingly minor changes in the evaluation criteria can cast long shadows in terms of their effects at the population level. A recent publication shows that in Chile the frequencies of babies with BW <3,000 g and birth length <50 cm increased markedly after 2005, coinciding with the replacement of the RM Chart by the AEA Chart (26). Thus, suggesting a causal relationship. The replacement of the RM Guidelines by the AEA Guidelines has meant a decrease capacity to correctly diagnose and treat mothers at risk of having newborns with low weight or excessive BW. This finding has important implications, including the well-known U-shaped relationship of BW with neonatal and infant mortality (27). Both children weighing <3,000 g and body length <50 cm at birth and those with a BW ≥4,250 or ≥4,000 g are at a higher risk of complications and dying than those of normal weight.

In addition, solid scientific evidence supports the possibility that inadequate fetal growth, manifested in a low or excessive BW (macrosomy), is associated with the early origin of chronic diseases of adults, such as obesity, arterial hypertension, and diabetes (28, 29). Hence the importance of early detection and effective treatment of mothers at nutritional risk of inadequate fetal growth. From this standpoint, because of its greater diagnostic sensitivity for mothers at nutritional risk, and because of its ease of use in populations that have an average height lower than the US population, the RM Guidelines offer significant advantages over other guidelines (1, 6).

AUTHOR CONTRIBUTIONS

FM and PR contributed to conception and design of the study. AE and MF organized the references of information. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Breastfeeding Contributes to Physiological Immune Programming in the Newborn

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The first 1,000 days in the life of a human being are a vulnerable stage where early stimuli may program adverse health outcomes in future life. Proper maternal nutrition before and during pregnancy modulates the development of the fetus, a physiological process known as fetal programming. Defective programming promotes non-communicable chronic diseases in the newborn which might be prevented by postnatal interventions such as breastfeeding. Breast milk provides distinct bioactive molecules that contribute to immune maturation, organ development, and healthy microbial gut colonization, and also secures a proper immunological response that protects against infection and inflammation in the newborn. The gut microbiome provides the most critical immune microbial stimulation in the newborn in early life, allowing a well-trained immune system and efficient metabolic settings in healthy subjects. Conversely, negative fetal programming by exposing mothers to diets rich in fat and sugar has profound effects on breast milk composition and alters the immune profiles in the newborn. At this new stage, newborns become vulnerable to immune compromise, favoring susceptibility to defective microbial gut colonization and immune response. This review will focus on the importance of breastfeeding and its immunological biocomponents that allow physiological immune programming in the newborn. We will highlight the importance of immunological settings by breastfeeding, allowing proper microbial gut colonization in the newborn as a window of opportunity to secure effective immunological response.

Keywords: breastfeeding, maternal programming, microbiome, newborn, immunity

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INTRODUCTION

In humans, the prenatal life (280 days) together with the following 2 years outside the womb (730 days) encompass "the first 1,000 days," which define a physiologically plastic and vulnerable time-window where adverse health outcomes that may affect life in the future are programmed (1, 2). Women nutritional state before and during pregnancy have profound and long-lasting consequences for the proper development of the fetus, which is known as "fetal programming" (1). After birth, nutrition of an infant is critical to define the optimal growth, development, and future health of the individual later in life (1). Defective fetal programming promotes

non-communicable chronic diseases in the newborn. Conversely, postnatal interventions such as breastfeeding during the first 1,000 days might mitigate risk factors and prevent metabolic and immune-related pathologies. In this regard, breast milk has been classified as the gold standard for infant nutrition during early postnatal life. According to the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), breastfeeding must provide nutritional support to the newborn no later than 1 h after birth and keep it as exclusive feed for at least the first 6 months, and then be supplemented with solid foods until 2 years of age or longer (2, 3). In fact, breastfeeding for the first 6 months of the life of an infant decreases the risk of overweight and obesity, type 2 diabetes (T2D), and other non-communicable chronic diseases in the infant (4–7).

Breast milk contains hundreds to thousands of distinct bioactive molecules that protect against infection and inflammation and contribute to immune maturation and proper organ development (8). Notably, breastfeeding also provides a source of bacterial colonization of the gut of the infant (9, 10). A healthy microbiota allows proper immune training in the newborn and immunogenic response under a future challenge in adulthood (9, 10). In contrast, using milk formula during lactation favors inadequate immune response and susceptibility to metabolic and immune-related pathologies in the newborn (1, 3). Also, maternal exposure to energy-dense foods might negatively change the immune composition of the milk and promote defective activation of the immunogenic response and immune maturation in the newborn (11–18). Overall, breastfeeding is a critical intervention that defines selective immunogenic programming settings and microbial colonization in the gut of the newborn, and prepares them to face several future health risks.

In the present contribution, we will focus on breast milk as the source of a plethora of bioactive molecules that provide immune maturation and healthy microbial gut colonization in the newborn. In a parallel scenario, we will provide compelling experimental evidence confirming that maternal exposure to energy-dense foods alter the immunogenic composition of breast milk and affect the microbiota in the newborn. We propose that immune acquisition through breast milk at early stages of life will provide functional microbial gut colonization preventing susceptibility to infection and negative outcomes of immunological activation.

PRIORITIZING BREASTFEEDING FOR HEALTHY NEWBORN MATURATION

Maternal breastfeeding has been practiced over millennia to secure good nutritional status for newborns (19). Breast milk is an extraordinarily complex, highly variable bioactive fluid, with changes in composition depending on the stage of lactation (from colostrum to late lactation), time of day, and physiological/nutritional state of the woman. Obstetric practices during labor play a critical role in initiation of effective breast feeding. For example, labor induced with oxytocin was negatively associated with effective breastfeeding initiation 36 h after birth, suggesting that induction of labor with oxytocin should be used judiciously (20). In fact, the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) recommend starting breastfeeding no later than 1 h after birth and that infants should be feed exclusively with breast milk for at least the first 6 months of life, and that breastfeeding should continue, supplemented with solid foods, until 2 years of age or longer (2, 3). Breastfeeding for the first 6 months of the life of an infant has enormous long-term health benefits, including prevention of the risk of metabolic-related comorbidities such as overweight and obesity, type 2 diabetes (T2D), and chronic diseases in the infant (4–7). Breastfeeding also improves positive metabolic outcomes in mothers (21–23).

Breast milk is a source of bioactive molecules, bacteria, and immune cells (8–10, 19). Immunogenic cells in breast milk program the immunogenic response in the newborn and incentivize healthy microbial colonization of the gut of the infant by training the immune system (8–10, 19). In this new scenario, breast milk protects the newborn against infection and inflammation at earlier stages of life and contributes to immune maturation (see below the section on *Breastfeeding contributes to physiological immunity in the newbor*). Notably, the role of breast milk in assisting physiological microbial colonization of the intestine of the infant occurs during the first 2 years of life (24), and there is evidence that altered gut microbiome in the newborn is found associated to metabolic compromise in children (25). Despite the many benefits of exclusive breastfeeding, only 40% of infants under 6 months are breast fed; only 23 countries have achieved exclusive breast feeding in at least 60% of infants <6 months old. Also, the Americas has one of the lowest breastfeeding rates worldwide, where only 6% of the countries have an exclusive breastfeeding rate above 60%. The rate of exclusive breast milk (EBM) in Mexico, according to the Encuesta Nacional de Salud y Nutrición (26), was 28% for infants under 6 months, one of the lowest in Latin America. We next add scientific evidence supporting the role of breastfeeding as a window opportunity to secure physiological immunity in the newborn preventing the risk to infection, immune tolerance, inflammatory immune profile, and microbiota disruption.

BREASTFEEDING CONTRIBUTES TO PHYSIOLOGICAL IMMUNITY IN THE NEWBORN

During the first weeks of postnatal life, the adaptative immune system of the newborn is immature, insufficient, and ineffective to protect against pathogens (27); multiple pathways have been proposed to explain defective immunity in the newborn, including immaturity of immune cells or lymphoid tissues. As a consequence, susceptibility to infections is elevated, and the probabilities of illness and death increase. In fact, birth is considered a dramatic and dangerous transition for the neonate, who is exposed to a new environment with a diverse microbial ecosystem compared with that *in utero*. Also, neonates experience enhanced susceptibility to infections while showing limited responsiveness to vaccination, particularly during the first months of life. Notably, the transfer of maternal immune components via breast milk allows the newborn to secure

immunity to respond to any dangerous external pathogens, increasing their fitness for survival (28). Initial reports propose that women provide passive immune protection by transmitting antibodies in the colostrum during the first 2–4 days of breastfeeding (29, 30). Also, experimental evidence in human and murine models confirms several mechanisms involved in maternal immune transference to the newborn, such as maternal leukocyte transfer (MLT) (31), and microchimerism, the infiltration of maternal cells in newborn tissues (32).

Additionally, host mechanism such as self-missing, mediated by the natural killer cells of the newborn and the *de novo* production of neonatal immunoglobulin A (IgA) maintain intestinal microflora and immune adaptation (33, 34). Notably, breast milk immune composition seems to integrate a local secretion from multiple cell types, as well as peripheral production that not always correlate with blood levels (35). We propose that physiological routes that contribute to the newborn immunity are assisted by maternal breastfeeding (**Figure 1**).

BREASTFEEDING CONTRIBUTES TO PROINFLAMMATORY CYTOKINE PROFILE IN THE NEWBORN

The components of breast milk and their role on proinflammatory profiles in the newborn have been described in recent years. Under homeostasis, proinflammatory cytokine profile in breast milk depends on gestational periods, maternal age, and maternal health (36). For instance, IL-6 and IL-8 were lower in breast milk at 36 weeks of gestational age (37), and TNF-α was observed only during the first few days of lactation (38). Some data report that colostrum in mothers with advanced age shows higher IL-1β and IL-6 levels when compared with adolescent mothers (36), confirming that aged mothers integrate a higher proinflammatory cytokine profile in their breast milk. There is also evidence that IL-6 accumulation in breast milk seems to depend on maternal IgA levels (29), suggesting that exposure to maternal infections or associated-cytokines might be accumulated in the breast milk to help the infant to survive. For instance, Type I-IFN accumulation in breast milk has been found after infection with influenza virus (39), whereas IL-10 and TGF-β decreased in mothers with allergies (40). In women with preeclampsia, high cytokine levels in breast milk persist for at least 30 days (41), and IL-1 and IL-6 increase, whereas IL-12 decreases in the colostrum (37). This evidence confirms that proinflammatory cytokine profile in breast milk is modulated by previous exposure to infections, allergies pathological traits, and aging (**Figure 1**).

Preclinical analysis in murine models have also confirmed the effect of breastfeeding on the proinflammatory cytokine profile in the newborn. Precisely, a high concentration of TGFβ-1 has been detected in the milk of mice and in various tissues in the mouse pups (42), whereas the low concentration of cytokines such as IFNγ, IL-2, IL-4, IL-5, TNFα, and IL-13 were detected under healthy condition (33). According to these data, proinflammatory cytokines are present in breast milk, and they are essential for healthy development in newborns; during aging, however, a swift

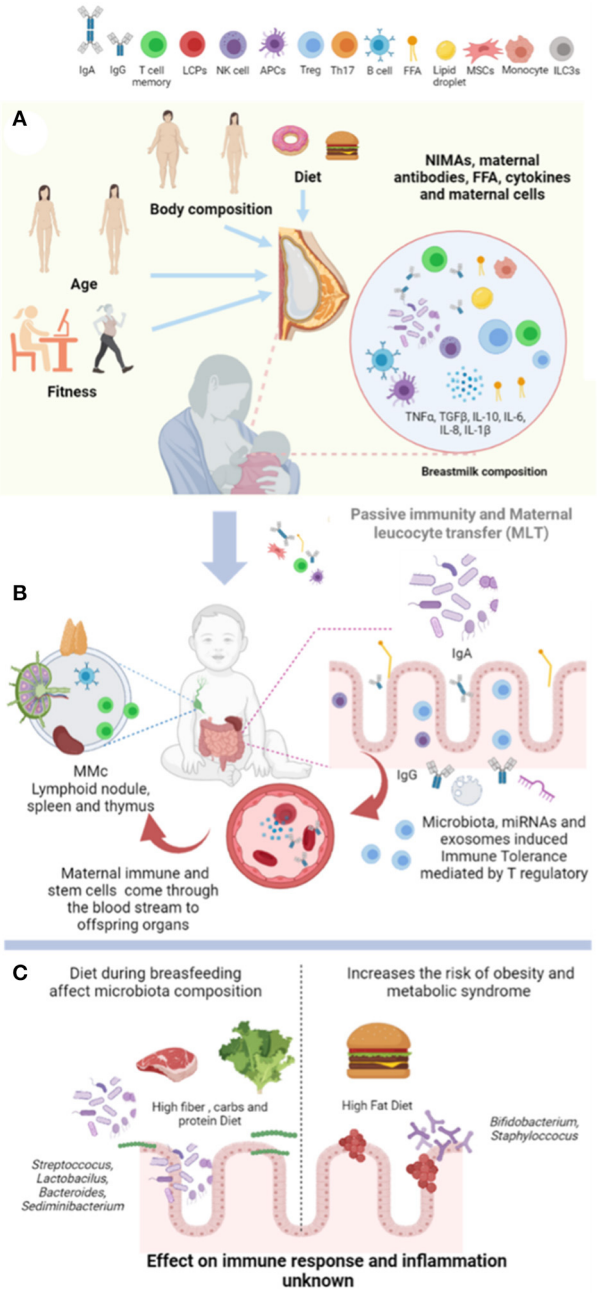

FIGURE 1 | Breastfeeding provides immunological programming in the newborn. **(A)** Body weight, age, lifestyle, and diet quality influence breast milk composition such as lipid species, microbiota, cytokines, and accumulation of immune cell types. **(B)** Maternal antibodies, non-inherited maternal antigens (NIMAs), and maternal leucocyte travel through the stomach and intestine of the offspring. Also, maternal immune and stem cells invade the newborn blood leading to maternal microchimerism (MMc) to generate immune tolerance. Finally, microbiota, mRNAi, and exosomes provide immune tolerance by T-cell accumulation in the gut of the offspring. **(C)** High fat, carbs, and protein diets intake disrupts microbiota composition by promoting Staphylococcus and Bifidobacterium accumulation. Whereas, high fiber, carbs, and protein leads to lactobacillus microbiota. However, the effect of diet during breastfeeding on immune response, MMc, immune tolerance, and offspring microbiota establishment has not been fully determined in humans. NIMAs, non-inherited maternal antigens; MMc, maternal microchimerism. Created by Biorender.

proinflammatory profile is exacerbated, which might provide adverse outcomes in the physiology of the newborn (**Figure 2**).

BREASTFEEDING-RELATED MICROCHIMERISM PROVIDES IMMUNOGENIC CELL TRANSFER TO THE NEWBORN

The transference of maternal cells such as immunogenic types, somatic tissue-specific cells, and stem cells to neonatal circulation and subsequent establishment in the newborn organs is known as maternal microchimerism (MMc) (43, 44). By itself, MMc establishes that the newborn displays a low frequency of immunogenic cells traveling and allocating in the tissues, but these cells might be retained for a long period of time (45). Notably, the MMc is involved in the tolerance, priming, and surveillance of the newborn, accounting as a major contributor of immunity after birth (46). Initial reports documented that maternal immune cells can be transferred to the fetus via the placenta during embryonic development (47). After birth, breastfeeding provides the newborn with immunogenic cell types that can remain until adulthood, as lymphoid and myeloid compartments of peripheral blood in healthy adult women (48, 49). Preclinical and clinical models documented that breast milk possesses several immunogenic cell types typically found in the blood, such as myeloid precursor cells, dendritic cells, and macrophages (33).

Additionally, innate lymphoid cells (ILCs) (34), natural killer (NK) (50), cytotoxic T cells (32), and T regulatory cells (51) have also been identified. Notably, recent evidence in humans shows that breast milk composition includes stem cells, specific memory CD4+ and CD8+ T-cells (52), and a large stem progenitor-like cell subset that expresses the CD45+ and CD45– markers (53). The CD45 marker is a transmembrane protein expressed in differentiated hematopoietic cells and seems to be an essential regulator for T- and B-cell antigen receptor signaling (53). On their own, murine models have confirmed findings in humans and demonstrated that breast milk from mice has B-cells with higher percentages of class-switched IgD-memory B-cells and plasma cells (PCs) (42, 43) and mammary gland IgA secretory cells (54), confirming immunogenic transmission to the newborn (**Figure 1**).

Although the mechanism involved in how immune cells travel from breast milk to the newborn circulatory system in humans is still not established, some potential pathways have been proposed. Evidence in humans suggests that breast milk components interact with the newborn saliva (31) protecting immune cells from the acidic pH of the stomach, and then, these cells infiltrate into the gut mucosa and travel to blood circulation within the newborn. Preclinical models have confirmed that the final allocation of maternal immune cells in mouse pups is an establishment of T-cell repository on the thymus, lymphatic nodes (55), spleen (56), Peyer's patches (32), brain (57), and gut (51).

Molecular and cellular regulation of MMc and elimination of non-inherited maternal antigens (NIMA) have been a

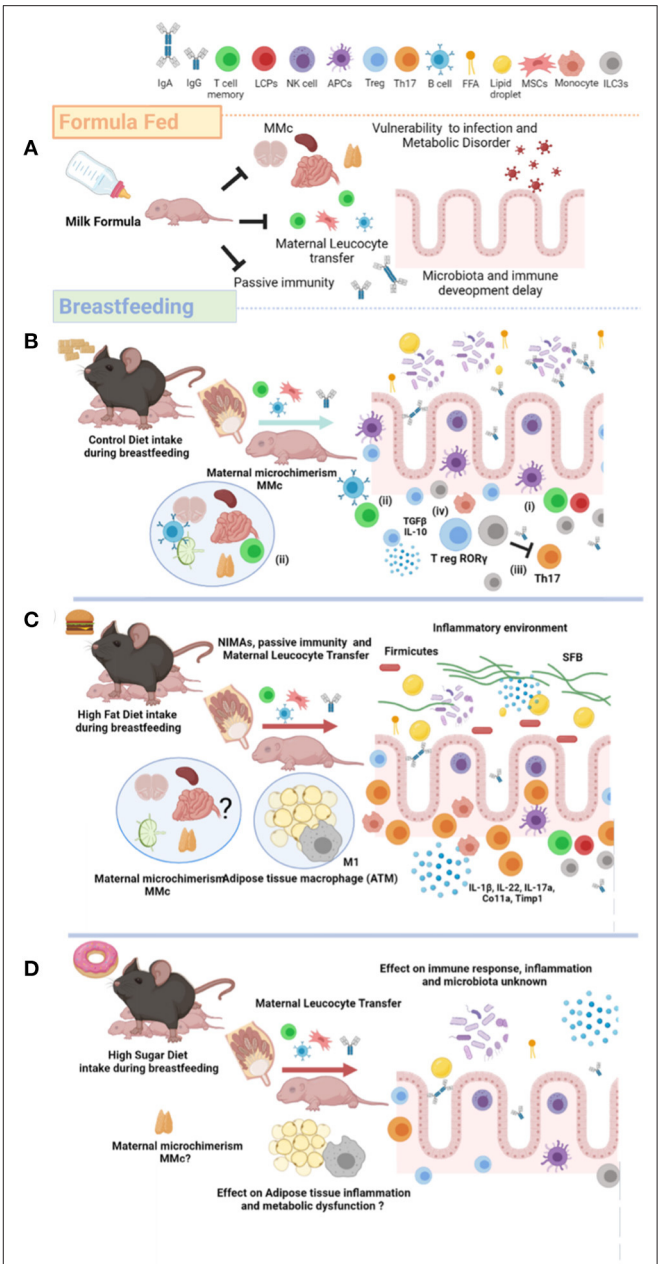

FIGURE 2 | Maternal nutrition modulates breastfeeding composition and metabolic failure in the newborn. **(A)** Artificial milk feed formula does not promote innate and adaptative immune activation, maternal MMc, and gut microbiome development in the newborn. Defective immunological system leads to offspring vulnerability against viral and bacterial infection. **(B)** Breastfeeding from lean mothers or healthy maternal nutrition induce maternal antibodies (passive immunity), NIMAs, and leucocyte maternal transfer. Additionally, breastfeeding favors several cell mechanisms involved in immunogenic tolerance: (i) altered antigen presenting, (ii) specific T- and B-subtypes on MMc, (iii) Th17 cells suppression by ILC3s, and (iv) accumulation of T-regulatory cells and microbiota invasion. **(C)** HFD exposure during breastfeeding reduces ILC3s and Treg, and increases the TH17 I the gut. Breastfeeding of mothers exposed to HFD also increase the inflammatory cytokine profile, and SFB colonization and *firmicutes* in the gut of newborn. Also, HFD exposure during breastfeeding increases the M1/M2 macrophages ratio in adipose tissue (ATM). The effect of high fat diet (HFD) exposure

(Continued)

FIGURE 2 | during breastfeeding on the MMc has not been totally described. **(D)** The effect of high sugar diet on immunological programming in the newborn has not been totally described. MMc, Maternal Microchimerism; NIMAs, Non-Inherited Maternal Antigens; ILC3s, Type 3 Innate Lymphoid Cells; HFD, High Fat Diet; ATM, Adipose Tissue Macrophage; SFB, Segmented Filamentous Bacteria; FFA, Free Fatty Acid; HSD, High Sugar Diet; Treg, T-regulatory; Th17, T-helper 17; MSCs, Mesenchymal Stem Cells. Created by Biorender.

matter of intense research. Preclinical murine models have provided important advances in the field of immunogenic transfer of maternal cell types to the newborn. Microchimerism and NIMAs were first reported in allogeneic transplantations. Reports propose several immune cell-dependent pathways of regulation: (i) antigen-presenting cells (APCs) NKs, B-specific phenotype and T-lymphocyte subset, and (ii) mesenchymal and stem cells. Molecularly, APCs from the newborn bind to the soluble antigen of maternal cells, allowing antigen processing and evasion of immune activation and systemic tolerance (44). Also, in a mice model of allogeneic hematopoietic stem cell transplantation (HSCT), breastfeeding generates $Foxp3^{+}$ regulatory T-cells that suppress anti-maternal immunity and persist into adulthood (58).

Additionally, neonatal NK cells favor MMc by mediating the recognition and elimination of maternal antibodies IgA and IgGs (59). This process is known as missing-self recognition antibodies, which involves the Fc and CD16 proteins (59). In addition, host dendritic cells and plasmacytoid dendritic cells process membrane alloantigen acquisition (mAAQ+), favoring tolerance mediated by a decrease in allopeptides-MHC complex presentation and PD-L1 and CD86 expression (60). MMc is also regulated by infiltration of lymphocyte precursors cells (61), and by selecting neonatal subsets of Th1, Th2, and Th17 lymphocytes (62). Finally, clinical and experimental evidence in humans and mice show NIMA exposure during pregnancy and breastfeeding potentiates transplantation tolerance later in life (58). On this context, the high mobility group box 1 (HMGB1) protein levels in maternal circulation favors tolerance in the newborn against mesenchymal and stem cells (MSCs)-derived NIMA transferred via breast milk (63). HMGB1 is a non-histone nuclear protein secreted as a proinflammatory factor by activated macrophages and monocytes, and reported in certain autoimmune diseases such as systemic lupus erythematosus (64). HMGB1 has been also involved in the activation and mobilization of MSCs in adult circulation (65), inducing immune tolerance toward MSC-specific antigens in the newborn (**Figure 2**).

This evidence confirms a bidirectional immune crosstalk between maternal breastfeeding to the newborn and highlights the role of MMc on immune tolerance in the newborn.

MATERNAL ANTIBODIES TRANSFERRED BY BREASTFEEDING ALLOW IMMUNE TOLERANCE IN THE NEWBORN

As previously commented, maternal antibodies in colostrum maintain the newborn immune defense against pathogens during their first weeks of life. In humans, IgA antibodies are grouped into IgA1 and IgA2 subclasses, which display tissue-selective expression (66). The IgA1 is the main antibody in the respiratory tract, saliva, serum, and skin, whereas the IgA2 is localized in the intestine (66). Maternal antibodies are mainly composed of two types of immunoglobulins: (i) secretory IgA antibodies (SIgA), involved in protection mediated by microorganism neutralization and agglutination (67) and, (ii) four subtypes that are expressed in human and mice as antigen-specific IgG antibodies (IgG1, IgG2, IgG3, and IgG4) (68) which are induced by maternal immunization (69). Physiologically, the mammary gland secretes dimeric IgA antibodies that bind to the polymeric Ig-receptor (pIgR) on the basolateral membrane of the mammary gland epithelium, and both are internalized via endocytosis. IgA antibodies-pIgR dimers are released by the apical membrane as secretory IgA (sIgA) to the breast milk (70).

The maternal antibody IgG1 displays a half-life of about 48.4 days in the human newborn; however, they might be found in the serum of 4- to 6-month-old infants (71). In contrast, IgA antibodies are continuously supplied through the breast milk from the mother to the newborn (72). Experimental evidence in mice has confirmed that maternal antibodies have a half-life from 7 to 16 days postnatal (73) and they even could be found in serum until 14 weeks of age (74). However, reports show that maternal antibodies (IgG) decline and do not protect the newborn at later stages (72). Time-dependent, antibody-producing B-cells have been found in the neonatal gut, which reaches a peak after 30 days of postnatal life (31), however, the adaptive response is still immature and has not had enough time to acquire immunogenic memory.

Additionally, recent evidence described a selective IgGs known as maternal natural antibodies produced by exposure to pathogens or maternal immunization (75), which might interfere with the humoral immune response of the infant (70). High concentrations of vaccine-induced maternal antibodies in the infant blunt the immune response after a challenge (76). In fact, the newborn experiences an inhibition of antibody generation, showing lower antibody count, and affecting neonatal immunity for up to more than 1 year (72). Of note, defective immunological response in the newborn is not dependent on the type of vaccine applied in the mother but it seems to integrate a common pathway that involves a cross-link interaction between the B-cell receptor (BCR) and the Fcγ receptor (FcγRIIB), both expressed on the surface of B-cells (72). In this context, maternal IgG antibodies in the newborn bind to the FcγRIIB receptor, blocking the antibody production in response to the BCR-antigen interaction in the B-cells.

This evidence confirms that early newborn immunity depends on maternal antibodies for protection (**Figure 1**), but how long does this protection last?

MATERNAL T-REGULATORY CELL TRANSMISSION TO THE NEWBORN BY BREASTFEEDING

Maternal transmission of T cells to the newborn is a topic of intense debate, and murine models have provided important

advances on this field. Initial reports suggested that a microbe-induced population of receptor-related orphan receptor gamma t (RORγ+) Tregs is essential in controlling gut inflammation, and they are able to be preserved up to day 7 after birth (77). Ramanan et al. (78) demonstrated that RORγ+ Treg percentages varied between C57BL/6 and BALB/c mice. C57BL/6 have relatively high percentages of RORγ+ among Foxp3+ Tregs (40–60%) in comparison with BALB/c mice (20%) (78). Other studies also determined that Treg cells are transmitted by the mother to the newborn after birth, remain stable for life, and become resistant to many microbial or cellular perturbations (79). In fact, Tregs transmission in breast milk and the abundance of RORγ+ Tregs in the newborn secure bacterial clearance and delayed inflammation (79). Other studies show that breastfeeding may duplicate Tregs compared with neonates who received milk formula, and that it promotes tolerance against non-inherited maternal antigens (51). This evidence suggests that T-regulatory cells pass through breast milk, favoring immunity in the newborn and second generations.

Together, this evidence supports the notion that breastfeeding sets physiological immunity in the newborn by transferring proinflammatory cytokines, immunogenic cell subtypes, T cells, and maternal antibodies. Besides, immunity after birth is also closely regulated by microbiota in the newborn, confirming a mutually dependent interface of maternal breastfeeding and microbiota ecosystem.

BREASTFEEDING–MICROBIOME INTERPLAY MODULATES IMMUNITY IN THE NEWBORN

It is well-recognized that the gut microbiome integrates the most critical immune microbial stimulation in the newborn. The establishment of a healthy gut microbiome plays a crucial role in early life, leading to a well-trained immune system, and an efficient metabolism in healthy subjects (80). Early reports suggest that the gut microbiome of an infant would attain an adult-like composition by the age of three, but recent studies have suggested that a well-developed microbiome may take a longer time (81). Our microbiome is abundant in the gut, skin, hair, ears, vagina, and the respiratory and urinary tracts; however, the gut by itself supports more than 90% of the total microbiome. Initially, it was considered that the uterus was sterile, but now it has been demonstrated that the microbiome establishment begins during intrauterine life. In fact, the placenta, amniotic fluid, fetal membranes, and umbilical cord blood contain live microorganisms, suggesting that bacteria in these tissues do not necessarily indicate a pathogenic state but a symbiotic interplay (82). These data challenge the assumption of a sterile environment in the womb and indicate that initial colonization in the intestinal tracts of the infant can begin before birth. However, the gut microbiota of an infant is established mainly after birth in two transition periods in infancy: the first transition period occurs immediately upon birth, during breastfeeding, and results in dominance of the gut microbiome by *Bifidobacterium*, which is found in large quantities in breast milk (83). The second transition period occurs during weaning and establishes an adult-type complex microbiome dominated by the Phyla *Bacteroidetes* and *Firmicutes* (84–86). However, many other environmental factors, including cesarean delivery, medication, antibiotics, and maternal diet (including varieties of fibers), can alter the gut microbiome [(82); **Figure 1**].

Breast milk contains as many as 600 different bacterial species, up to 10^3-10^4 CFU/ml (87). It was proposed that bacteria may be transferred from mother to infant via breast milk through an "entero-mammary pathway" (88–94) to establish a healthy gut microbiome and populate the upper respiratory tract of the infant (88). The development of this respiratory tract microbiome, like that in the gut, is affected by the birth mode and the feeding practiced in childhood (88). Reports have documented that breast milk microbiome includes *Staphylococcus* and *Streptococcus*, as the most frequently cited taxa; however, additional taxa have been identified, including *Corynebacterium*, *Bifidobacterium*, *Propionibacterium*, *Bacteroides*, *Enterococcus*, *Faecalibacterium*, *Lactobacillus*, *Veillonella*, *Serratia*, *Ralstonia*, *Acinetobacter*, *Rothia*, and several members of the *Lachnospiraceae* and *Ruminococcaceae* families (95). Notably, *Staphylococcus*, *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* found in breast milk microbiota are shared between mother-to-infant (10). In contrast, substituting breast milk with formula promotes a dramatic alteration of healthy gut microbiome establishment [(89, 90); **Figure 1**].

The microbiota in breast milk also modulates immunity in the newborn. Colonization of intestines by a diversity of bacteria in early life stimulates the differentiation and activation of T- and IgA-producing B cells that integrate the immune system in the newborn (91). Additionally, commensal microbiota is coated by IgA as a homeostatic IgA response, whereas humans express two subtypes (IgA1 and IgA2), mice express a single IgA subtype (92). Furthermore, maternal antibodies such as IgA transferred by breastfeeding stimulate the maturation of the innate mucosal immune system in the newborn in both humans and mice (80). IgA is a critical regulatory mechanism in this process of training and maturation of immunity. For instance, IgA-bacteria binding efficiently colonizes the small intestine (83), and according to Bunker and Bendelac (93), the bacteria are bound to a specific IgA, in the small, but not in the large intestine. By itself, maternal IgA in human has a relevant role on microbiome composition in the early months of life and is required for a healthy intestinal barrier and immune homeostasis. Also, maternal acquisition of antibodies in the newborn includes anti-commensal IgG2b and IgG3 by breast milk allowing activation of T-cell-independent and Toll-like receptor-dependent antibodies against their gut microbiota (59). This mechanism limits mucosal T-follicular helper response and germinal B-cell responses against new commensal antigens in the newborn (59). Also, maternal gut-associated lymphoid tissue (GALT) allows IgA accumulation stimulating the mammary gland to induce IgA secretion in the breast milk [(94); **Figure 1**].

Some studies in mice have identified selective microbiome species on acquired immunity in the newborn. Lactobacillus reuteri from the maternal microbiota is also found in breast milk and stimulates type 3 innate lymphoid cells (ILCs) in the

lamina propria of the neonatal small intestinal to enhance IgA production (96). By itself, IgA plays a critical immunological role on the intestinal mucosa, in Peyer's patches, and in mesenteric lymph nodes of the newborn (97). Breastfeeding also favors the development of GALT by impairing mucosal immunity related to reduction of IgA levels through decreasing the IL-4 and IL-10 levels and the adhesion molecule MAdCAM-1 (98). CD4 T-regulatory cells (Tregs) subsets are essential to maintain self-tolerance in adult life as well in the neonatal period in humans and mice. Recently, reports documented that human breast milk promotes FoxP3$^+$ Treg cell differentiation, increasing the number of FoxP3$^+$CD4$^+$CD25$^+$ Tregs cell subtype and generating FoxP3 T-cell responses in the small intestine through microbiota (*Bifidobacterium breve*, *B. adolescentis*, *B. bifidum*, and *Lactobacillus plantarum*) (99). This evidence confirms a close relationship between secretory IgA and microbiota, allowing proper intestinal immune development in the newborn [(59); **Figure 2**].

Breast milk also contains hundreds of complex oligosaccharides and galactooligosaccharides, which contribute to the stability of the microbiome (100). Oligosaccharides in breast milk are more concentrated in the early stages of lactation, reaching up to 20–25 g/L in colostrum to 5–15 g/L in mature milk (101). Oligosaccharides-related molecules reach the colon of the infant, and are fermented mainly by *Bifidobacterium* to produce short-chain fatty acids (100). Oligosaccharides and probiotic components of breast milk modulate immune development, gut inflammation, and microbiome of the infants, conditioning their susceptibility to allergies (102).

Finally, altered early microbiome, called "dysbiosis," might affect the development of the immune system of the host. Physiologically, the gut microbiome also maintains a constant crosstalk with the gut epithelia, inducing cell differentiation, and tight junction enhancement (103, 104); however, an imbalanced microbiome might destabilize the tight junction of epithelia, resulting in a leaky gut. The intestinal mucosal surface in the newborn shows differences with adult mucosa. For instance, the epithelium of the respiratory and gastrointestinal tracts of newborns has higher permeability (leaky) than those in adults, which increases the risk of tissue damage (80). A leaky epithelium allows an increased passage of toxic substances, bacteria, and viruses that might harm the body and increase susceptibility to pathological diseases. Besides, the epithelia of the newborn do not secrete enzymes or anti-microbial peptides, and the pH of the stomach and the composition and glycosylation of the secreted mucus layer also differ (105–107). By itself, dysbiosis increases the recruitment of immunological cell types, and activates the Toll-like receptors and nucleotide-binding oligomerization domain receptors, which exacerbate the release of inflammatory cytokines to the circulatory system (108–112).

Conversely, a healthy gut microbiome, from intrauterine life through the first 1,000 days, decreases the risk of suffering infectious and non-infectious diseases in early and late life. However, high-energy diets favor dysbiosis and negatively impact the gut microbiota (**Figures 1**, **2**). We next add scientific evidence supporting the negative role of energy-dense diets or obesity in mothers on the immune programing of the newborn.

MATERNAL DIET MODULATES BREAST MILK–GUT MICROBIOME INTERPLAY AND IMMUNITY IN THE NEWBORN

Maintaining proper nutrition during lactation secures positive developmental and health outcomes in the newborn and in his adult life. Very few studies, however, have directly assessed the effect of maternal diets on immunogenic breast milk composition, and it remains as a very poorly understood topic. Also, the contribution of energy-dense diets on immune identity in breast milk and its effects on the microbiome of the newborn has not been completely explored. As Bravi et al. commented in their review, "the direct relation between the dietary intake of single nutrients and their presence within human milk has not been studied satisfactorily, for many reasons" (113). Some preclinical models have started to decode the impact of maternal diet on breast milk composition. Reports have documented that maternal diet during lactation modulates the composition of breast milk, glucose tolerance, and weight of the infant (7). Also, it had been confirmed that negative physiological conditions such as obesity, overweight, or overnutrition with energy-dense diets are associated with a pro-inflammatory profile and immune activation in the plasma of the infants after birth (114–118). In humans, 25% of calorie intake in obese people comes from snacks and junk food (119), which could have a negative contribution on breastfeeding composition and health in the newborns from obese mothers. Initial reports documented that supplementing the diet of lactating women with docosahexaenoic acid increases the concentration of docosahexaenoic and eicosapentaenoic acids in breast milk' however, there were no changes in IL-6 and TNF-α cytokines (11), or TGF-beta (12). In addition, supplementing the diet with black currant seed oil (BCSO) during pregnancy decreased IL-4 and increased IFN-γ levels in breast milk, whereas no significant differences were observed in IL-5, IL-10, IL-12, and TNF-α levels. Conversely, dietary intervention to increase consumption of fruits and vegetables during lactation in women decreases IFN-γ, TNF-α, IL-6, IL-8, and IL-1β (13).

Preclinical animal models have confirmed the deleterious effect of high-energy diets on pro-inflammatory cytokines accumulation in newborns. For instance, we and others have reported that maternal exposure to energy-dense diets in murine models increase peripheral pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1β release and induce neuroinflammation in the newborn [(114, 118); **Figures 1**, **2**]. Exposure to energy-dense diets programs maternal immune activation in mothers (120) and also shapes the microbiota in breast milk (15). As we commented, the breast milk microbiome harbors *Staphylococcus* and *Streptococcus* as the major families, as well as *Corynebacterium*, *Bifidobacterium*, *Propionibacterium*, *Bacteroides*, *Enterococcus*, *Faecalibacterium*, *Lactobacillus*, *Veillonella*, *Serratia*, *Ralstonia*, *Acinetobacter*, *Rothia*, and several members of the *Lachnospiraceae* and *Ruminococcaceae* families (95). Some reports suggest that maternal exposure to energy-dense diets modulates microbiota and immune profile in the breast milk, leading to major metabolic outcomes in the newborn (16). In addition, diets

high in plant protein, fiber, and carbohydrates promote the presence of *Lactobacillus*, *Bacteroides*, *Sediminibacterium*, and *Streptococcus* in the microbiota of the breast milk, high intake of animal protein and HFD show accumulation of *Staphylococcus* and *Bifidobacterium* (15). Murine models also confirm that mothers exposed to HFD develop a selective microbiota profile by expanding firmicutes, a Gram-positive bacteria associated with promoting IL-17-producing type 3 innate lymphoid cells (ILC3s) in the lamina propia of the newborn, which seem to favor an increased susceptibility to intestinal injury (16). Notably, mice fed with energy-dense diets activate the aryl hydrocarbon receptor, a nuclear receptor/transcription factor involved in xenobiotic response that disrupts fat metabolism (17). Inhibition of ILC3s promotes intestinal inflammation mediated by increases in Th17 and IL-22 and colonization of segmented filamentous bacteria (SFB) (121). SFB are Gram-positive commensal, spore-forming bacteria found in mice and rat ileum, promoting the robust differentiation of T-helper-17 cells (Th17) (18). This suggest that diet components might also be recognized as xenobiotic elements and disrupt basal physiological settings, allowing intestinal inflammation. While these reports confirm that exposure to energy-dense diets modulates the immune response in the newborn by Th17/ILC3s-Treg balance in mice and microbiota profile in both, the effect of high sugar intake during breastfeeding is unknown (**Figure 2**). In an elegant recent report, Taylor et al. documented a new deleterious outcome associated to intestinal function in high sugar diets (122). The authors reported that exposure to dietary fructose improves the survival of intestinal cells, favoring the expansion of the surface area of the gut, and increasing nutrient absorption and adiposity in mice exposed to energy-dense diets.

Together, this evidence confirms that intake of energy-dense diets modulates the microbiome in the breast milk, allowing immune activation in the newborn.

BREAST MILK FROM OBESE MOTHERS AND THEIR EFFECTS IN NEWBORN IMMUNITY

Breast milk from obese mothers displays a proinflammatory profile and contributes to neurodevelopmental alterations in the newborn. Reports confirm that maternal body mass index correlates with higher omega-6 to omega-3 fatty acid ratio and lower concentrations of lutein and docosahexaenoic, eicosapentaenoic, and docosapentaenoic acids in the breast milk (123). The authors confirm that concentrations of saturated fatty acids and monounsaturated fatty acids in breast milk were positively associated with maternal inflammation (123). In fact, breast milk from obese mothers is positively associated with a pro-inflammatory profile (123), suggesting that obesity contributes to breast milk composition and susceptibility to negative outcomes in the newborn.

Preclinical models have confirmed that breast milk from obese mothers partially protects the newborn against a challenge of high-fat diets (124). These data confirm that breast milk from obesity-prone dams fed with a high fat diet (HFD-OP) shows a decrease in the total lipid content and reduction in levels of the precursors of inflammatory lipids. Also, macrophage marker (F4/80), a marker of inflammation (TNF-α), a marker of tissue fibrosis, collagen-1 (Col1a), and tissue inhibitor of metalloproteinase-1 (Timp1) increase in the adipose deposits of 20-week old offspring mice exposed to HFD from obesity-resistant (OR) mothers (124). Conversely, newborn breastfed from mothers exposed to high fat diet and re-challenged to HFD in adulthood showed an increase in total CD11c+ proinflammatory and CD11c– anti-inflammatory markers in adipose tissue macrophages (ATMs), and M1:M2 proinflammatory ratio [(125); **Figure 2**]. These results show that HFD exposure during lactation promotes controversial results associated with inflammatory mechanism involved in newborn physiology and metabolism. For a deep understanding of the immunological properties in breast milk of obese mothers, see (126).

CONCLUSION

Our proposal adds experimental evidence confirming the contribution of breastfeeding as a rationale to set immunity in the newborn during the first 1,000 days. We propose that breastfeeding secure physiological immunity in the newborn preventing the risk to infection, immune tolerance, inflammatory immune profile, and microbiota disruption. We propose that the physiological crosstalk of breastfeeding–microbiota assists proper immunological programming after birth, providing a mutual interface for healthy outcomes. However, conditions such as obesity or maternal exposure to energy-dense diets disrupts the physiological microbiome in the breast milk, favoring microbiota imbalance in the gut, and defective immunity in the newborn. Together, we conceive that breastfeeding supports an early priming stage of postnatal immune maturation and microbiome colonization, integrating a window of opportunity for preventive and interventional measures. Future studies are warranted to explore the long-term benefits of external factors assisting proper immune performance in the newborn to prevent obesogenic pathologies later in life.

AUTHOR CONTRIBUTIONS

MC, AC-M, MG-J, MDC-F, RV-C, and CM-V contributed to conception, design of the manuscript, and wrote sections of the paper. AC-M and MG-J design the figures. All authors contributed to the article and approved the submitted version.

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Prenatal and Early Childhood Exposure to Lead and Repeated Measures of Metabolic Syndrome Risk Indicators From Childhood to Preadolescence

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Background: Exposure to lead (Pb) during the early life stages has been associated with the development of metabolic syndrome (MetS). Longitudinal studies of Pb exposure in critical developmental windows in children are limited.

Methods: Our study included 601 mother–child dyads from the PROGRESS (Programming Research in Obesity, Growth, Environment and Social Stressors) birth cohort. Blood lead levels (BLLs) were assessed during the second and third gestational trimesters, in cord blood at delivery, and at ages 1, 2, and 4 years. Bone lead levels in the patella and tibia were assessed at 1 month postpartum and evaluated in separate models. To account for cumulative exposure (prenatal, postnatal, and cumulative), we dichotomized the BLLs at each stage visit and determined the following: "higher" if a BLL was at least once above the median (HPb) and "lower" if all BLLs were below the median (LPb). We analyzed fasting glucose, HbA1c, triglycerides (TGs), total cholesterol (TC), high-density lipoprotein cholesterol (cHDL), low-density lipoprotein cholesterol (cLDL), body mass index, waist circumference (WC), body fat percentage, and systolic (SBP) and diastolic blood pressure (DBP) at two study visits between 6 and 12 years of age and created cutoff points based on the clinical guidelines for each indicator. Mixed effects models were used to analyze each outcome longitudinally for each BLL score, adjusting for child's sex, size for gestational age, child's age, maternal parity, mother's age, and socioeconomic status.

Results: We observed associations for HPb exposure and TC in all stages (OR = 0.53, 95%CI = 0.32–0.86) and postnatally (OR = 0.59, 95%CI = 0.36–0.94) and for prenatal HPb and TGs (OR = 0.65, 95%CI = 0.44–0.95). HPb at all stages was associated with WC (OR = 0.27, 95%CI = 0.08–0.86), BMI (OR = 0.33, 95%CI = 0.11–0.99), SBP (OR = 0.53, 95%CI = 0.32–0.85), and DBP (OR = 0.57, 95%CI = 0.34–0.95). Pb levels

in the patella were associated with cHDL (OR = 1.03, 95%CI = 1.00–1.07) and those in the tibia with TGs (OR = 0.95, 95%CI = 0.91–0.99).

Conclusion: Early life exposure to Pb may alter early indicators of MetS. A follow-up of these children will allow for more definition on the impact of longer-term exposures.

Keywords: lead, prenatal exposure, metabolic syndrome, early childhood, heavy metals

INTRODUCTION

Exposure to lead (Pb) has been declared second on the list of 10 highest priority toxic substances to public health due to established multisystem toxicity and widespread exposure by the World Health Organization (WHO) and the Agency for Toxic Substances and Disease Registration (ATSDR) (1, 2).

During pregnancy, Pb has potential impacts on fetal health due to its ability to cross the placental barrier (3); additionally, endogenous Pb exposure increases due to bone resorption (4, 5). Postnatally, Pb can also be transferred to the newborn through breast milk (3, 6, 7). Developmental windows, including *in utero* and early childhood, are key to study the effects of Pb exposure on organ growth and development, which may contribute to adverse health outcomes later in life (7).

In Mexico, the principal route of exposure to Pb is gastrointestinal, *via* the consumption of food prepared, served, or stored in lead-glazed low-temperature ceramics as lead will leach into food, especially with acidic food frequent in Mexican cuisine (1, 3, 8). Once inside the human body, Pb can traverse to the brain, heart, liver, and kidneys, altering their normal biological functions (9). One of the mechanisms of Pb toxicity is the production of reactive oxygen species, resulting in various adverse health effects such as oxidative damage to DNA, proteins, and lipids and increased lipid peroxidation in the cell membrane (9, 10). In the cardiovascular system, Pb is capable of displacing divalent cations, particularly Ca^{2+}, affecting the ion channels and other receptor functions in the brain, such as the *N*-methyl-D-aspartate receptor. In the cardiovascular system, it might modify the permeability of blood vessels, leading to vascular damage, cardiac toxicity, cardiac dysfunction, and hypertension. Another mechanism of action is its role as an endocrine disruptor, functioning as endogenous hormones and mimicking endocrine effects (4, 11). Moreover, results from *in vitro* and animal models suggest that Pb can modify the differentiation of progenitor cells, increase adipogenesis, and alter glucose homeostasis, accounting for some of the observed comorbidities present in metabolic syndrome (MetS) (2, 12).

MetS is defined as a set of physiological, biochemical, clinical, and metabolic factors that increase the risk of cardiovascular diseases such as myocardial infarction, atherosclerosis, systemic arterial hypertension, cerebrovascular disease, and diabetes mellitus type 2 (13, 14). The International Diabetes Federation (IDF) defines the diagnostic criteria for MetS in the adult population as the presence of at least three out of five factors: abdominal obesity, elevated triglycerides, low levels of high-density lipoprotein cholesterol, high blood pressure, and altered fasting glucose (2, 15). However, there is no consensus on the applicability of these cutoff points in children and adolescents (13, 14, 16). According to the IDF definition, MetS should not be formally diagnosed in children under the age of 10. However, there is evidence that the abdominal circumference, body mass index, blood pressure, lipoprotein blood levels, and blood glucose can be altered beginning in early childhood and that these profiles may track into adolescence and adulthood (13, 14, 17).

The presence of MetS during childhood and preadolescence significantly increases the risk of diabetes mellitus type 2 and cardiovascular disease in adulthood (18, 19). The prevalence of MetS can be attributed to factors such as poor diet, sedentary lifestyle, and social or genetic factors (20). Intriguingly, there is growing evidence supporting a link between Pb exposure and the development of MetS (21) and various pathologies (17, 20, 22). For example, exposure to Pb during pregnancy and lactation, in animal models, was associated with the development of insulin resistance and obesity, and this association was greater among males than females (18). However, there is conflicting evidence of Pb exposure during the prenatal stage and the subsequent levels of cholesterol (total, high density, and low density) in pediatric cohorts (17, 20, 21). Some studies in children have reported sexually dimorphic sensitivity between the effect of exposure to Pb and the presence of different indicators of MetS (20, 21).

Despite growing evidence supporting a link between Pb exposure and MetS risk factors, there are only a few existing longitudinal studies among pediatric populations that examined perinatal Pb exposure and its relationship with increased risk of MetS in childhood and preadolescence (17, 18). The aim of this study was to explore the association between prenatal and early childhood exposure to Pb and repeated measures of MetS risk indicators in children between 6 and 12 years of age.

MATERIALS AND METHODS

Study Population

The participants of this study are part of the PROGRESS (Programming Research in Obesity, Growth, Environment and Social Stressors) prenatal cohort from Mexico City. Pregnant women receiving medical and prenatal care in the Mexican Social Security Institute (IMSS) were invited to participate. The recruitment period took place between July 2007 and February 2011. To be eligible for the study, women had to be at $<$20 weeks gestation, $\geq$18 years of age, without current or prior pathologies of heart or kidney disease, accessibility to a phone, plan to reside in Mexico City for the next 3 years after their admission to the study, not use steroid drugs (including glucocorticoids) or antiepileptic medications, and not consume alcohol daily. The institutional boards and ethics committees of Harvard School

of Public Health, Icahn School of Medicine in Mount Sinai, and the National Institute of Public Health in Mexico approved the project, as well as the collaborating institutions: National Institute of Perinatology (INPer), IMSS, and the American British Cowdray (ABC) Medical Center. Participants granted their informed consent for participation in the study. There were 948 women followed during pregnancy and who gave birth to a live child; children had follow-up visits at 1, 6, 12, 18, 24, 48, and 72, and 96 months of age. The cohort had most of its dropouts between birth and at the stage 24 visit and has maintained stable retention at around $n = 600$ mother–child dyads since.

The inclusion criteria for this study were: having data of at least one blood lead level (BLL) during pregnancy and one postpartum (i.e., second and third trimesters of pregnancy, birth, and at 12, 24, and 48 months of age) and outcome data (i.e. MetS indicators) at 72 and 96 months. Some of the children's blood samples from stages 12 and 24 were randomly lost during transportation from Mexico to the laboratory in the USA, leaving a total of $n = 174$ and $n = 247$, respectively. For stage 48, we have the data for 501 children. Children who had an extremely low birth weight equivalent to <1,500 g and gestational age <32 weeks were excluded ($n = 21$). On average, 601 children had data for at least one incidence of MetS for stage 72 and 540 had data for stage 96, with varying covariate data during the study follow-up that are indicated in the corresponding results table.

Pb Exposure

Prenatal exposure was evaluated with blood measurements from women in the second and third trimesters of pregnancy and in umbilical cord blood. Postnatal Pb exposure was assessed from children's blood samples collected at 12, 24, and 48 months of age. All blood samples were drawn in trace metal-free tubes and refrigerated at 4°C until shipment to the laboratory where they were frozen at −20°C until analysis. Pb concentration was measured by external calibration using the Agilent 8800 ICP Triple Quad (Agilent, Santa Clara, CA, USA) in MS/MS mode in the Trace Metals Laboratory at the Icahn School of Medicine at Mount Sinai. The limit of detection was <0.2 μg/dl and the instrument precision (given as %RSD) was ∼5%. Good precision and accuracy were shown using blinded quality control samples obtained from the Maternal and Child Health Bureau and the Wisconsin State Laboratory of Hygiene Cooperative Blood Lead Proficiency Testing Program. BLLs were analyzed as continuous variables (given in micrograms per deciliter) and as categorical variables classified according to the median exposure per stage, described below.

To account for cumulative exposure, at the prenatal, postnatal, and cumulative stages, we dichotomized the BLLs using the median for each study visit as the cutoff point. Two categories were obtained: "higher," when a BLL was at least one time above the median (HPb), and "lower," if all BLLs were below the median (LPb).

We measured bone Pb levels at 1 month postpartum in maternal tibia (mid-tibial shaft, cortical bone) and patella (trabecular bone) using a K-shell X-ray fluorescence instrument for 30 min in each leg. The measures were computed, averaged, and weighted by the inverse of the proportion of the measurement error corresponding to each determination. Negative values, produced when the true values are below the detection limit of the instrument, were imputed with random draws from a uniform distribution between 0 and the lower limit of 2 μg lead/g bone mineral (23).

Risk Factor Indicators of MetS

Metabolic syndrome risk factors (IMetS), which were assessed at the 72- and 96-month visits, included the following: glucose, glycated hemoglobin (HbA1c), total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (cHDL), low-density lipoprotein cholesterol (cLDL), body fat percentage (BF%), waist circumference (WC), body mass index (BMI), systolic blood pressure (SPB), and diastolic blood pressure (DBP).

Fasting venous blood was drawn to obtain blood levels of TC, cHDL, cLDL, TGs, glucose, and HbA1c. Enzyme methods (Roche Diagnostics, Indianapolis, IN, USA) were used to obtain measures of TC, cHDL, cLDL, and TGs. The levels of HbA1c were determined using the Miura 200 automated analyzer (ISE S.r.l., Rome, Italy). The weight, height, and waist circumference were measured during both follow-ups in triplicate and the average of each measurement obtained. The weight and height data were used to calculate the BMI (weight/size^2) and the z-score with BMI for age indicator using the WHO "Anthro" software (24). Total adipose tissue was measured using the InBody 370 or 230 tetrapolar bioelectric impedance equipment (Biospace Co., Ltd., Los Angeles, CA, USA). BF% was obtained by estimating the total adipose tissue divided by the total body mass. WC was measured at the midpoint between the last floating rib and the iliac crest. Standardized personnel performed all anthropometric measurements.

SBP and DBP were measured at rest using an automated Spacelabs Healthcare monitor, Ultralite Ambulatory 90217 (Snoqualmie, WA, USA). This measurement was taken in duplicate with a 3-min difference between each, and the average of the measurements was used.

IMetS were categorized according to the different cutoff points (24, 25) considering high levels in blood if: glucose was ≥100 mg/dl, HbA1c was ≥5.7%, TC was ≥170 mg/dl, cHDL was <45 mg/dl, cLDL was >110 mg/dl, and TGs were >75 mg/dl for children 9 years of age or younger and >90 mg/dl for children 10 years of age or older. The cutoff point for BF% and WC was the 80th percentile. BMI z-scores were classified according to the WHO Child Growth Standards (24): underweight, less than −2 SD; normal weight, greater than −2 to less than +1 SD; overweight, greater than +1 SD; and obese, greater than +2 SD. The cutoff point for SPB and DBP was the 90th percentile.

Covariates

The following variables were considered for model adjustment: maternal age at recruitment, socioeconomic status (SES), parity collected through standardized questionnaires during the second trimester of pregnancy, child's size for gestational age (Fenton score), sex, and age at the 72- and 96-month visits. SES was measured using the Mexican Association of Market Intelligence and Opinion Agencies questionnaire. With reference to our study population, which belongs to a middle–lower SES, we

collapsed the variable into three categories: lower, medium, and higher. Parity considered two categories (including the current pregnancy): primiparous (less than or one pregnancy) and two or more pregnancies.

Statistical Analysis

Descriptive analyses were carried out for the evaluation of extreme or implausible values in the database. In the case of continuous variables, the mean and standard deviations were obtained, as well as the ranges or the median and interquartile range, as appropriate. Frequencies and percentages were obtained for categorical variables. Student's *t*-test and the Mann–Whitney *U* test were used for continuous variables and the chi-square test used for categorical variables.

To assess the association between exposure to Pb and IMetS, linear mixed effects models and logistic mixed effects models were utilized. BLLs were evaluated both as continuous and dichotomous. IMetS were assessed as continuous and categorized. The models were adjusted for sex, maternal and child ages, parity, SES, and the Fenton score. Secondary analyses were additionally adjusted for the BMI *z*-scores to ensure that obesity did not impact the results. Models were utilized considering three developmental windows: prenatal, postnatal, and cumulative. We stratified our models by sex to assess possible effect modifications of the associations. Outliers of the IMetS were excluded ($n = 4$), three with biologically implausible BF% data and one with a BMI *z*-score for age ≥5 SD. All analyses were performed using Stata version 14 software (StataCorp LLC, College Station, TX, USA).

As a sensitivity analysis, mixed effects models were utilized with a subsample that had the complete follow-up data to verify that loss to follow-up and the presence of missing values did not affect the results.

RESULTS

General characteristics of the 601 mother–child dyads included in this study are described in **Table 1**. Women's mean age at enrollment was 27.1 ± 5.5 years. More than half of the participants had two or more previous pregnancies (62%), and most had lower SES. At birth, children were an average of 3 ± 0.5 kg in weight, with a gestational age of 38.1 weeks. There were 48 children with low birth weight and 39 who were born preterm. The median BLLs were higher in the prenatal stages compared to those in the postnatal stages.

The characteristics of the 601 participants included in these analyses were compared to those of non-participants, and the differences were not statically significant (results not shown).

Table 2 shows the descriptive characteristics of the IMetS for boys and girls at the 72- and 96-month study visits. Most indicators showed statistically significant differences between visits, and we observed a considerable increase in the prevalence of overweight and obesity. Boys had higher glucose and lower triglycerides than did girls at the 96-month study visit ($p < 0.05$).

The main finding for IMetS classified according to the cutoff points was the presence of at least one IMetS in children: 61.6% for the stage 72 study visit and 63.7% for the stage 96 study visit. The prevalence of glucose ≥100 mg/dl was found to be higher in boys than that in girls. Most of the IMetS showed an increase in prevalence between stages 72 and 96 and were more prevalent among overweight and obese participants. Between stages 72 and 96, increases of 6 percentage points for overweight and 13.6 percentage points for obesity were found in boys. Similar increases were found in girls, 8.6 percentage points for overweight and 7.5 percentage points for obesity (**Table 3**).

Table 4 shows the results of the BLL mixed effects logistic models for prenatal, postnatal, and all stages (i.e., IMetS categorized according to the cutoff points). According to the results, we found that children with HPb in the prenatal stage were 47% (OR = 0.53, 95%CI = 0.31–0.99) less likely to have TC > 170 mg/dl compared to children with LPb. Similar results were observed for postnatal BLLs (OR = 0.59, 95%CI = 0.36–0.94). We observed an association between prenatal HPb and TGs (OR = 0.65, 95%CI = 0.44–0.95). Children with postnatal HPb were 73% (OR = 0.27, 95%CI = 0.08–0.86) less likely to have a WC above the 80th percentile. For the "all stages" model, children with HPb were less likely to have SBP (OR = 0.53,

TABLE 1 | Basal characteristics in mother–child dyads from the PROGRESS cohort.

Characteristics	Mean (SD) or median (Q1–Q3)		
	Total ($N = 601$)	Boys ($n = 308$)	Girls ($n = 293$)
Maternal characteristics			
Maternal age (years)[a]	27.1 (5.5)	27.3 (5.4)	26.9 (5.7)
Socioeconomic status[b]			
Lower	52.6%	52.9%	52.2%
Medium	37.3%	36.7%	37.9%
Higher	10.1%	10.4%	9.9%
Parity (2)			
Primiparous	38.4%	36.4%	40.6%
Multiparous	61.6%	63.6%	59.4%
Children's characteristics			
Birth weight (kg)*	3.1 (0.4)	3.1 (0.4)	3.0 (0.4)
Gestational age (weeks)[c]	38.8 (1.5)	38.8 (1.6)	38.8 (1.5)
BLLs in the prenatal stage			
Second trimester (μg/dl)[c*]	2.9 (1.9–4.4)	3.1 (2.0–4.6)	2.7 (1.9–4.2)
Third trimester (μg/dl)[c]	3.1 (2.0–4.8)	3.1 (2.0–4.9)	3.0 (1.9–4.6)
At birth in umbilical cord (μg/dl)[c]	2.2 (1.4–3.7)	2.4 (1.4–3.7)	2.1 (1.4–3.8)
Bone Pb levels: patella	3.4 (1.3–8.9)	3.0 (1.2–8.8)	4.3 (1.4–9.5)
Bone Pb levels: tibia	3.0 (1.1–7.5)	3.1 (1.4–7.4)	2.9 (0.9–7.6)
BLLs in the postnatal stage[d]			
1 year (μg/dl)[c]	2.0 (1.6–2.9)	2.1 (1.5–3.2)	2.0 (1.6–2.6)
2 years (μg/dl)[c]	2.2 (1.6–3.1)	2.4 (1.7–3.2)	2.0 (1.5–3.0)
4 years (μg/dl)[c]	1.7 (1.3–2.5)	1.7 (1.3–2.5)	1.7 (1.3–2.6)

BLLs, blood lead levels.
**$p < 0.05$ for the difference between sex categories (Student's t-test or Mann–Whitney U test for numerical variables and Pearson's test of independence for categorical variables).*
[a]With values shown as mean (SD).
[b]Categorical variables with values shown as frequency (%).
[c]With values shown as median and interquartile ranges.
[d]BLLs at 1 year: N = 139, n = 67 boys and n = 72 girls; at 2 years: N = 203, n = 99 boys and n = 104 girls; at 4 years: N = 475, n = 242 boys and n = 233 girls.

TABLE 2 | Children's metabolic syndrome risk factors by sex and study visit.

Indicators	Boys			Girls		
	Median		*p-value*	Median		*p-value*
	Stage 72	Stage 96		Stage 72	Stage 96	
Glucose (mg/dl)**	88.6	87.1	0.157	85.7	85.3	0.822
HbA1C (%)	5.4	4.9	<0.001	5.4	5.1	<0.001
TC (mg/dl)	161.0*	154	0.092	166*	157	<0.001
TGs (mg/dl)**	68.0	75.0	<0.001	77.5	86.0	<0.001
cHDL (mg/dl)	50.9	51.1	0.064	49.4	46.7	<0.001
cLDL (mg/dl)	92.8*	87.7	<0.01	97.1*	89.9	<0.001
Body fat (%)	22.6*	29.6	<0.001	24.4*	30.5	<0.001
WC (cm)	54.3	65.6	<0.001	54.9	65.1	<0.001
BMI (kg/m^2)	15.7	18.1	<0.001	15.8	18.0	<0.001
Systolic blood pressure (mmHg)	102.0*	112.3	<0.001	100.5*	110.6	<0.001
Diastolic blood pressure (mmHg)	61.5	70.7	<0.001	60.5	69.5	<0.001

For stage 72, glucose, TC, TGs, cHDL, and cLDL: n = 265 boys and n = 260 girls; WC and BMI: n = 308 boys and n = 293 girls; systolic and diastolic blood pressure: n = 286 boys and n = 272 girls; HbA1c: n = 264 boys and n = 261 girls; BF%: n = 277 boys and n = 269 girls.
For stage 96, glucose, TC, TGs, cHDL, and cLDL: n = 259 boys and n = 250 girls; WC and BMI: n = 275 boys and n = 265 girls; systolic and diastolic blood pressure: n = 265 boys and n = 253 girls; HbA1c: n = 257 boys and n = 251 girls; BF%: n = 274 boys and n = 264 girls. The p-values shown represent the differences between stages.
TC, total cholesterol; TGs, triglycerides; cHDL, high-density lipoprotein cholesterol; cLDL, low-density lipoprotein cholesterol; WC, waist circumference; BF%.
**p < 0.05 for differences between sex at stage 72 (Mann–Whitney U test for numerical variables); **p < 0.05 for differences between sex at stage 96 (Mann–Whitney U test for numerical variables).*

TABLE 3 | Children's metabolic syndrome risk factors according to the cutoff points by sex and study visit.

Indicators	Boys		Girls	
	Stage 72 *n* (%)	Stage 96 *n* (%)	Stage 72 *n* (%)	Stage 96 *n* (%)
Glucose, ≥100 mg/dl	25 (9.4)	26 (10.1)	18 (6.9)	15 (6.0)
HbA1C, ≥5.7%	32 (12.5)	17 (6.6)	27 (10.8)	23 (9.2)
Total cholesterol, >170 mg/dl	65 (24.9)	78 (30.6)	98 (38.7)	76 (30.6)
Triglycerides, >75 or >90 mg/dl	104 (39.7)	96 (37.9)	134 (51.7)	120 (48.6)
cHDL, <45 mg/dl	74 (27.9)	78 (30.1)	83 (31.9)	109 (43.6)
cLDL, >110 mg/dl	36 (13.6)	44 (17.0)	58 (22.3)	48 (19.3)
Body fat percentage, >80th percentile	27 (9.7)	27 (9.8)	27 (10.0)	26 (9.8)
Waist circumference, >80th percentile	61 (19.8)	55 (20.0)	58 (19.8)	53 (20.0)
BMI for age, *z*-scores				
Underweight, less than−2 SD	7 (2.3)	4 (1.4)	1 (0.3)	3 (1.1)
Normal, greater than −2 to less than +1 SD	214 (69.7)	140 (50.9)	212 (72.4)	147 (55.5)
Overweight, greater than +1 SD	46 (15.0)	58 (21.1)	50 (17.1)	68 (25.7)
Obesity, greater than +2 SD	40 (13.0)	73 (26.6)	30 (10.2)	47 (17.7)
Systolic blood pressure, >90th percentile	27 (9.4)	25 (9.4)	26 (9.6)	23 (9.1)
Diastolic blood pressure, >90th percentile	27 (9.4)	26 (9.8)	27 (9.9)	25 (9.9)

cHDL, high-density lipoprotein cholesterol; cLDL, low-density lipoprotein cholesterol.

95%CI = 0.32–0.85) and DBP (OR = 0.57, 95%CI = 0.34–0.95) above the 80th percentile. We also observed this association in the prenatal model (OR = 0.60, 95%CI = 0.37–0.98).

Body fat percentage models only adjusted for maternal and infant ages because of the limited number of each category in categorical covariates. The glucose model in the postnatal stage was adjusted for socioeconomic status, sex, maternal age, infant age, and parity. Glucose, TC, cHDL, and cLDL: $n = 586$ for all stages, $n = 585$ for the prenatal stage, and $n = 509$ for the postnatal stage. Glycosylated hemoglobin and TGs: $n = 583$ for all stages, $n = 582$ for the prenatal stage, and $n = 508$ for the postnatal stage. WC and BMI: $n = 601$ for all stages, $n = 600$ for the prenatal stage, and $n = 519$ for the postnatal stage. Systolic and diastolic blood pressure: $n = 599$ for all stages, $n = 598$ for the prenatal stage, and $n = 517$ for the postnatal stage. The prenatal stage included measurements of the blood lead levels during the second and third trimesters of pregnancy. The postnatal stage included measurements of the blood lead levels at birth and at 1, 2, and 4 years of age.

Supplementary Table S2 shows the results of the longitudinal association models between the BLLs at each stage (i.e., prenatal, postnatal, and all stages) and continuous IMetS between the 72- and 96-month study visits. We observed a statistically significant inverse association between children with HPb during all stages and TC levels ($\beta = -5.40$, 95%CI = −9.75 to −1.04). Similarly, HPb had an inverse association with SBP ($\beta = -1.92$, 95%CI = −3.72 to −0.11) at all stages. Additionally, we observed suggestive inverse associations between HPb and cLDL and BF%, which did not reach statistical significance, during the prenatal stage ($\beta = -3.26$, 95%CI = −7.03 to 0.51; $\beta = -1.45$, 95%CI = −2.95 to 0.05).

Table 5 shows the results of the regressions assessing the relationships with bone Pb measures as continuous variables. We observed that higher Pb levels in the patella were positively associated with cHDL levels <45 mg/dl (OR = 1.03, 95%CI = 1.00–1.07). We also observed an inverse association among children with higher tibia Pb levels and TGs: children with higher tibia Pb levels were 5% less likely to have elevated TGs (OR = 0.95, 95%CI = 0.91–0.99).

The Pb data available decreased to 75.6% for stage 12 and to 64.3% for stage 24. To assess the effect of loss to follow-up, we assessed the associations with a subsample of children who had complete data for all visits. The results were similar to those reported in the initial sample. Our results did not change in the models using continuous BLLs (results not shown) or in the sensitivity analysis including children with complete

TABLE 4 | Association of higher blood lead exposure levels (above the median) and indicators of metabolic syndrome categorized according to the cutoff points.

Indicators	All stages[a]		Prenatal[a]		Postnatal[a]	
	OR	95% CI	OR	95% CI	OR	95% CI
Glucose, ≥100 mg/dl	1.22	0.64–2.33	0.79	0.46–1.37	0.97	0.56–1.68
HbA1c, ≥5.7%	0.87	0.50–1.48	0.90	0.55–1.44	1.47	0.85–2.52
Total cholesterol, >170 mg/dl	**0.53**	**0.32–0.86**	*0.67*	*0.43–1.02*	**0.59**	**0.36–0.94**
Triglycerides, >75 or >90 mg/dl	0.72	0.46–1.11	**0.65**	**0.44–0.95**	*0.69*	*0.46–1.04*
cHDL, <45 mg/dl	1.46	0.88–2.44	1.37	0.88–2.12	1.08	0.66–1.75
cLDL, >110 mg/dl	0.75	0.42–1.33	0.81	0.48–1.34	0.93	0.53–1.61
Body fat percentage, >80th percentile	0.78	0.20–2.97	1.62	0.53–4.93	0.65	0.20–2.05
Waist Circumference, >80th percentile	**0.27**[b]	**0.08–0.86**	0.77	0.29–1.99	*0.37*	*0.13–1.07*
BMI for age, *z*-scores	**0.33**	**0.11–0.99**	*0.43*	*0.16–1.14*	0.60	0.20–1.68
Systolic blood pressure, >90th percentile	**0.53**	**0.32–0.85**	*0.66*	*0.41–1.02*	0.76	0.46–1.24
Diastolic blood pressure, >90th percentile	**0.57**	**0.34–0.95**	**0.60**	**0.37–0.98**	0.76	0.45–1.26

cHDL, high-density lipoprotein cholesterol; cLDL, low-density lipoprotein cholesterol; TC, total cholesterol; WC, waist circumference.
[a]Models adjusted for maternal characteristics (socioeconomic status, maternal age, and parity) and characteristics of infants (sex, size for gestational age, and infant age).
[b]Results in bold with statistically significant differences ($p < 0.05$).
[c]Results in italics with marginally significant differences ($p < 0.09$).

TABLE 5 | Associations between the lead levels in the trabecular (patella) and cortical (tibia) bone and indicators of metabolic syndrome categorized according to the cutoff points.

	Patella[a]		Tibia[a]	
	OR	95%CI	OR	95%CI
Glucose, ≥100 mg/dl			1.02	0.97–1.07
HbA1C, ≥5.7%	0.99	0.95–1.03	0.99	0.94–1.03
Total cholesterol, >170 mg/dl	1.00	0.96–1.03	0.95	0.91–0.99
Triglycerides, >75 or >90 mg/dl	0.98	0.95–1.01	**0.95**[c]	**0.91–0.99**
cHDL, <45 mg/dl	**1.03**[c]	**1.00–1.07**	1.00	0.96–1.04
cLDL, >110 mg/dl	1.01	0.97–1.05	0.97	0.92–1.03
Body fat percentage, >80th percentile				
Waist circumference, >80th percentile	0.99	0.92–1.07	0.99	0.90–1.09
BMI for age, *z*-scores	0.93	0.85–1.01	0.98	0.89–1.09
Systolic blood pressure, >90th percentile	0.99	0.95–1.02	0.99	0.95–1.04
Diastolic blood pressure, >90th percentile	0.97	0.93–1.01	0.98	0.93–1.03

cHDL, high-density lipoprotein cholesterol; cLDL, low-density lipoprotein cholesterol.
[a]Models adjusted for socioeconomic status, sex, maternal age, size for gestational age, infant age, and parity.
[b]Results in bold with statistically significant differences ($p < 0.05$).
[c]Results in italics with marginally significant differences ($p < 0.09$).

data (**Supplementary Table S3**). Finally, the results for the sex-stratified models evaluating possible effect modifications showed no statistically significant differences in the association between boys and girls (**Supplementary Table S4**).

DISCUSSION

Our study found associations between Pb exposure in early life and the different early-stage risk indicators of MetS. Although in some cases the direction of such associations was opposite that of our hypotheses, these suggest a disruption of the expected normality and should be evaluated. Pre- and postnatal HPb exposures were inversely associated with children's TC, WC, BMI, elevated TGs, and blood pressure at ages 6–12 years. Pb in cortical bone was associated with higher odds of having cHDL <45 mg/dl, and Pb in trabecular bone showed lower odds of elevated TGs. Evidence from studies in children remains controversial, and our results are in line with some, but not all. To illustrate this, below, we present a summary of the associations reported in other studies; details can be found in **Supplementary Table S5**. In general, the differences between our study and others reported here could be due to the study design (many were cross-sectional), the Pb biomarkers used, differences in ethnicity and age, and the use of different cutoff points for IMetS. Contrary to most prior literature (20, 21, 26, 27), we observed no evidence of sex-specific associations. A similar null sex-specific association was found between BLLs and TC (20).

Total Cholesterol

Our study suggests that HPb, both postnatally and during all stages, was associated with higher odds of having lower TC levels. This result could be explained by the effect of prenatal exposure to Pb in the regulation of cholesterol metabolism (20, 28). According to the study by Liu et al., exposure to Pb was inversely associated with the TC levels in boys (20), and the direction of the association was similar to what we have observed in the present study. Kupsco et al. found no association between the BLLs in pregnancy and TC in 4- to 6-year-old children (17). In the study by Poursafa et al. (21), positive associations were found across quartiles of the BLLs and TC in children and among girls.

cHDL

In our study children with higher levels of Pb in the patella were more likely to have cHDL levels <45 mg/dl. These results were similar to prior evidence, which showed that prenatal BLLs were

inversely associated with cHDL at age 10–18 years (20) although a null association was reported by Poursafa et al. (21) in Iranian pre- and adolescents.

Triglycerides

Prenatal BLLs and tibia Pb levels were inversely associated with TGs. Most of the evidence has reported null associations (17, 20), yet one study on Iranian boys and girls found that an increase in the quartiles of BLLs was positively associated with TGs (21). In our study, concordance between the direction of the associations between BLLs in the prenatal stage and the cumulative Pb levels measured in the tibia may suggest that exposure during pregnancy could impact the TGs levels in 6- to 12-year-old children.

BMI

Not surprisingly, we observed that an increased prevalence of overweight and obesity added to the increased WC measures and BF% at the stage 96 study visit, which could be due to changes in body composition associated with age (29).

Similar to what have been reported, we found an inverse association between HPb and BMI for age (30–34). This evidence shows that both prenatal (32, 34) and postnatal (33) exposures to Pb adversely impact children's growth. In the study by Shao et al., negative associations were found between urinary levels of Pb and overweight and obesity, being stronger among 6- to 12-year-olds compared to those in 13- to 19-year-olds (30). In the study by Liu et al., Pb levels in the patella were negatively associated with lower child BMI z-scores ($\beta = -0.02$, 95%CI = 0.03 to -0.01). In prior studies of the PROGRESS cohort, Renzetti et al. (Pb during the last two trimesters of pregnancy, delivery and postpartum, with BMI in children at 4–6 years) (32) and Kupsco et al. (BLLs during the third trimester of pregnancy and the BMI z-scores at 48 months) (17) reported null associations. Similarly, null associations were found by Afeiche et al. between longitudinal exposure to Pb and BMI in 48-month-old Mexican children. However, other findings showed that children with high exposure in all windows of development (prenatal, infancy, and early infancy) were almost 1 cm ($\beta = -0.98$, 95%CI = -1.86 to -0.10) shorter than children with low exposure during those periods, in birth cohorts in Mexico City (35).

Although the evidence is not conclusive, we propose three main biological mechanisms that could be involved. Firstly, the impact of Pb exposure *in utero* resulting in lower weight (36, 37) and height at birth (37): this effect can continue during early childhood (38). A second mechanism is the action of Pb as an endocrine disruptor by reducing the response of hormones such as insulin like-growth factor (39) and the action of cortisol that could affect the hypothalamus–pituitary–adrenal axis (40). The third and most reported mechanism involves the main effect of Pb on bone growth by impairing the function of bone cells (osteoblasts), the mineralization of bone, and the ability of the bone to respond to hormonal regulation, which can also affect the bone cartilage (41, 42).

Waist Circumference and Body Fat Percentage

We found a similar association between HPb and WC to that reported in the study by Liu et al., where the Pb levels in the patella were inversely associated with WC ($\beta = -0.12$ cm, 95%CI = 0.22 to -0.03) and BF% ($\beta = -0.09$%, 95%CI = 0.17 to -0.01) (34). Similar associations were also reported by Deierlein et al. in a study of girls (33). In the case of BF%, null associations were found in our study, similar to results previously reported in the PROGRESS cohort with children in other developmental stages (17, 32).

Systolic and Diastolic Blood Pressure

Contrary to what we expected, our results showed that HPb was associated with less likelihood to have elevated SBP and DBP (above the 90th percentile). Positive associations have been shown between Pb exposure and SBP using different biomarkers such as maternal toenail (27), maternal tibia bone (26), and children's BLLs (21). Null associations between maternal exposure to Pb and blood pressure were reported by Skröder et al. in a cohort of 4.5-year-olds rural in Bangladesh (43) and by Kupsco et al. in the same study population as ours, children 4–6 years old (17). Poursafa et al. showed positive associations between the quartiles of exposure to Pb and SBP and DBP. More studies are needed to explain the biological mechanisms that could produce these changes on vascular systems.

We are aware of the possibility of a type 1 error due to multiple comparisons; however, after Bonferroni adjustments, the presented associations between HPb and TC were still statistically significant. One of our study limitations is having missing BLLs in the 12- and 24-month postnatal stages, but the results in the models indicated that these losses did not influence the associations found.

Exposure to lead continues to be a public health problem in Mexico since the main source of exposure is the use of lead-glazed low-temperature ceramics to prepare, serve, and store food. Lead will leach into food with each use, even in very old and worn-out dishes. This type of ceramics is widespread in Mexico, and recent studies using representative national data have shown a clear association between their use and BLLs in 1- to 4-year-old children (44, 45).

The strengths of this study include it being the first epidemiological study that evaluated the association between repeated measures of exposure to Pb (separately during prenatal, postnatal, and cumulative early life stages) and the risk of IMetS longitudinally at two different time points, between ages 6 and 12 years. A particular strength is our extensive data on Pb exposure in both blood (acute exposure, with a half-life of 25 days) and bone [indicator of chronic exposure (half-life of decades) and endogenous exposure during pregnancy and long-term exposure in mothers]. The PROGRESS cohort is a prospective study that collected data on 11 risk factors of IMetS evaluated at two different ages, as well as covariates for model adjustment.

CONCLUSION

In this study, a longitudinal comprehensive assessment of early risk indicators of MetS in children, we observed small changes according to the biomarkers and developmental windows of Pb exposure. These changes should be assessed across adolescence and early adulthood.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Institute of Public Health, Mexico Icahn School of Medicine at Mount Sinai, New York, USA. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KM-S and MT-O: conceptualization, methodology, formal analysis, writing—original draft, writing—review and editing, and visualization. AA and EO-P: conceptualization, methodology, formal analysis, writing—original draft, and writing—review and editing. MP-Z: investigation and writing—review and editing. AM-G: investigation. RW and MM: writing—review and editing, supervision, project administration, and funding acquisition. AS: methodology, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.750316/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Pre-conceptional Maternal Vitamin B12 Supplementation Improves Offspring Neurodevelopment at 2 Years of Age: PRIYA Trial

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Background: The first thousand days window does not include the pre-conceptional period. Maternal pre-conceptional health has a profound influence on early embryonic development (implantation, gastrulation, placentation etc). Nutrition provided by B-complex vitamins is important for fetal growth, especially neural development. We report effects of a maternal pre-conceptional vitamin B12 and multi micronutrient (MMN) supplementation on offspring neurodevelopmental performance.

Methods: In the Pune Rural Intervention in Young Adolescents trial (PRIYA), adolescents ($N = 557$, 266 females) were provided with vitamin B12 (2 μg/day) with or without multiple micronutrients, or a placebo, from preconception until delivery. All groups received mandatory iron and folic acid. We used the Bayley's Scale of Infant Development (BSID-III) at 24–42 months of age to investigate effects on offspring neurodevelopment.

Results: Participants had similar baseline B12 levels. The levels improved in the B12 supplemented groups during pre-conception and pregnancy (28 weeks gestation), and were reflected in higher cord blood holotranscobalamin (holo-TC) levels compared to the placebo group. Neurodevelopmental outcomes in the B12 alone group ($n = 21$) were better than the placebo ($n = 27$) in cognition ($p = 0.044$) and language ($p = 0.020$) domains (adjusted for maternal baseline B12 levels). There was no difference in neurodevelopmental outcomes between the B12 + MMN ($n = 26$) and placebo group. Cord blood Brain Derived Neurotrophic Factor (BDNF) levels were highest in the B12 alone group, though not significant.

Conclusion: Pre-conceptional vitamin B12 supplementation improved maternal B12 status and offspring neurodevelopment at 2 years of age. The usefulness of cord BDNF as a marker of brain development needs further investigation. Our results highlight the importance of intervening during pre-conception.

Keywords: vitamin B12, pre-conception, supplementation, neurodevelopmental outcome, offspring

INTRODUCTION

The developing fetus is dependent on its mother for its nutrition. Maternal nutrition before and during pregnancy affects fetal growth and development, and maternal malnutrition may predispose the offspring to undesirable outcomes in later life. This concept is called "fetal programming." This is the backbone for the Developmental Origins of Health and Disease (DOHaD) paradigm which expanded the idea to include "health" as a programmed state (1, 2). Pregnancy and the first 2 years of life (1,000 days) are considered the most crucial window for programming (3).

Maternal nutritional factors (both macro and micronutrients) influence neurodevelopmental processes *in utero*, such as neurogenesis, myelination, synaptogenesis, and cortical brain growth (3).Vitamins B12 and folate are of special interest due to their role in the one carbon metabolism pathway. This represents a series of biochemical reactions involving the methionine and folate cycles. The methylation of homocysteine involves the transfer of a methyl group from 5-methyl tetra-hydro folate (THF) by methionine synthase (MS). Vitamin B12 is a cofactor for this reaction. This transfer in turn generates S-adenosyl methionine (SAM) which is a universal methyl donor. One carbon metabolism supports important cellular processes such as DNA synthesis, repair, and methylation, which is important for epigenetic regulation of gene expression (4). Offspring of mothers with low maternal vitamin B12 and folate status during pregnancy have a higher risk of neural tube defects and neurodevelopmental disorders [Autism, Attention Deficit Hyperactivity Disorder (ADHD)], poorer cognitive development, and smaller brain volumes in childhood (5–8). In animal models (rats), offspring of mothers exposed to a high folate and low vitamin B12 diet show lower levels of Brain Derived Neurotrophic Factor (BDNF) in the brain, and poorer cognitive function (9, 10).

In India, vitamin B12 deficiency is widely prevalent in pregnant women (50–70%) (11, 12) and is attributable to the socio-cultural practice of vegetarianism and poor economic status (13–16). This deficiency is associated with a range of adverse pregnancy and offspring health outcomes (17). In prospective birth cohorts from western India, we have earlier shown that exposure to low maternal vitamin B12 *in utero* is associated with poorer cognitive functioning at the age of 2 and 9 years in the offspring (18, 19). However, public health policy in India mandates only iron and folic acid supplementation to women in the reproductive age group, and during pregnancy and lactation. A randomized controlled trial in South India showed that supplementing 50 μg/day oral B12 from 14 weeks of pregnancy until 6 weeks postpartum improved B12 concentrations in breast milk, the vitamin B12 status of infants at 6 weeks and infant cognitive function at 30 months of age (20, 21).

Important milestones in fetal neural development such as neural tube closure are completed by 26–28 days of gestation (22). The majority of pregnancies in India are unplanned, and by the time pregnancy is detected (typically between 10 and 14 weeks gestation) this early developmental window is lost. Pre-conceptional supplementation will ensure that the mother has improved vitamin stores during the early neurodevelopmental period. The success of pre-conceptional folic acid supplementation in preventing neural tube defects is well-known (23–25). Few studies have examined the effects of pre-conceptional maternal micronutrient supplementation on offspring neurodevelopment in India. This approach will expand the 1,000 days concept to include the pre-conceptional period.

The Pune Rural Intervention in Young Adolescents (PRIYA) is a pre-conceptional vitamin B12 and multi micronutrient supplementation trial in adolescent participants of the Pune Maternal Nutrition Study. Here we report neurodevelopmental outcomes at 2 years of age in the offspring of female participants in the trial. We hypothesized that pre-conceptional B12 supplementation in the mothers would contribute to better neurodevelopmental outcomes in their offspring.

MATERIALS AND METHODS

PRIYA Trial

The PRIYA trial methods have been published previously (26). Briefly, The Pune Maternal Nutrition Study (PMNS) is a pre-conceptional observational birth cohort set up in 1993 (**Figure 1**). Married non-pregnant women were recruited from six villages around Pune and those who became pregnant were followed up. Seven hundred and sixty-two children were born and followed up serially. At ∼17 years of age, 690 participants from the PMNS cohort (11) were screened for inclusion in the PRIYA trial. Of these, 117 were excluded due to severe vitamin B12 deficiency (<100 pmol/L) because of the ethical imperative of a placebo-controlled trial. Sixteen were excluded due to systemic illnesses. Five hundred and fifty-seven (266 females) participants were randomized (**Figure 1**) to receive either a placebo, B12 (2 μg/day) + multiple micronutrients (MMN) or B12 alone (2 μg/day). The composition of the MMN tablet (**Supplementary Table 1**) was guided by the WHO/UNICEF/UNU international multiple micronutrient preparation (UNIMMAP). We excluded Iron and Folic Acid because the mandated IFA tablets (Iron and Folic Acid) were given to all participants as per Government of India recommendations (100 mg elemental Iron and 500 μg folic acid once a week during adolescence, and at least 100 tablets during pregnancy). The investigational supplements (vitamin B12 containing) were continued for the female participants daily until their first delivery. They and the study team were blinded to the vitamin/micronutrient supplementation.

This paper describes the findings in the female participants and their children. Participants were followed up regularly for health problems, and marriages were recorded. Married women were monitored to detect pregnancy which was confirmed by a urine pregnancy test. At 24–28 weeks gestation, mothers visited the Diabetes Unit, KEM Hospital Research Center Pune, for a fasting oral glucose tolerance test (as per international guidelines) and clinical and biochemical evaluations. The clinical evaluation included anthropometric measurements, an obstetric consultation and estimate of fetal growth by ultra-sonography. We also obtained socio demographic information (assessed using

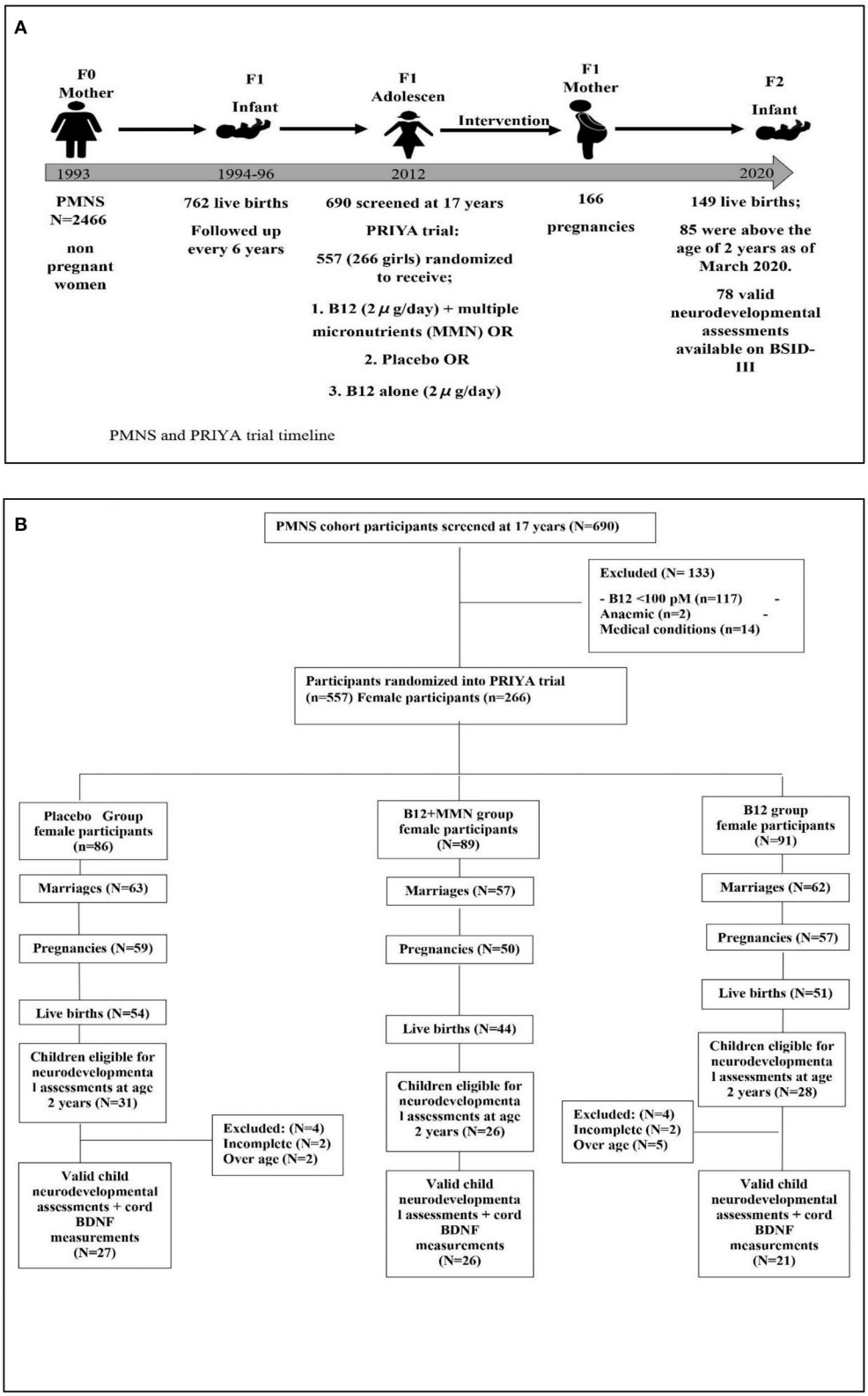

FIGURE 1 | Diagram depicting the study timeline **(A)** and recruitment of study participants **(B)** for neurodevelopmental follow up as of February 2020. Further collection of data discontinued due to COVID-19 pandemic.

the Standard of Living Index questionnaire from the National Family Health Survey of India (NFHS).

Details of deliveries were recorded (gestational age and type of delivery). Cord blood samples were collected and processed for hematological and nutrient measurements. We performed detailed anthropometric measurements on the baby within 72 h of birth.

We measured circulating concentrations of vitamins B12, holotranscobalamin (holo-TC), folate, and total homocysteine at baseline, 6–12 months after the start of the supplementation (at ∼18 years of age), 28 weeks gestation, and in cord blood (**Table 1**). We additionally measured B2 and B6 levels in mothers at 28 weeks gestation and in offspring cord blood.

Hemogram was measured on a Beckman Coulter analyzer (AC.T diffTM Analyzer, Florida, USA) on the day of the collection. Plasma vitamin B12 and folate were measured using a microbiological assay and total homocysteine, vitamin B2 and B6 by HPLC (PerkinElmer 200 Series, PerkinElmer, Shelton, CT, USA). Plasma holo-TC was measured by a two-step immunoassay using CMIA technology (Architect, Abbott GmbH & Co. KG, Germany). This represents the fraction of vitamin B12 transported on transcobalamin-II and is available for the peripheral tissues, hence also called "active" vitamin B12. It is increasingly used as a more sensitive marker for B12 deficiency. The remaining vitamin B12 (70–80%) is attached to haptocorrin and is not available for peripheral tissues. Total vitamin B12 (called vitamin B12) is the sum of the two. Plasma BDNF was measured in cord blood using ELISA kit (XpressBio, Frederick, USA).

Neurodevelopmental Assessments

The offspring born in the trial were followed up every 6 months until 2 years of age for measurements of their growth. Once they reached 24 months of age, the parents were approached regarding participation in the neurodevelopment study, and their written informed consent was obtained. The neurodevelopmental assessment was performed at the Child Development Center (TDH center), KEM Hospital, Pune.

The neurodevelopmental assessment was performed using the Bayley's Scale of Infant Development (BSID-III) (27). The BSID-III assesses the developmental status of infants from 1 to 42 months of age. The scales assess five domains across three main subscales: (1) cognitive (2) language—receptive and expressive language and (3) motor—which assesses gross and fine motor skills. The assessment was performed by trained clinical psychologists certified to perform the BSID-III. Testing was carried out in a quiet room, with a parent or guardian present, and instructions were provided in a language that was comfortable for the child. All children were assessed between 24 and 42 months of age. Each test protocol was independently reviewed and scored by two raters. The BSID-III test yields raw scores based on the performance of the child on test items for cognitive, expressive, and receptive communication, and fine and gross motor skills. The raw scores were converted into age standardized scaled scores as recommended in the manual. Summation of the scaled scores yields 3 composite scores for the cognitive, language and motor skills domains. We used the composite scores in our analysis. Composite scores were categorized into average, below or above average performance, based on standardized criteria provided in the manual, where the average is 100 with SD of 15 and a score of <85 is considered to be below average (27).

As part of ongoing assessments in the PMNS cohort, maternal intelligence [determined by the mothers' Intelligence Quotient (IQ) score] was assessed in some of the mothers at age 22–24 years using the Weschler's Adult Intelligence Scale-IV (WAIS-IV).

Ethical Considerations

Details of community participation in the planning of this trial have been described earlier (26). The original PRIYA trial was approved by the KEM Hospital Research Centre Ethics committee and monitored by a Data Safety Monitoring Board (DSMB) and a Scientific Advisory Committee (SAC). The trial was registered with the CTRI (2012/12/003212) and ISRCTN (32921044). Neurodevelopmental follow up of the offspring was approved by the KEM Hospital Research Centre Ethics committee and registered in (clinical trials.gov ID: NCT03088189). Written informed consent was obtained from the parents of the children before conducting the neurodevelopmental assessment.

Statistical Analysis

The purpose of our analysis was to see if pre-conceptional B12 and micronutrient supplementation in the mothers led to improvement in offspring neurodevelopmental performance (composite BSID-III scores) at 2 years of age. We also investigated the effect of supplementation on circulating vitamin levels in the mother and cord blood, and on cord blood BDNF levels.

We first examined whether randomization had equally distributed potential confounders such as parental education and standard of living index, maternal age, IQ, and anthropometry, length of supplementation and compliance across the three supplementation groups.

All data were represented as either mean and standard deviation (for normally distributed variables) or median and 25–75th percentile (for skewed variables). The skewed outcome variables (maternal and child biochemical measures, birth outcomes and neurodevelopmental measures) were log transformed. We used Pearson's correlation coefficient to test associations between the length of supplementation and biochemical measures at 28-week gestation and offspring cord blood. We compared differences in outcome variables between B12 alone or B12+MMN groups and the placebo group using the *t*-test. Adjustments for additional covariates (e.g., maternal B12 levels at screening) were performed using ANCOVA. We also examined longitudinal changes in the logarithmic values of vitamin B12 concentrations between time points and treatment groups using a two-way repeated measures ANOVA. Further, we examined the additional effect of duration of supplementation and compliance in the same model. We used a non-parametric test (Mann-Whitney *U*-test) to test the significance of difference

TABLE 1 | Maternal characteristics at baseline and in pregnancy, and child characteristics.

Variables	*n*#	Placebo group	*n*	B12 + MMN group	*n*	B12 group	*p*-values	
Parental sociodemographic characteristics								
Maternal age at 28 weeks gestation (years)	25	19.8 (1.0)	25	19.4 (1.1)	21	19.7 (1.1)	0.555	
Maternal education (years)	26	12.5 (11.0, 13.0)	26	12.0 (10.0, 13.0)	21	12.0 (11.0, 13.5)	0.639	
Maternal height (cms)	25	158.2 (5.2)	25	158.7 (5.0)	21	157.8 (4.8)	0.852	
Maternal weight at 28 weeks gestation (kgs)	25	55.4 (48.6, 59.3)	25	51.2 (49.4, 54.4)	21	52.9 (47.0, 60.7)	0.656	
Maternal IQ	21	76.6 (9.5)	15	74.4 (8.8)	17	75.8 (7.2)	0.751	
Standard of Living Index	26	36.0 (30.5, 40.5)	26	38.0 (31.0, 40.0)	21	37.0 (32.0, 40.0)	0.923	
Paternal Education (years)	25	14.0 (10.5, 15.0)	24	12.0 (10.0, 15.0)	19	12.0 (10.0, 15.0)	0.656	
Duration of supplementation (months)	27	34.0 (22.0, 45.0)	26	36.0 (21.0, 45.2)	21	35.0 (23.0, 49.5)	0.937	
Compliance (percentage)	27	89.4 (76.0, 94.8)	26	77.5 (67.8, 88.5)	21	81.5 (72.3, 87.8)	0.649	
Maternal micronutrients							***p*-values (B12 + MMN vs. Placebo)**	***p*-values (B12 vs. Placebo)**
At screening								
Vitamin B12 (pM)	27	151.0 (122.0, 193.0)	26	159.5 (134.0, 219.0)	21	138.0 (125.0, 190.0)	0.350	0.860
Holo-TC	27	11.0 (8.3, 13.4)	26	11.1 (6.2, 15.8)	21	7.9 (5.3, 11.4)	0.87	0.30
Folate (nM)	27	20.9 (15.3, 24.6)	26	15.7 (11.3, 26.6)	21	20.8 (15.3, 29.1)	0.357	0.698
Homocysteine (μmol/L)	27	20.1 (15.1, 38.0)	26	18.6 (15.3, 30.3)	21	27.5 (17.0, 39.6)	0.646	0.434
At 18 years								
Vitamin B12 (pM)	25	162.0 (125.9, 192.5)	26	285.0 (205.8, 368.7)	18	274.7 (224.7, 388.2)	<0.001***	<0.001***
Holo-TC	27	9.5 (6.2, 14.9)	26	22.6 (8.7, 29.2)	19	25.2 (8.1, 36.0)	<0.001***	<0.001***
Folate (nM)	26	23.0 (17.2, 29.8)	26	21.2 (15.3, 28.8)	18	20.4 (14.9, 28.3)	0.734	0.925
Homocysteine (μmol/L)	27	16.7 (11.7, 28.3)	26	9.60 (8.30, 13.4)	18	10.6 (9.22, 16.0)	<0.001***	0.013*
At 28 weeks gestation								
Hemoglobin (gm/dl)	25	10.4 (9.5, 11.0)	25	10.2 (9.4, 11.0)	21	10.4 (9.1, 10.7)	0.491	0.638
Vitamin B12 (pM)	25	134.0 (95.5, 163.0)	25	164.0 (149.0, 218.5)	21	204.0 (173.5, 261.0)	0.007**	<0.001***
Holo-TC (pM)	25	14.8 (8.85, 25.1)	25	21.9 (15.3, 36.5)	21	21.3 (16.9, 36.8)	0.027*	0.012*
Folate (nM)	25	47.9 (18.0, 71.5)	25	20.6 (10.2, 49.7)	21	28.5 (16.6, 51.4)	0.043*	0.302
Vitamin B2 (pM)	25	244.0 (210.5, 273.0)	25	276.0 (229.5, 304.5)	20	244.0 (221.7, 269.5)	0.028*	0.852
Viamin B6-pyridoxal-5-phospate (pM)	24	3.5 (2.3, 4.6)	25	4.6 (3.3, 7.4)	21	3.1 (2.6, 4.8)	0.117	0.357
Vitamin B6-pyridoxal (pM)	15	1.0 (0.8, 1.6)	12	1.1 (0.9, 1.3)	12	1.3 (1.0, 1.7)	0.786	0.922
Homocysteine (μmol/L)	25	7.0 (5.0, 9.2)	25	6.3 (4.3, 8.1)	21	5.1 (3.9, 7.2)	0.559	0.550
Child characteristics								
Child age at assessment (months)	27	27 (26, 34)	26	29 (27, 36.2)	21	29 (26, 32)	0.623	0.901
Gender	27	Boys = 18 (66.7%)	26	Boys = 13 (50%)	21	Boys = 11 (52.3%)		
Birth anthropometry								
Gestation age (weeks)	27	39.0 (38.0, 40.2)	26	39.0 (38.0, 40.2)	21	39.4 (38.8, 40.2)	0.920	0.936
Birth weight (gm)	27	2,908.6 (412.5)	26	2,809.2 (458.6)	21	2,788.9 (315.9)	0.411	0.277
Birth length (cm)	27	49.1 (46.8, 49.8)	26	48.2 (47.4, 49.8)	20	48.5 (47.2, 49.3)	0.990	0.328
Head circumference (cm)	27	33.4 (1.0)	26	33.1 (1.0)	20	33.0 (0.9)	0.237	0.142
Cord micronutrients								
Vitamin B12 (pM)	27	226.0 (138.0, 289.0)	26	275.5 (181.7, 313.7)	21	289.0 (167.0, 446.0)	0.240	0.200
Holo-TC (pM)	27	40.7 (23.3, 81.9)	26	79.4 (39.2, 125.0)	21	96.1 (39.4, 125.0)	0.021*	0.048*
Folate (nM)	27	55.9 (37.9, 70.8)	26	52.0 (36.8, 68.1)	21	42.7 (31.3, 80.0)	0.473	0.278
Vitamin B2 (pM)	26	357 (73.7)	25	316 (73.5)	20	314 (67.3)	0.053	0.924
Vitamin B6-pyridoxal-5-phospate (pM)	12	29.3 (18.6, 42.7)	13	25.0 (19.1, 39.5)	13	17.5 (11.6, 43.9)	0.494	0.298
Vitamin B6-pyridoxal (pM)	26	4.80 (3.6, 7.9)	25	5.70 (4.6, 8.3)	21	4.80 (3.2, 6.9)	0.187	0.806
Homocysteine (μmol/L)	27	8.30 (6.8, 11.6)	26	6.30 (4.8, 9.8)	21	6.60 (4.6, 11.9)	0.134	0.342
BDNF (pg/ml)	27	70.0 (31.0, 299.0)	26	106.0 (31.0, 412.2)	21	195.0 (31.0, 512.0)	0.620	0.364

Values represented as Mean (SD), Median (25th, 75th) or n (%).

**p < 0.05, **p < 0.01, ***p < 0.001 p-values calculated by t-test.*

IQ, Intelligence Quotient; Holo-TC, holotranscobalamin; BDNF, Brain Derived Neurotrophic Factor; MMN, multi micronutrient.

#The number of women attending the follow up differed across the three time points.

in cord BDNF values between the supplementation groups because BDNF values could not be normalized by various transformations. Statistical analysis was performed using SPSS 25.0 and R statistical software 4.1.1.

RESULTS

Of the 266 women randomized in the trial, 182 were married, 166 became pregnant, and 149 delivered a live baby (**Figure 1**). Between May 2017 and February 2020, we approached the parents of 85 children who had attained the age of 2 years, for participation in the neurodevelopmental study. We had to halt the assessments after February 2020 due to the COVID-19 pandemic. None of the children had significant neurodevelopmental disorders (cerebral palsy, seizure disorders, or neural tube defects). Seven children who were above the inclusion age of 42 months as per the BSID norms, were excluded from analysis after confirming that they had achieved appropriate neurodevelopment for 42 months of age. Assessment could not be completed in 4 children. Our analysis is based on the remaining 74 children. The median age of the children at the time of performing the BSID was 29 months (**Table 1**). There were 42 boys and 32 girls; of these, 27 were in the placebo group, 26 in the B12 + MMN and 21 in the B12 alone group. There were no differences in gestational age at delivery, birth weight, length or head circumference amongst the offspring in the three supplementation groups. Similarly, there were no differences in parental education, standard of living index, maternal age, or IQ (**Table 1**).

The children who were not invited for the study because they were below 24 months of age differed from those studied; they had higher socio-economic status and parental education, higher maternal and cord B12 and holo-TC, and lower cord homocysteine compared to the study group (**Supplementary Table 2**).

Effect of Supplementation on Maternal and Newborn Micronutrient Status, and Birth Measures

At baseline, maternal B12 and holo-TC levels were similar across the three supplementation groups (**Table 1**). Fifty one percent of the participants had vitamin B12 deficiency at screening (B12 <150 pM), and this reduced to 22% at 6–12 months after starting supplementation. There was a rise in vitamin B12 and holo-TC levels in the B12 supplemented groups compared to the placebo group, both pre-conceptionally (18 years of age) and at 28 weeks of gestation. There was no significant association between length of supplementation (from start of supplementation till date of 28 weeks gestation and date of delivery) and circulating concentration of vitamin B12 in either group. There was a significant association between length of supplementation and holo-TC concentrations in the cord blood of the B12 alone group ($r = 0.462$ $p = 0.035$).

Repeated measures ANOVA showed a significant effect of time ($F = 18.517$, $p < 0.001$) and treatment ($F = 9.363$, $p < 0.001$) on log serial B12 concentrations. Addition of length of supplementation and compliance did not change the result. *Post hoc* comparisons using Bonferroni correction, showed that the log B12 concentrations in both B12 + MMN (95% CI = 0.14, 0.56, $p < 0.001$) and B12 alone (95% CI = 0.17, 0.63, $p < 0.001$) groups were significantly higher than the placebo group. There was no difference in vitamin B12 concentrations between B12 + MMN and B12 alone groups. Cord blood levels of holo-TC were significantly higher in both the B12 supplemented groups compared to the placebo group, though vitamin B12 levels were similar.

Baseline plasma homocysteine concentrations were high but similar in the three supplementation groups, and fell substantially in the vitamin B12 supplemented groups pre-conceptionally. During pregnancy, as expected, plasma homocysteine concentrations fell in all groups. They were similar in the three groups during pregnancy and in the cord blood.

Circulating folate concentrations were similar at baseline in the three groups and increased during pregnancy (due to supplementation). Folate levels were significantly lower at 28 weeks gestation in the B12 + MMN group compared to those in the placebo group. Folate levels were similar in the cord blood across the groups. Circulating B2 levels were higher in the B12 + MMN group as compared to the placebo group during 28 weeks gestation.

Hemoglobin concentrations were similar in the mother and the offspring across all the groups.

Comparison of BSID Scores and Cord BDNF Between Supplementation Groups

Age standardized composite scores for the domains of cognition, motor and language development were obtained on 74 children. There was no difference in performance between males and females (**Supplementary Table 3**). No significant developmental delays were observed in any of the children (score <69). Few children showed a below average performance on the cognitive (4.1%, $n = 3$), motor (4.2%, $n = 3$), and language domain (8.3%, $n = 6$) (score <85) (**Supplementary Table 4**).

The offspring of mothers in the B12 alone group performed the best in the cognitive and language domains, and significantly better than the placebo group (**Table 2**, **Figure 2**). This difference persisted after adjusting for the baseline plasma vitamin B12 concentrations. Cognition and language composite scores were 5–7% higher in the B12 alone group than the placebo group.

There were no significant differences between the B12 + MMN group and the placebo group on any of the neurodevelopmental domains.

The two supplementation groups had higher cord BDNF values than the placebo group, the B12 alone group had the highest values, however the difference was non-significant (**Table 1**). Cord blood BDNF values did not show significant associations with any of the BSID-III composite scores.

TABLE 2 | Comparison between placebo and supplemented groups in BSID-III domains.

BSID-III domains	Placebo group	B12 + MMN group	B12 group	#*p*-value		$*p*-value	
				Group (B12 + MMN vs. Placebo)	Group (B12 vs. Placebo)	Group (B12 + MMN vs. Placebo)	Group (B12 vs. Placebo)
Cognitive	90.0 (85.0, 95.0)	90.0 (85.0, 96.2)	95.0 (90.0, 100)	0.969	0.034*	0.781	0.044*
Motor	94.0 (91.0, 100.0)	95.5 (90.2, 100.0)	97.0 (91.0, 107.0)	0.687	0.818	0.522	0.384
Language	92.2 (7.8)	93.7 (9.87)	98.6 (10.1)	0.556	0.020*	0.633	0.020*

Values represented as mean (SD) or Median (25th, 75th).
$^{*}p < 0.05$.
#P-value calculated by t-test.
$P-value calculated by ANCOVA; adjusted for maternal baseline B12 levels.
Language performance was normally distributed, and mean (SD) are reported. MMN, multi micronutrient.

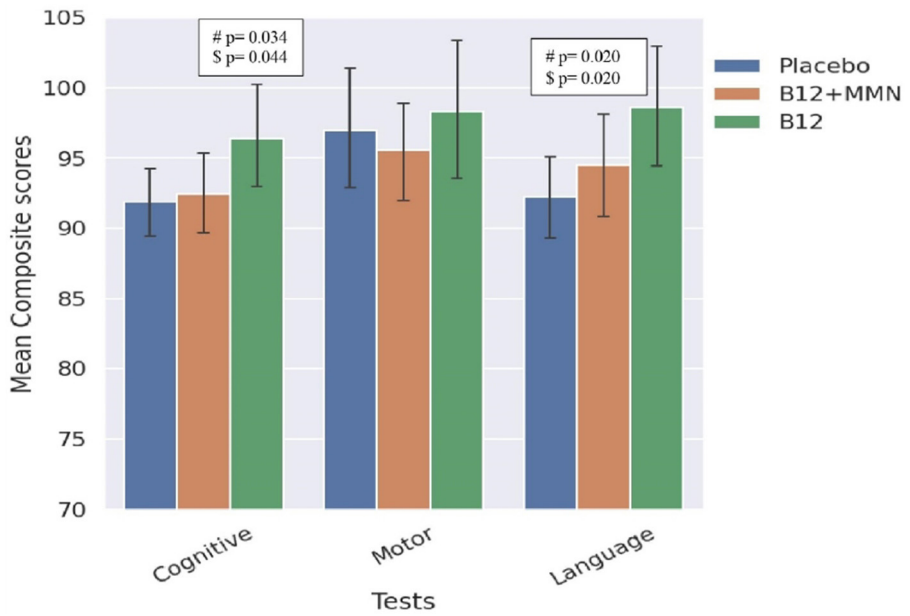

FIGURE 2 | Bar graph comparing scores in BSID-III domains between treatment and placebo groups. Comparison between placebo and treatment groups on BSID-III domains. #significance at $p < 0.05$, $significance $p < 0.05$ value adjusted for maternal baseline B12 levels. Error bars represent 95% confidence intervals.

DISCUSSION

In this rural Indian population with a substantial prevalence of B12 deficiency, we found that supplementation of adolescents with 2 μg/day of B12 significantly improved their own B12 status (total B12 and holo-TC) and offspring cord blood holo-TC. Offspring whose mothers received vitamin B12 alone performed better than offspring of mothers in the placebo group in neurodevelopmental assessments (cognitive and language domain of the BSID-III test at 24–42 months of age). Offspring whose mothers received B12 + MMN performed similarly to the placebo group.

The role of pre-conceptional folic acid supplementation in preventing NTDs is well-established, especially in western (mainly non-vegetarian) populations (23, 24). In vegetarian populations like India, vitamin B12 is likely to play a similar role, because both folate and B12 act as cofactors for the enzyme methionine synthase, in methylation reactions. Studies in India have highlighted an association of both maternal vitamin B12 and folate with different outcomes in the offspring including neurodevelopmental performance. Studies in Pune showed an association of low maternal vitamin B12 status (low holo-TC concentrations and TCN2 polymorphisms) with an increased risk of NTD, and a positive association between maternal vitamin B12 status during pregnancy and offspring neurocognitive performance at 2 and 9 years of age (5, 18, 19). A study in North Indian children aged 12–18 months found that both vitamin B12 and folate status had significant associations with cognitive performance (28) while a study in Mysore found that higher maternal folate concentrations, but not vitamin B12, during pregnancy were associated with better cognitive ability in children at 9–10 years of age (29). Adequate status of both vitamins is likely to be important for brain development and function. Recent systematic reviews,

including both observational and interventional studies, provide a moderate level of evidence for a role of maternal B12 status in determining offspring cognitive function, and highlight a need for more studies from developing countries (17, 30). Studies in Mexico and Singapore have also reported an association between maternal dietary intake of vitamin B12 and offspring cognitive abilities (31, 32). Observations in the ALSPAC cohort in the UK suggests a weak association of a maternal genetic determinant of circulating vitamin B12 concentrations (*FUT*2) and offspring IQ at 8 years of age (33). On the other hand, a cohort study in Canada showed no significant associations between maternal vitamin B12 concentrations and BSID-III outcomes in their offspring at 18 months (32). This may be due to a lack of significant variation in maternal vitamin B12 status, given the low prevalence of vitamin B12 deficiency in their population (34).

Our findings from this pre-conceptional maternal micronutrient supplementation trial fills an important gap in the literature. Our observations are supported by a maternal B12 supplementation study from south India, which supplemented mothers with 50 μg vitamin B12 from the 1st trimester of pregnancy until 6 weeks postpartum. Supplementation improved maternal B12 levels in the third trimester (20) and offspring had better neurodevelopmental scores (language domain) at 30 months of age (21). In another trial, vitamin B12 (1.8 μg) and/or folic acid (150 μg) supplementation in 6–30-month-old children for a period of 6 months showed improvement in their neurocognitive performance (35). In this study B12 alone group showed improvement in gross motor functioning and the B12 + folic acid group in gross motor as well as problem-solving functioning compared to the placebo; folic acid alone had no effect.

A high prevalence of B12 deficiency is unique to the Indian context due to the socio-cultural practice of vegetarianism. In a previously published systematic review (17), we found a high prevalence of B12 deficiency during pregnancy reported from southern (51%), western and northern India (70–74%). Severe absorption defects (i.e., pernicious anemia) are rare and vitamin B12 deficiency is largely a low dietary intake problem (15–18). This offers a unique opportunity to control a modifiable risk factor at the public health scale to improve neurodevelopment and human capital in the next generation. The utility of this approach in populations with a high consumption of fish and meat (e.g., in coastal areas of India) will need to be examined, keeping in mind that folate may be more important than B12 in these populations. Our choice of a near- recommended dietary allowance (RDA) dose of B12 (2 μg/day) was based on our earlier studies showing adequate absorption of oral B-12 (36) in this population and the demonstration in a pilot study of improvement in B-12 and homocysteine status after oral supplementation with 2 or 10 μg/day for 1 year (37). In another study of severely B12 deficient girls (plasma B12 <100 pmol/l), we demonstrated an improvement in hematological parameters and peripheral and autonomic nerve functions after supplementing 2 μg/day of vitamin B12 for 11 months (38). In the present study we found a rise in both total B12 and holo-TC levels in the supplemented groups within a few months of starting supplementation at 28 weeks gestation, and in the cord blood. Use of a small dose of vitamin B12 makes our results important for public health actions. Thus, we believe that our current study fills an important gap to help public health policy to include supplementation with a physiological dose of vitamin B12 among adolescents and reproductive age women. This may improve not only their own health, but that of the next generation's as well. Being aware of the difficulties of achieving long term compliance with tablet supplementation in relatively asymptomatic individuals, we have recently reported the efficacy of commonly eaten vitamin fortified food items (a nutrient bar and yogurt) to achieve better vitamin B12 status (39). All these approaches are usable in the national programmes to improve micronutrient nutrition of children, adolescents and pregnant mothers. The improved cognitive outcomes were seen specifically in the B12 alone supplemented group and not in the B12 + MMN group. We are unsure about the reasons for this. Though the circulating levels of vitamin B12, holo-TC, and BDNF appeared higher in the B12 alone group, the difference from the B12 + MMN group was not significant. There was no difference in the compliance and length of supplementation in different groups. Maternal IQ, parental education, and socio-economic status which may influence child neurodevelopment were also similar. It has been postulated that administration of a combination of multiple micro nutrients may interfere with actions of each other (40), such a mechanism could operate in the B12 + MMN group. It is notable that the effects of maternal multiple micronutrient supplementation on offspring outcomes are inconsistent. A systematic review from 9 trials (6 of which used the UNIMMAP micronutrient formulation) did not find favorable effects on child mortality, birth size, or offspring cognition (41).

Vitamins B12 and folate participate in the one-carbon metabolism pathway to stimulate synthesis of precursor nucleotides for DNA synthesis, and also generate the universal methyl donor S-Adenosyl methionine (SAM) which is involved in methylation of DNA (an important epigenetic mechanism), proteins and lipids and generating neurotransmitters (42, 43). These mechanisms are reputedly involved in fetal growth and differentiation and a deficiency or imbalance of these vitamins may result in a permanent change in the structure and function of developing tissues which may manifest as disorders in later life ("fetal programming") (1). We have demonstrated alterations in adiposity and insulin resistance in children whose mothers had an imbalance of these vitamins (low B12—high folate) during pregnancy (11). Animal studies have shown differences in the expression of neurotrophic factors such as BDNF in the brains of fetuses whose mothers were exposed to low vitamin B12 status (9). In our study, though we did not find significant differences in cord blood BDNF concentrations between supplementation groups, the values tended to be higher in the B12 alone group. Further studies are required to understand the utility of cord BDNF levels as a neurodevelopmental marker in humans.

Neurodevelopment is a dynamic process that involves neurogenesis, neuronal migration, cortical growth and gyrification, starting in early pregnancy and lasting until infancy (first 1,000 days). The pre- and periconceptional

period is an important window within this broader window because of "epigenetic reprogramming" of the conceptus which happens within 48–72 h of conception (44). The majority of pregnancies are unplanned, and women approach the healthcare system after this window. Our supplementation was specifically started in adolescence to ensure adequate micronutrient stores in the mother from before conception, in time to support gametogenesis, conception, embryogenesis, organogenesis, and placentation (43, 45). The success of pre-conceptional folic acid supplementation in preventing NTDs is well-known (23–25). Thus, we propose that the 1,000-day window should be expanded to include the preconception period. This would shift the action from the clinic to the community and will fit well into a multitude of adolescent and reproductive age programs across the world.

Additional strengths of the PRIYA is that it is a trial within a cohort in which original observations were made. The randomized controlled trial design ensured that potential confounders were similarly distributed between allocation groups. High rates of participation in the trial, high rates of follow up, and of sample collection at delivery are also noteworthy. Exclusion of women with severe B12 deficiency (<100 pM) from a placebo-controlled trial on ethical grounds reduced the power of the study because they and their offspring could have benefited the most from the B12 supplementation. The COVID pandemic also interfered with our ability to test more children for neurodevelopment and meant that we missed the children of women who became pregnant later. Despite these limitations we were able to see the beneficial effects of the supplementation. We expect that the performance on the Bayley's scale will reflect in neurodevelopmental indices at a later age. This will be tested during subsequent follow ups.

SUMMARY AND CONCLUSION

We found that pre-conceptional maternal supplementation with a near RDA dose (2 μg/day) of vitamin B12 exposed their offspring to higher vitamin B12 status peri-conceptionally and during pregnancy. This was associated with better neurodevelopmental performance in the children, in cognitive and language domains, between 24 and 42 months of age. Our study highlights an important role for maternal vitamin B12 in offspring neurodevelopment. We urge that the first 1,000 days window be extended to include the pre-conceptional period. Our findings have strong implications for public health policy to improve the vitamin B12 status of young adolescents and reproductive age women in populations with a sizable prevalence of vitamin B12 deficiency. Utility of this approach in non-vegetarian populations, needs to be documented. We foresee benefits of such a policy to many national nutrition programmes in India.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by KEM Hospital Research Center Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CSY and RVB designed the neurocognitive follow-up study. ND and RVB analyzed the data and wrote the first draft. BP and MD performed and reported the neurocognitive assessments. DB performed the biochemical measurements. SB and AB contributed to the statistical analysis. SS and RS conducted the follow-up of the participants. KK, RL, and PY contributed to conducting the PRIYA trial. CSY and CF designed the original PRIYA trial. CSY and CF edited the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: SKB was employed by Cytel Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
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Corrigendum: Pre-conceptional Maternal Vitamin B12 Supplementation Improves Offspring Neurodevelopment at 2 Years of Age: PRIYA Trial

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A Corrigendum on

Pre-conceptional Maternal Vitamin B12 Supplementation Improves Offspring Neurodevelopment at 2 Years of Age: PRIYA Trial

by D'souza, N., Behere, R. V., Patni, B., Deshpande, M., Bhat, D., Bhalerao, A., Sonawane, S., Shah, R., Ladkat, R., Yajnik, P., Bandyopadhyay, S. K., Kumaran, K., Fall, C., and Yajnik, C. S. (2021). Front. Pediatr. 9:755977. doi: 10.3389/fped.2021.755977

In the original article, there was a an error in the number of females given in the **Abstract,** sub-section ***Methods***. The sentence "the Pune Rural Intervention in Young Adolescents trial (PRIYA), adolescents (N = 557, 226 females)" should instead read "the Pune Rural Intervention in Young Adolescents trial (PRIYA), adolescents (N = 557, 266 females)."

The corrected sub-section appears below:

Methods: In the Pune Rural Intervention in Young Adolescents trial (PRIYA), adolescents (N = 557, 266 females) were provided with vitamin B12 (2 μg/day) with or without multiple micronutrients, or a placebo, from preconception until delivery. All groups received mandatory iron and folic acid. We used the Bayley's Scale of Infant Development (BSID-III) at 24–42 months of age to investigate effects on offspring neurodevelopment.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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ORIGINAL RESEARCH
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Parental Feeding Styles and Their Association With Complementary Feeding Practices and Growth in Mexican Children

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Background: Complementary feeding practices and corresponding parental feeding styles influence nutritional status in later stages of childhood. Findings on the association of these variables with infant growth remain inconsistent; in Mexico, a research gap exists in this area.

Research Aims: (1) To characterize parental feeding styles and complementary feeding practices, and (2) to evaluate the association of parental feeding styles with complementary feeding practices and infant growth at 6 and 9 months of age.

Methods: Data were collected from a prospective Mexican birth cohort. Parental feeding styles, complementary feeding practices, and anthropometric data from 263 to 234 mother-child pairs (infants of 6 and 9 months of age, respectively) were analyzed. Logistic and linear regression models were used to determine the associations between variables.

Results: The predominant parental feeding style was the "responsive style" (90%). Only 43.7 and 8.1% of 6- and 9-month-old infants, had adequate complementary feeding practices, respectively. At 6 months, mothers who were responsive to satiety signals had 11% lesser possibilities (OR = 0.89, 95% CI [0.80, 0.98]) of their infant having inadequate complementary feeding practices than their counterparts and "pressuring to finish" and "pressuring to eat cereal" sub-constructs were associated with lower weight for length and body mass index Z-scores ($p = 0.02$).

Conclusions: A high proportion of infants (>40%) did not meet international recommendations. The "pressuring" parental feeding style sub-constructs were associated with growth indicators in 6-month old infants. This emphasizes the importance of promoting parental responsiveness to infant appetite and satiety signals to achieving adequate complementary feeding practices.

Keywords: infant feeding practices, parental feeding styles, complementary feeding, breastfeeding, growth

INTRODUCTION

Complementary feeding (CF) is a transitional period in which an infant passes from breastfeeding (BF) to the family diet (solid food). Adequate complementary feeding practices (CFP) during the first 2 years of life are key to proper infant growth, nutrition, and development. During the first year of life, BF and CF coincide with a period of behavioral modeling which determine long-term eating habits, growth, and development outcomes, as well as future metabolic responses linked to non-communicable diseases like type 2 diabetes (1).

In 2019, globally around 44% of infants between zero and 5 months of age were exclusively breastfed, 71% of infants between 6 and 8 months received CF, and 28% met food diversity recommendations (2). In Mexico, 28.6% of infants under 6-month-old received exclusive BF, 91.2% of infants between 6 and 9 months received CF, and 70.9% met food diversity recommendations (3).

The interruption of exclusive BF coincides with an early introduction of CF and of ultra-processed foods, including infant formulas and sugar-sweetened beverages (SSB) (4). In Mexico, 35% of infants between 6 and 11.9 months consume SSB and ∼20% consume desserts and unhealthy snacks (5). In recent years, the availability and consumption of energy-dense foods and SSB has risen among children in low- and middle-income countries. Consumption of these products can affect the health and nutrition of children by displacing nutrients and leading to inadequate dietary intake (6). Studies in animal and human models have shown that introduction of CF before 4 months of age and high protein intake, were associated with greater weight gain and obesity during childhood (7) which may lead to increased risk of cardiovascular disease in later stages of life (8).

During the CF period, the age of food introduction, genetic predisposition, and parental feeding styles (PFS), determine both food preferences and consumption patterns which may influence dietary habits throughout life (9). PFS, the attitudes that characterize parental actions to maintain or modify child eating behaviors, are based on degree of parental control and responsiveness shown during child feeding (10). These styles influence CFP by establishing the quantity, quality, and frequency of foods offered to infants. Controlling PFS (i.e., the restrictive or pressuring style), can lead to poor infant self-regulation of intake by overriding appetite and satiety mechanisms that can lead to an increased risk of overweight and obesity across the life cycle (11). Responsive PFS, in which the caregiver adequately interprets the appetite and satiety signals of the infant, can lead to healthy eating habits and promote infant growth (12).

Empirical findings on the association of PFS with CFP and growth remain inconsistent, and in Mexico a notable research gap exists in this area (13). Therefore, this study aims to characterize PFS and CFP, and to evaluate the association of PFS with CFP and infant growth at 6 and 9 months of age.

MATERIALS AND METHODS

Design

Data was used from an open ongoing prospective cohort study of mother-child pairs called *MAS-Lactancia,* whose overall goal is to examine appetite and satiety self-regulation as a mediator of maternal and infant health outcomes. All study procedures were approved by the Research, Biosafety, and Ethics Committees of the National Institute of Public Health of Mexico (CI-1281-2016).

Sample

A total of 2,874 women attending to a federal government healthcare facility (HF) were screened based on: having no personal history of high blood pressure, hypertensive diseases of pregnancy, endocrine disorders, and diseases of the kidney, liver, heart, or vascular system (**Figure 1**). Mothers were residents of Cuernavaca city; Mexico. Infants with intrauterine growth restriction (or low weight-for-gestational-age), or conditions that affected appetite, food intake, or growth (e.g., congenital diseases, epilepsy, cleft palate, and food allergies), were excluded from the study as well as infants with malformations that prevented accurate anthropometric measurements (14). Of the 2,874 women screened, the *MAS-Lactancia* cohort recruited 980 women between 18–39 years old at the 16–20 week stage of a singleton pregnancy. Of these women, 42.4% were lost to follow-up due to work or school responsibilities, death of the infant, or because the mother could not be located despite repeated telephone calls and messages, as well as home visits. All the participants received personalized BF counseling from recruitment to 18 months of infant life in order to promote adequate CFP.

At 6 and at 9 months, respectively, 88 and 63 infants with incomplete information regarding CFP, PFS, and anthropometric measurements were excluded from the analysis. Eventually, this study analyzes and presents the findings of 263 and 234 participants at 6 and 9 months, respectively (**Figure 1**).

Measurement

Exposure Variables

Parental Infant Feeding Styles

Data analyzed included three PFS and their respective sub-constructs of the Infant Feeding Style Questionnaire (IFSQ): (1) Pressuring style: pressuring to finish, pressuring to eat cereal, and pressuring to soothe, (2) Restrictive style: restrictive in dietary quantity, and quality, and (3) Responsive style: responsive in attention and responsive to satiety. These PFS were selected based on their previous association with diet and weight in children (12), and adequate model fit (15). The "indulgence" and "laissez-faire" PFS were not used in this study due to limited information, reliability on their use within Latino population, and lack of an adequate model fit (15).

A 5-point Likert scale was used to assess response options of the IFSQ and scored PFS and their sub-constructs (1 = never, 2 = rarely, 3 = half the time, 4 = most of the time, 5 = always). An additive score was calculated for each PFS and corresponding sub-constructs, and used as a continuous variable. Higher scores were interpreted as having greater affinity with the corresponding PFS or sub-construct (16).

Outcome Variables

Complementary Feeding Practices at 6 and 9 Months of Age

We classified CFP according to two components: BF and CF. The BF component was further classified into

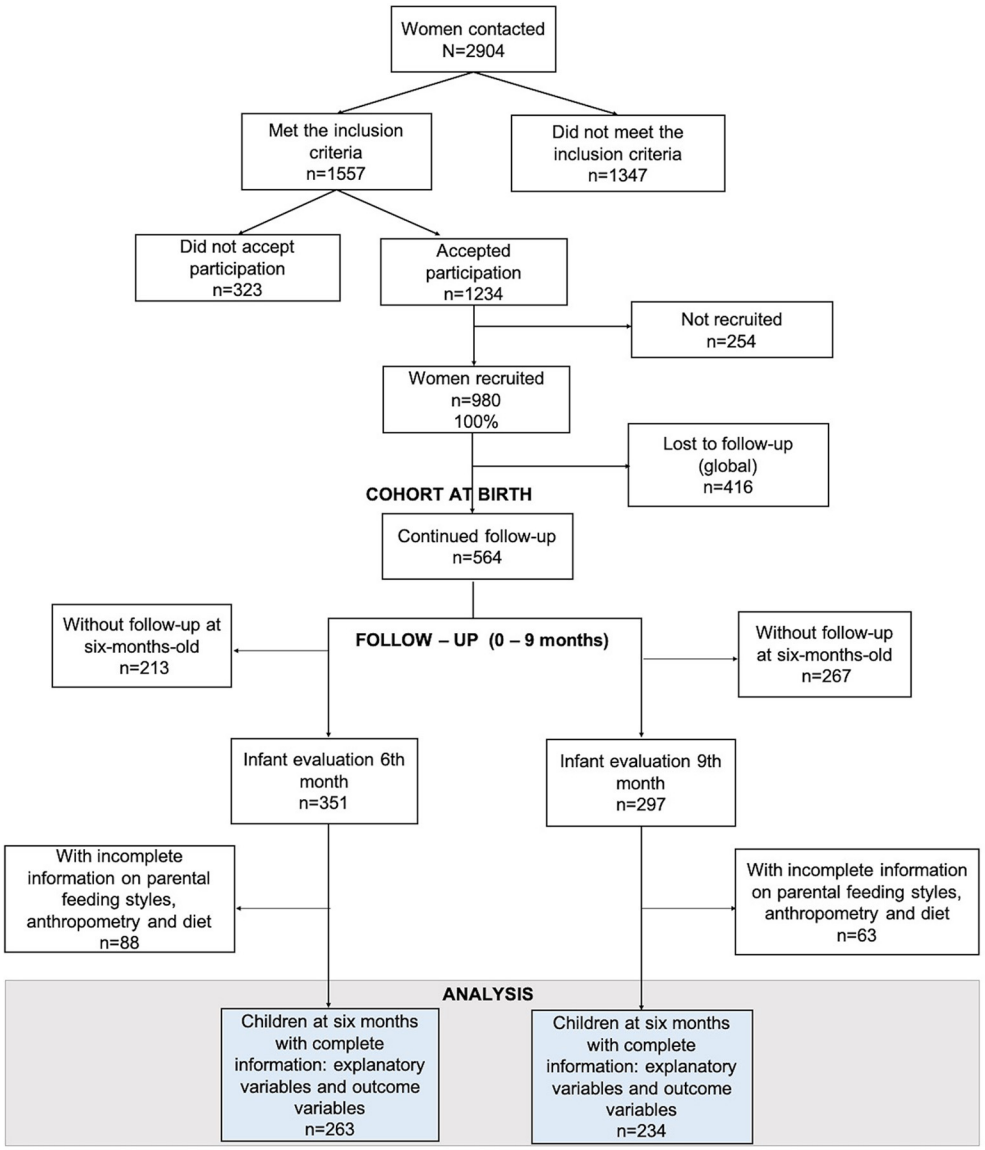

FIGURE 1 | Study sample from the *MAS-Lactancia* birth cohort.

two categories (adequate or inadequate, according to international recommendations) according to the infant's age and type of BF received (**Supplementary Table 1**). The CF component was evaluated with four indicators based on the WHO recommendations for infant feeding: (1) age at first introduction of solid foods (adequate or inadequate), (2) minimum food diversity (yes or no), (3) consumption of SSB (yes or no), and (4) consumption of ultra-processed foods (yes or no) (4). Then, three categories of CFP based on BF and CF practices were defined in accordance with international recommendations: adequate, moderately adequate, and inadequate (**Supplementary Table 1**).

Infant Growth

Weight-for-length (W/L) and body mass index (BMI) Z-scores at 6 and 9 months of age were obtained with the statistical software STATA® version 14.0 (StataCorp, TX, USA, 2015), according to WHO recommendations. Abdominal circumference was measured in centimeters.

Covariates

We considered for inclusion in multivariate analyses: (1) infant characteristics: weight at birth in grams, morbidity (e.g., acute respiratory infections and gastrointestinal infections, and/or hospitalizations history before follow-up), and type of primary caregiver (e.g., parents, grandmother, aunt, babysitter/teacher,

nursery staff), and (2) mother and household characteristics: number of children, formal education (years), and BMI at 16–20 weeks of gestation. A Household Wealth Index (HWI) was generated using principal component analysis, which included housing conditions (housing type, floor, walls, and roofing construction materials), water and sanitation services, ownership of home appliances, electronics, and number of rooms (17). The first component explained 30% of the variability, which was interpreted as a proxy of socioeconomic index, divided into tertiles, the lowest reflecting the poorest conditions.

Data Collection

From March 2016 to December 2019, study staff invited women to participate during prenatal care sessions at HF. Written informed consent was obtained for all women who chose to participate in the study. PFS data was collected with the IFSQ, previously validated in a Latino population (15) and adapted to the Mexican population. The IFSQ was applied to mothers and main caregivers of infants with 6 and 9 months of age, and used a self-report format (**Supplementary Table 2**).

Information on feeding practices was obtained through three methodologies. The first two, *status-quo* and recall as recommended by the World Health Organization (WHO) (18), were supplemented with a 24-h recall questionnaire of multiple-iterative steps, to characterize infant food intake with greater precision (14).

Anthropometric measurements were taken (19) by trained and standardized personnel (20). Training and standardization were carried out before data collection and staff re-standardizations were performed every 6 months. To measure infant weight, a pediatric scale (model Tanita® 1584 Baby Scale) with an accuracy of 10 g was used. Scales were calibrated daily with a known reference weight, and all measurements were taken in duplicate. Length was measured using a wooden stadiometer with an accuracy of 1 mm (Schorr). Abdominal circumference was measured with a Lufkin® model W606PM tape.

Covariate data was obtained from the recruitment and screening questionnaires, infant caregiver questionnaire, morbidity questionnaire, and socioeconomic and demographic characteristics questionnaire. The interviewers were previously trained and standardized to apply all the questionnaires.

Data Analysis

Means and standard deviations were obtained for continuous variables, and percentages for categorical variables. Student *t*-tests, chi-square, and Fisher's exact tests were performed to estimate differences between participants and between the baseline characteristics of study mothers. Also, we compared the distributions of CFP by parental feeding style at 6- and 9-months using Fisher's exact tests.

To evaluate the association between CFP and PFS, multinomial logistic regression models were performed using adequate feeding practices as the reference category. Relative risk ratios and 95% confidence intervals (CI) were interpreted as the odds ratio (OR). To evaluate the association between PFS and growth, multiple linear regression models were used, where β coefficients and 95% CIs were obtained. Regression analyses were performed for each PFS sub-construct and were adjusted for covariates selected *a priori* based on the directed acyclic graph methodology. A supplementary analysis was also performed to compare age at CF introduction and formula consumption category. In addition, we replicated our main multinomial regression analysis using repeated measures from both 6 and 9 months, defining the exposure as increased, decreased, or without change in PFS sub-construct scores. We also replicated our main linear regression analysis, using as outcome the difference of W/L between 9 and 6 months. All analyses were conducted with STATA 14.0 (StataCorp, TX, USA, 2015).

RESULTS

We analyzed a subset of 263 and 234 mother-child pairs at 6 and 9 months, respectively, with complete information on PFS, anthropometry, and diet. A total of 564 participants were lost to follow-up; however, there were no differences in sociodemographic characteristics between the study and the sample subset excluded from analysis, at 6 and 9 months ($p > 0.05$), except for the mother's age ($p = 0.01$; **Supplementary Table 3**).

Participant Characteristics

Infant characteristics, PFS, and PFS sub-constructs are shown in **Table 1**. Mean birthweight of the infants was 3.1 ($SD = 0.4$) kilograms. Mean W/L Z-scores at 6 and 9 months were 0.11 ($SD = 1.0$) and −0.03 ($SD = 1.0$), respectively. At both 6 and 9 months, the "responsive" PFS scored the highest (mean 4.1, $SD = 0.5$; and 4.2, $SD = 0.4$, respectively), as well as the "responsive to satiety" sub-construct (mean 4.4, $SD = 0.4$, at each follow-up event) and the "responsive in attention" sub-construct (mean 3.6, $SD = 1.0$, at 6 months and 3.8, $SD = 0.9$ at 9 months). More than 50% of the infants at each follow-up event were females and had a history of illness or hospitalization prior to the staff visits (morbidity). The parents were the main caregivers of the infants (86.3 and 84.2% at 6 and at 9 months, respectively). Other caregivers were grandparents, uncles, teachers or neighbors (information not shown).

On average, mothers were 27.1 ($SD = 5.1$) years old, with 13.0 ($SD = 3.2$) years of formal education, and the mean BMI at recruitment was 26.1 ($SD = 4.0$). More than 50% of the mothers had a partner, were first-time mothers, and were employed. Around 30% of mothers were in the highest tertile of the HWI, and 34% were in the lowest tertile of the HWI (information not shown).

Infant Feeding Practices

Adequacy of CFP by BF and CF at 6 and 9 months of age is shown in **Table 2**. At 6 and 9 months, 43.7 and 8.1% of infants, respectively, had adequate CFP. Infants older than 6 months received mixed BF both with and without infant formula and BF was adequate in 87.1% of infants at 6 months and 76.5% of infants at 9 months. The mean age of CF introduction was 4.3 months ($SD = 2.2$), and occurred on average 2.6 months

TABLE 1 | Descriptive characteristics and parental feeding styles of the study sample[a].

Infant characteristics	6 months		9 months	
	n	M (SD)	*n*	M (SD)
Age (months)	263	6.5 (0.4)	234	9.3 (0.5)
Birthweight (kg)	259	3.1 (0.4)	230	3.1 (0.4)
Weight (kg)				
Females	136	7.2 (0.8)	121	7.90 (0.9)
Males	127	7.6 (0.8)	113	8.41 (0.9)
Length (cm)				
Females	136	65.0 (2.0)	121	68.5 (2.3)
Males	127	66.2 (2.0)	113	70.0 (2.2)
Weight-for-length z-score[b]	263	0.1 (1.0)	234	−0.0 (1.0)
BMI for age z-score[b]	263	0.0 (1.0)	234	−0.0 (1.0)
Abdominal circumference (cm)	236	42.4 (2.4)	156	43.1 (3.3)
Parental feeding styles scores[c]				
Restrictive	263	2.9 (0.4)	234	2.9 (0.5)
Pressuring	263	3.1 (0.5)	234	3.0 (0.5)
Responsive	263	4.1 (0.5)	234	4.2 (0.4)
Parental feeding style sub-construct scores				
Restrictive in quantity	263	3.5 (0.8)	234	2.9 (0.8)
Restrictive in quality	263	2.4 (0.5)	234	1.9 (0.5)
Pressuring to finish	263	3.1 (0.7)	234	3.2 (0.8)
Pressuring to eat cereal	263	2.9 (0.6)	234	3.0 (0.6)
Pressuring to soothe	263	3.0 (0.8)	234	2.9 (0.8)
Responsive to satiety	263	4.4 (0.4)	234	4.4 (0.4)
Responsive in attention	263	3.6 (1.0)	234	3.8 (0.9)

BMI, body mass index.
[a]MAS-Lactancia cohort 2016–2018, differences in the number of observations per contact and covariates.
[b]Weight-for-length and BMI indicators according to WHO growth standards.
[c]Parental feeding styles assessed by the Infant Feeding Style Questionnaire.

earlier for those consuming formula, compared to those who were still receiving BF (**Supplementary Table 4**). The mean age of CF introduction on infants with adequate CFP was higher ($p = 0.012$) compared to those with inadequate CFP (mean 5.9, $SD = 0.4$ and 2.4, $SD = 2.1$, respectively). Approximately 50% of infants had inadequate food diversity (<4 food groups in formula-fed infants) at 9 months of age. Nearly one of every three infants and one of every two infants at 6 and at 9 months of age, respectively, consumed ultra-processed foods. Around one of every 10 infants at 6 months of age, and one of every three infants at 9 months, consumed SSB. Nearly one of every three infants at 6 months of age, and one of every two infants at 9 months, consumed both ultra-processed foods and SSB (**Table 2**).

Parental Feeding Styles and Complementary Feeding Practices

The association between PFS and CFP is shown in **Table 3**. Mothers who were responsive to signals of the "satiety" sub-construct had an 11% lower possibility ($p = 0.02$) of their infant having inadequate CFP at 6 months of age than their counterparts. There were no significant differences ($p > 0.05$) for the others sub-constructs.

Parental Feeding Styles and Growth

Highest scores from sub-constructs "pressuring to finish" and "pressuring to eat cereal" were associated with infants at 6 months of age with lower W/L and BMI Z-scores ($p \leq 0.02$), and were marginally associated with a lesser abdominal circumference ($p = 0.05$). Likewise, the "responsive to satiety" sub-construct was marginally associated with lesser abdominal circumference ($p = 0.09$). At 9 months of age, a marginal association was present between "pressuring to finish" and "pressuring to eat cereal" sub-constructs, with lower W/L Z-score and BMI Z score ($p = 0.06$ and $p = 0.07$, respectively). No differences were identified from others sub-constructs (**Table 4**).

DISCUSSION

Our results show that the "responsive" style was the predominant PFS for Mexican mothers. Nevertheless, more than 40% of the infants evaluated had a suboptimal CFP and not compliant with international feeding recommendations. Also, infants had inadequate food diversity and already consumed ultra-processed foods and SSB as a part of their diets. Likewise, between 87.1 and 76.5% of infants evaluated (at 6 and 9 months, respectively) received age-appropriate BF.

Mothers of 6-month-old infants who were "responsive to satiety" signals had lower possibilities of their infants having inadequate CFP than their counterparts; in contrast, the "pressuring to finish" and "pressuring to eat cereal" sub-constructs were associated with lower W/L and BMI Z-scores, as well as lesser abdominal circumference, among 6-month-old infants. This difference was statistically not significant ($p < 0.05$) at 9 months of age, possibly due to the reduction of the study sample at follow-up. However, the results presented the same direction compared to those obtained at 6 months.

Related to the existing literature, there are no recent studies and data on PFS in Mexican mothers of children under 1 year of age. However, PFS have been explored in low income Hispanic mothers of preschool-age children (10) and in low income African-American and Latino mothers of infants (15, 21), both groups residing in the United States. Additionally, PFS have been studied in Latin America but focused on preschool-age children (13). Also the results in Latino and African-American mothers showed that the "responsive style" was the predominant PFS. However, in our sample at 9 months, the second predominant PFS was "pressuring style." This result highlights the importance of early identification of controlling eating behaviors in parents, since it could cause poor regulation of infant intake, and affect weight gain during childhood.

In our sample, CF was introduced earlier than is recommended, particularly in formula-fed infants. Similar results were found in a birth cohort from Brazil (1) and in a randomized controlled trial in the Netherlands (22), where semi-solid and liquids different from BF were introduced before 6-months of age. According to Schneider et al., infants who introduced CF before 4 months of age were found to have a

TABLE 2 | Infant feeding practices by breastfeeding and complementary feeding components, at 6 and 9 months of infant age.

Infant feeding practices	6 months ($n = 263$)					9 months ($n = 234$)				
	Global	Adequate	Moderately adequate	Inadequate	*p*-value‡	Global	Adequate	Moderately adequate	Inadequate	*p*-value‡
	%	%	%	%		%	%	%	%	
Complementary feeding practices (global)	100.0	43.7	11.8	44.5		100.0	8.1	40.6	51.3	
Type of breastfeeding[a]										
Mixed (BF y CF)	42.2	67.6	18.9	13.5	**<0.01**	41.0	10.4	50.0	39.6	**<0.01**
Mixed (BF, F y CF)	44.9	33.9	8.5	57.6		35.5	10.8	56.6	32.5	
No breastfeeding (F y CF)	12.9	0.0	0.0	100.0		23.5	0.0	0.0	100.0	
Assessment of BF[b]										
Adequate	87.1	50.2	13.5	36.2	**<0.01**	76.5	10.6	53.1	36.3	**<0.01**
Inadequate	12.9	0.0	0.0	100.0		23.5	0.0	0.0	100.0	
Food diversity										
Yes	14.8	23.1	28.2	48.7	**<0.01**	48.7	16.7	15.8	67.5	**<0.01**
No	85.2	47.3	8.9	43.8		51.3	0.0	64.2	35.8	
SSB consumption										
Yes	12.2	0.0	0.0	100.0	**<0.01**	35.1	0.0	0.0	100.0	**<0.01**
No	87.8	49.8	13.4	36.8		65.0	12.5	62.5	25.0	
UP food consumption										
Yes	29.3	0.0	40.3	59.7	**<0.01**	53.8	0.0	29.4	70.6	**<0.01**
No	70.7	61.8	0.0	38.2		46.2	17.6	53.7	28.7	
UP+SSB consumption	31.9	0.0	36.9	63.1	**<0.01**	58.6	0.0	27.0	73.0	**<0.01**
Age at introduction of CF										
Adequate	66.9	65.3	17.6	17.1	**<0.01**	–	–	–	–	–
Inadequate	33.1	0.0	0.0	100.0		–	–	–	–	–
Mean (SD) years	4.3 (2.2)	5.9 (0.4)	5.6 (0.7)	2.4 (2.1)	**0.012**	–	–	–	–	–

p-values are bolded $p < 0.05$. SSB, sugar-sweetened beverages; UP, ultra-processed foods; BF, breastfeeding; CF, complementary feeding; F, infant formula.
[a] *Type of breastfeeding based on WHO classification.*
[b] *Breastfeeding assessment according to infant age.*
‡ *Fisher exact test.*

TABLE 3 | Association between parental feeding style sub-constructs and complementary feeding practices.

	6 months ($n = 263$)						9 months ($n = 234$)					
	Complementary feeding practices						Complementary feeding practices					
	Moderately adequate†			Inadequate†			Moderately adequate†			Inadequate†		
Parental feeding style sub-constructs	OR	CI 95% (LL, UL)	*p*-value	OR	CI 95% (LL, UL)	*p*-value	OR	CI 95% (LL, UL)	*p*-value	OR	CI 95% (LL, UL)	*p*-value
Pressuring to finish	0.98	0.90, 1.07	0.65	1.03	0.98, 1.10	0.26	1.00	0.86, 1.16	1.00	1.02	0.88, 1.18	0.81
Pressuring to eat cereal	1.04	0.86, 1.27	0.66	0.92	0.81, 1.04	0.19	0.85	0.60, 1.21	0.37	0.87	0.61, 1.23	0.43
Pressuring to soothe	0.92	0.79, 1.07	0.29	1.04	0.94, 1.14	0.44	0.97	0.73, 1.30	0.87	1.02	0.77, 1.37	0.86
Restrictive in quality	1.06	0.89, 1.25	0.52	1.05	0.94, 1.17	0.40	1.00	0.74, 1.34	0.99	0.97	0.72, 1.31	0.85
Restrictive in quantity	0.92	0.80, 1.07	0.28	1.01	0.91, 1.13	0.82	0.94	0.79, 1.12	0.49	0.91	0.77, 1.09	0.31
Responsive to satiety	1.04	0.88, 1.24	0.61	0.89	0.80, 0.98	**0.02**	0.82	0.57, 1.17	0.27	0.89	0.63, 1.28	0.54
Responsive in attention	0.98	0.87, 1.09	0.68	1.01	0.94, 1.09	0.73	0.90	0.70, 1.15	0.40	0.93	0.72, 1.19	0.56

Assessment of the seven parental feeding style sub-constructs corresponding to pressuring, restrictive and responsive feeding styles. p-values are bolded if $p < 0.05$.
† *Versus category of reference of adequate complementary feeding practices.*
OR, odds ratio; CI, confidence intervals; LL, lower limit; UL, upper limit. Logistic regression models adjusted by morbidity, birthweight, type of caregiver, breastfeeding, educational background, number of children, BMI at recruitment, and mother's Household Wealth Index (HWI) tertile.

TABLE 4 | Association between parental feeding style sub-construct scales and growth indicators.

Sub-construct scales[a] Growth indicators[b]	6 months				9 months			
	n	β	CI 95%LL, UL	*p*-value	*n*	β	CI 95%LL, UL	*p*-value
Pressuring to finish (score)								
Weight-for-length z-score	238	−0.03	−0.05, 0.00	**0.02**	214	−0.02	−0.04, 0.00	0.06
Body mass index z-score	238	−0.03	−0.05, 0.00	**0.02**	214	−0.02	−0.05, 0.00	0.07
Abdominal circumference (mm)	236	−0.06	−0.13, 0.00	0.05	211	−0.04	−0.12, 0.04	0.34
Pressuring to eat cereal (score)								
Weight-for-length z-score	238	−0.07	−0.13, −0.02	**0.01**	214	0.00	−0.05, 0.05	0.92
Body mass index z-score	238	−0.07	−0.12, −0.02	**0.01**	214	0.00	−0.06, 0.05	0.91
Abdominal circumference (mm)	236	−0.20	−0.34, −0.02	**0.01**	211	−0.01	−0.19, 0.16	0.88
Pressuring to soothe (score)								
Weight-for-length z-score	238	−0.03	−0.07, 0.01	0.15	214	−0.01	−0.05, 0.03	0.67
Body mass index z-score	238	−0.03	−0.07, 0.01	0.21	214	−0.01	−0.06, 0.03	0.63
Abdominal circumference (mm)	236	−0.02	−0.13, 0.09	0.75	211	0.06	−0.08, 0.21	0.40
Restrictive in quality (score)								
Weight-for-length z-score	238	0.01	−0.04, 0.05	0.82	214	−0.01	−0.06, 0.04	0.64
Body mass index z-score	238	0.00	−0.04, 0.05	0.89	214	−0.01	−0.06, 0.04	0.61
Abdominal circumference (mm)	236	−0.01	−0.13, 0.11	0.88	211	0.05	−0.12, 0.22	0.54
Restrictive in quantity (score)								
Weight-for-length z-score	238	−0.02	−0.06, 0.02	0.39	214	−0.01	−0.05, 0.03	0.49
Body mass index z-score	238	−0.02	−0.05, 0.02	0.43	214	−0.01	−0.05, 0.03	0.53
Abdominal circumference (mm)	236	0.03	−0.07, 0.13	0.55	211	−0.02	−0.16, 0.11	0.77
Responsive to satiety (score)								
Weight-for-length z-score	238	0.01	−0.03, 0.05	0.66	214	0.00	−0.05, 0.04	0.86
Body mass index z-score	238	−0.01	−0.05, 0.02	0.43	214	0.00	−0.03, 0.04	0.89
Abdominal circumference (mm)	236	−0.08	−0.16, 0.01	0.09	211	−0.05	−0.17, 0.07	0.42
Responsive in attention (score)								
Weight-for-length z-score	238	−0.01	−0.04, 0.02	0.44	214	0.00	−0.03, 0.04	0.96
Body mass index z-score	238	0.01	−0.03, 0.05	0.63	214	0.00	−0.05, 0.04	0.84
Abdominal circumference (mm)	236	0.02	−0.09, 0.13	0.73	211	−0.04	−0.20, 0.12	0.64

p-values are bolded if significant a $p < 0.05$.

[a] *Assessment of the seven parental feeding style subscales corresponding to pressuring, restrictive, and responsive feeding styles.*

[b] *Lineal regression models adjusted for morbidity, birthweight, type of caregiver, breastfeeding, educational background, number of children, BMI at recruitment, and mother's Household Wealth Index (HWI) tertile.*

CI, confidence intervals; LL, lower limit; UL, upper limit.

higher risk of childhood overweight (1). The early initiation of CF displaces breast milk consumption with other foods or liquids (e.g., processed juices, SSB, or non-human milk), which poses a nutritional and health risk for infants (7, 12).

Results from the National Health and Nutrition Survey 2018–2019 (ENSANUT by its acronym in Spanish) showed that food diversity is around 70.9% in Mexican infants between 6 and 23 months-old (23). In our study sample <50% of 6- and 9-month-old infants comply with this indicator. Additionally we found, consistent with previous research on Mexican infants (5), that from 6-month of age, infants were already consuming SSB and ultra-processed foods, and continued this practice as they grew older. Consumption of SSB and ultra-processed foods at early age represent a major public health concern, due to the low nutritional quality of these products and their potential of altering the regulatory mechanisms of appetite and satiety (6, 8). Sound infant feeding policies (i.e., those currently in place in Mexico), must be implemented to prevent consumption of processed and ultra-processed foods, increase food diversity, and avoid non-nutritional diets in young children (3, 5).

According to our results, between 76.5 and 87.1% of infants received age-appropriate BF; this proportion was higher than previously reported in Mexican infants (40.2% for infants between 6 and 11.9 months-old) (5). These relative high rates of BF may be attributed to counseling on infant feeding, and particularly on BF, provided to mothers at follow-up. Individual counseling may increase parental knowledge, confidence and self-efficacy, promoting positive feeding behaviors like BF (24).

Recent data from ENSANUT 2018–2019 in Mexico showed that infant under 12 months of age had a consumption of infant formula around 43% (23). Our results are comparable with these national data since around 45% of 6-month-old infants and 35% of 9-month-old infants reported consuming infant formula in addition to BF.

In accordance with Thompson et al., we found that the "responsive" feeding style and specifically, the "responsive

to satiety" sub-construct was associated with lower odds of inadequate CFP at 6 months of age (21). "Responsive" feeding styles has been associated with healthy eating patterns and with a lower risk of obesity (16). Promoting the "responsive feeding" style is particularly important during early infancy to achieve an adequate transition to CF, since at around 6 months of age infants should start the process of incorporating healthy new foods into the diet to develop healthy eating habits (12, 25). Also, like previously reported, it is plausible that the study mothers who practiced BF (75%), would have supportive infant feeding attitudes and beliefs, and better identify their infants' signs of appetite; thereby, favoring positive CFP (1, 16). However, at 9 months, no association was observed between categories of CFP and parental feeding style sub-constructs ($p > 0.05$). One possible explanation is that unlike the 9-month-old infants are already immerse in the family diet, infant around 6 months are going through the period of transition to family diet and we speculate that mothers could be more responsive and careful of infant feeding which allow us to identify associations between "responsive" sub-construct and CFP. Another possible explanation of the lack of associations at 9 months is that the maternal behavior with regard to infant feeding could variate according to specific feeding situations like refusal to eat some kind of food. It is possible that as the infant grows more controlling practices appear, creating a unfavorable feeding environment (12).

In relation to W/L indicator, similar results were presented in a systematic review, where the authors found that "pressuring style" is related with lower weight gain or weight status on infants (26). Another finding was that mothers are concerned about their infant's size, therefore they pressure their children to eat (10, 26). Also our findings are similar with a previous cross-sectional study that reported an association between the "pressuring" feeding style and lower W/L Z-scores (9). Furthermore, Milanaik et al. found that mothers of under 9-month-old infants were more likely to add cereal to the infant's bottle, believing that this practice would improve growth and sleep patterns (27). Another study found that Mexican mothers offered cereal to young children to promote growth, development, and wellbeing, as considering it healthy, nutritious, practical, and easy to prepare (28).

"Pressuring" during childhood may mark the beginning of overfeeding behaviors in children and overweight in later stages of life. These findings highlight the importance of discouraging the "pressuring" feeding style to prevent childhood obesity, and the need for continued research in PFS and child growth.

Limitations

Some limitations of the present study should be considered. Due to the lack of variability in some PFS (90% of mothers practiced the "responsive" style), it was not possible to describe the characteristics of CFP and growth through each PFS. Additionally, the potential for residual confusion cannot be ruled out, given that there were unmeasured variables (i.e., paternal role and social desirability bias, as mothers may have responded according to expected standards). We present here cross-sectional statistical analyses at 6 and 9 months, although a longitudinal analysis was also performed to evaluate the 6 to 9-month period which identified the expected direction of results (similar to the findings of the cross-sectional study), however, the associations were not statistically significant (**Supplementary Table 5**), possibly due to the short study period, low variability between outcome variables within these periods, or the reduction of the study sample. For this reason, the main findings of this study are derived in the cross-sectional analysis with the aim to contribute evidence around the association between PFS, and CFP and infant growth across different phases of early childhood.

Some strengths of our study should also be considered. To our knowledge, this study is among the first to examine the association of PFS with CFP and growth among Mexican infants below 1 year of age. In Mexico, evidence around factors which influence CFP is scarce, and to our knowledge there are limited studies focused on PFS. Additionally, our data come from the "*MAS-Lactancia*" study, a current open prospective birth cohort, relevant in the present epidemiological and nutritional context in Mexico. Likewise, the methodology used in the study was designed to collect detailed information on feeding practices, PFS, and growth indicators during infancy which allowed to provide evidence around the characterization of CFP and PFS. Another strength of the present study is that we assessed PFS with the IFSQ, which has been previously validated in Latino populations (15) and adapted to the Mexican population (14).

CONCLUSIONS

The results of this study show a high proportion of infants with suboptimal CFP, and that some PFS sub-constructs (i.e., "responsive to satiety") were associated with adequate CFP only at 6 months, while some others (i.e., "pressuring to finish") were negatively associated with anthropometric indicators of growth and adiposity. Our findings suggest that promoting "responsive" PFS and identifying signals of appetite and satiety in early childhood may have a positive impact in CFP at 6 months of age. Further research is needed to better understand the impact of PFS on infant diet, growth, and development in Mexico and beyond. Nonetheless, our findings can be used to improve infant feeding guidelines and policies and can be used to improve current early dietary counseling to parents of toddlers focusing on responsive feeding that sensitize parents to infant's cues during feeding to improve infant and complementary feeding practices, promote a self-regulation of energy intake and a healthy weight and prevent overweight and obesity on childhood.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data comes from an ongoing prospective cohort that continues to be updated. Requests to access the datasets should be directed to the corresponding author IR-S.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética en Investigación. Instituto

Nacional de Salud Pública. Registro ante CONBIOÉTICA: 17CEI00120130424 Registro ante COFEPRIS: 13 CEI 17 007 36 FWA: 00015605. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EK-H performed the formal analysis and wrote the paper. JR-D and IR-S conceptualized the study. IR-S and EO-P performed and reviewed the statistical analysis. EK-H and IR-S wrote the initial drafts. GR-O, MS-E, MR-P, RP-E, and JR-D provided substantive inputs, which were incorporated in the final draft. All authors had the responsibility for final content, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.786397/full#supplementary-material

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Maternal Sweeteners Intake Modulates Gut Microbiota and Exacerbates Learning and Memory Processes in Adult Male Offspring

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Background: There is increasing evidence that gut microbiota in offspring is derived in part from maternal environment such as diet. Thus, sweeteners intake including caloric or non-caloric during perinatal period can induce gut dysbiosis and program the offspring to develop cognitive problems later in life.

Objective: To determine the effect of maternal high-sweeteners intake during gestation and lactation on gut microbiota shifts in adult male offspring rats and the impact on cognitive dysfunction.

Methods: Thirty-four male pups from dams fed standard diet (Control-C, $n = 10$), high-sucrose diet (HS-C, $n = 11$), high-honey diet (Ho-C, $n = 8$), and high-stevia diet (HSt-C, $n = 5$) were fed standard diet after weaning, and body weight and food intake were recorded once a week for 26 weeks. Learning and memory tests were performed at week 23 of life using the Barnes maze. Fecal samples from the breastfeeding and adulthood periods were collected and analyzed by sequencing the 16S rRNA V3-V4 region of gut microbiota.

Results: Maternal high-sucrose and stevia diets programmed the male offspring, and changes in microbial diversity by Shannon index were observed after weaning ($p < 0.01$). Furthermore, maternal high-stevia diet programming lasted into adulthood. The increase of *Firmicutes* abundance and the decrease in phylum *Bacteroidetes* were significant in HS-C and HSt-C groups. This led to an increase in the Firmicutes/Bacteroidetes index, although only in HS-C group was statistically significant ($p < 0.05$). Of note, the downstream gram-negative *Bacteroidales* and the upregulation of the gram-positive *Clostridiales* abundance contribute to cognitive dysfunction.

Conclusion: These results suggest that dams fed a high-sucrose and stevia diets during gestation and lactation favor a deficient memory performance in adult male offspring rats through shifts gut microbiota diversity and relative abundance at several taxa.

Keywords: honey, sucrose, steviol glycosides, memory, gut microbiota

INTRODUCTION

In recent years, the gut–brain axis has been highlighted as a key pathway in the development of mental illnesses such as anxiety and depression (1), as well as cognitive disorders, including memory flexibility (2). In addition, recent studies indicate that bacteria colonization of the gastrointestinal tract through the microbial transmission of dams to offspring during early life modulates health in adult life (3). In fact, the deepest shifts on gut microbiota composition take place in childhood (4). During the first days of life, the newborn's intestine is colonized by Gram negative bacteria, which by consuming oxygen, generate an anaerobic environment and the relative abundance of *Lactobacillus* and *Bifidobacterium* genera (5). Thus, relatively minor changes occur, maintaining a stability of gut microbiota, although with the possibility of reshaping by the environment, including diet (6). This means that maternal nutritional exposures during pregnancy and breastfeeding could affect the microbial transmission to offspring modifying the microbiota ecosystem and setting health alterations associated with a dysbiotic microbiota (3).

Currently, the development of chronic diseases has been associated with the increase in sugar sweetened beverages (SSB) consumption (7). In a recent study of Hispanic families living in New York, the authors reported that 89% of parents and 66% of child aged 1–2 years old used SB regularly (8). In fact, more than 30% of pregnant women consume SSB, resulting in metabolic disorders not only in mothers but also in the childhood health (9). Thus, some authors report that the amount of sugar added in women from 25 to 34 years is between 7 and 11% of the total energy intake (7). Thereby, the non-caloric sweeteners consumption has been increasing due to low caloric content and its great acceptability as a substitute for sucrose (10). However, to date, there is controversy in the adverse health effects derived from both sucrose consumption and non-caloric sweeteners, including the steviol glycosides (11, 12). Precisely, murine models exposed to different non-nutritive sweeteners such as sucralose and aspartame develop glucose intolerance (13). In addition, diet with high glycemic index (GI) has an impact on learning and memory processes (14, 15). Nevertheless, other natural sweeteners such as bee honey have recently been highlighted despite having a caloric content; it is credited with beneficial health effects due to the phenolic compounds content (16, 17). For instance, a long-term honey consumption (52 wk) prevents memory loss compared to sucrose-fed rats (18). A tentative hypothesis proposes that the beneficial effects of bee honey consumption are related to shifts on gut microbiota. In fact, a prebiotic effect of honey allows changes in phyla *Actinobacteria* (genus: *Bifidobacterium*) and *Firmicutes* (genus: *Lactobacillus*) (19, 20). The most common phyla in the gut microbiota are *Firmicutes* and *Bacteroidetes*; however, other bacteria occur in lower quantities such as *Proteobacteria*, *Cyanobacteria*, *Verrucomicrobia*, and *Elusimicrobia* (21). Thus, considering the potential influence of maternal diet on cognitive performance in offspring (22, 23), we hypothesized that maternal high-sweeteners intake during gestation and lactation might promote gut microbiota dysbiosis leading to memory loss susceptibility in adult male offspring rats. Different experimental studies have shown the impact of maternal diet on bacterial diversity of the offspring, as well as specific changes in bacterial species (3, 24). Therefore, our objective was to evaluate the impact of maternal high-sweeteners intake during gestation and lactation on diversity and microbial composition of adult male offspring rats and its association with cognitive performance.

MATERIALS AND METHODS

Diets

Dams were fed during the pregestational period with a cafeteria diet (372 kcal/100 g) which consisted of a mix of liquid chocolate, fried potatoes, bacon, biscuits, standard chow diet, and pork pate based on a 1:1:1:1:1:2 ratio. During gestation and lactation feeding period, all the diets were made with sweeteners mixed with standard chow diet formula (Rodent Lab Chow diet 5001; LabDiet, St. Louis, MO 63144). High-sucrose diet (HSD) consisted of a standard diet and condensed milk based on a 1:1.5 ratio (339 kcal/100 g). High-honey diet (HHD) consisted of a standard diet and bee honey based on a 1:1.15 ratio (339 kcal /100 g). High-stevia diet (HStD) consisted of a standard diet and organic steviol glycosides extract diluted in water based on a 1:1 ratio (335 kcal/100 g) (15). Honey from the flower of the avocado plant was used, which contains 38 g of fructose and 31 g of glucose per 100 g. The honey was obtained from the company Hermes Honey S.A. de C.V. located in Aguascalientes, Mexico. Condensed milk and organic steviol glycosides extract were obtained through a local supermarket. Otherwise, male offspring received the standard chow diet formula containing 335 kcal/100 g (Rodent Lab Chow diet 5008; LabDiet, St. Louis, MO 63144, USA).

Animal Models

Dams' experiments were performed with 12 female Wistar rats, 6 wk old, weighing 200–250 g from Tetrarium (Scientific, Technological, and commercial services S.A. de C.V. Monterrey, Mexico). The animals were acclimatized and fed a control diet for 2 wk. All animals were housed in polypropylene boxes in an environment of 21–22°C with 12-h light/dark cycles. Animals were randomly allocated in four groups: control-C group (*n*

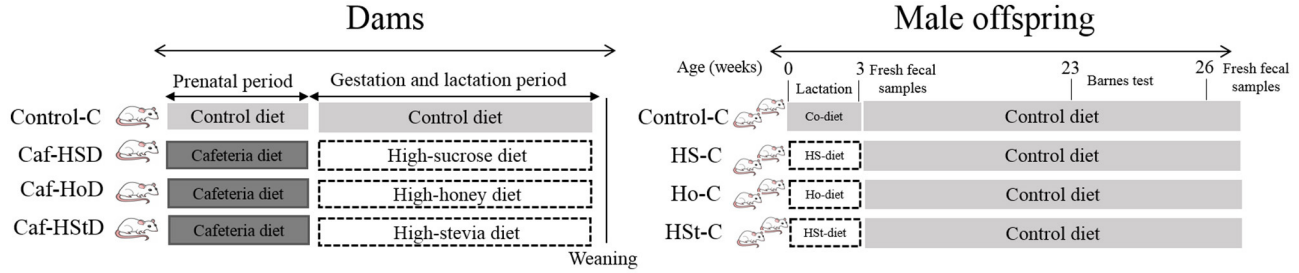

FIGURE 1 | Experimental design.

= 3), cafeteria-HSD group (n = 3), cafeteria-HHD group (n = 3), and cafeteria-HStD group (n = 3). The control-C group was fed a standard diet, and the cafeteria-HSD, cafeteria-HHD, and cafeteria-HStD groups were fed a cafeteria diet for 4 wk (pregestational period). Immediately after mating period, dams continued with the same diet (Control-C group) and the other rats were fed the high-sweetener isocaloric diets: high-sucrose diet (HSD), high-honey diet (HHD), and high-stevia diet (HStD) for 7 wk (gestation and lactation) (**Figure 1**). The body weight and food intake of the dams were recorded once a week.

Thirty-four male pups from dams fed standard diet (Control-C, n = 10), high-sucrose diet (HS-C, n = 11), high-honey diet (Ho-C, n = 8), and high-stevia diet (HSt-C, n = 5) were fed standard diet after weaning. Offspring rats had *ad libitum* water and food access, while body weight and food intake were recorded once a week. The Barnes maze test was performed at week 23 of life. Fresh fecal samples were collected by weeks 3 and 26 of life (**Figure 1**). At the end of the experimental period (week 26), pups were fasted overnight one night on an empty stomach and the next day fasting glucose was measured from a drop of blood collected from the tail vein. Finally, rats were sacrificed by decapitation and trunk blood was collected to obtain serum for further analyses. All the procedures were performed according to the national and institutional guidelines of the Animal Research Bioethics Committee of the Faculty of Public Health and Nutrition (CE 2/2019-13).

Barnes Maze Test

The Barnes maze test (BMT) was used to assess spatial learning and memory performance, as previously described (15). The BMT was placed in a lighted and cold (16°C) room. Male rats were randomized to perform the tests in the maze at week 23 of life. The training lasted 5 days, and rats were trained with four trials per day. After efficient acquisition training, the short-term test was performed on the sixth day. Finally, a week later, the long-term test was conducted. Spatial learning in the BMT was assessed using escape latency (time to find the escape hole) and total errors (number of incorrect holes that were checked before the first encounter with the escape hole) on the platform. To analyze the number of errors, a semi-quantitative error scale was used as previously described (25).

Fecal Sampling, DNA Extraction, and 16S Gene PCR Amplification

Fresh fecal samples were collected at the end of breastfeeding (week 3 of life) and during adulthood (week 26 of life) periods, early in the morning and after the overnight fasting period, by abdominal massage. Samples were collected in 15-ml Falcon tubes and immediately frozen at −80°C for further analyses. DNA from fecal samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following supplier's instructions with a few modifications. DNA was measured and quantified by NanoDrop 8,000 (Thermo Scientific) and Picogreen fluorometer following the protocol. A linear regression was performed to calculate the final DNA concentration of each sample. All samples were quantified in triplicate. Values were expressed as ng/μl.

Variable regions 3–4 of the 16S rRNA gene were amplified using specific forward (5′ TCGTCGGCAGCGTCAGA TGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′) and reverse primers (5′ GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGACTA CHVGGGTATCTAATCC 3′) containing the Illumina adapter overhang nucleotide sequences. PCRs were carried out using the following parameters: 3 min 95°C predenaturation; followed by 25 amplification cycles consisting of denaturation (30 s at 95°C), alignment (30 s at 63°C), and elongation (30 s at 72°C). The final elongation consisted of 5 min at 72°C. DNA concentration of amplicons of interest was determined by gel electrophoresis. DNA of each sample was pooled and purified with AMPure XP to remove primer dimers and other small mispriming products according to the manufacturer's specifications. An index PCR was then carried out to attach dual indices using a Nextera XT v2 kit.

Illumina Mi-Seq Sequencing

Sequencing was performed on the Illumina MiSeq platform according to the manufacturer's instructions (Illumina, 16S Metagenomic Sequencing Library Preparation). Libraries were demultiplexed using Illumina's bcl2fastq 1.8.4 software (Illumina), and reads were processed with custom Python scripts to sort them into samples, removing barcode and amplicon primers sequence. For taxonomic composition analysis, custom Python scripts in the Quantitative Insights into Microbial

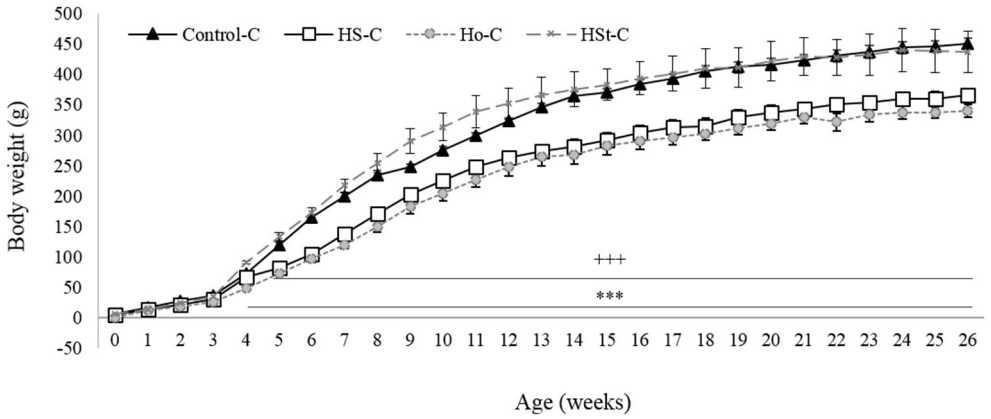

FIGURE 2 | Effect of maternal high-sweeteners diet on body weight in 26-week-old male offspring. All the results are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test *post-hoc*. Control-C ($n = 10$), HS-C ($n = 11$), Ho-C ($n = 8$), HSt-C ($n = 5$). $^{+++}$ $p < 0.001$ (Control-C vs. HS-C); *** $p < 0.001$ (Control-C vs. Ho-C).

Ecology (QIIME, San Diego, CA, USA) software pipeline 1.9 were used to process the sequencing files. The sequence outputs were filtered for low-quality sequences (defined as any sequences that are <200 bps or >600 bps, sequences with any nucleotide mismatches to either the barcode or the primer, sequences with homopolymer runs >6, sequences with an average quality score of <30, and sequences with ambiguous bases >0) and were truncated at the reverse primer. Sequences were chimera checked with Chimera Slayer, and chimeric sequences were filtered out. Analysis started by clustering sequences within a percent sequence similarity into operational taxonomic units (OTUs) with a 97% similarity threshold. Thus, 100, 100, 100, 90.77, and 73.42% of the reads were assigned to the phylum, class, order, family, and genus level, respectively. Alpha diversity measurements (Shannon) were calculated. Weighted and unweighted UniFrac distances were used to perform the principal coordinate analysis (PCoA) for beta diversity.

Statistical Analysis

Statistical analyses were conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± SEM. Statistically significant differences between experimental groups (Control-C, HS-C, Ho-C, and HSt-C) were determined by one-way analysis of variance (ANOVA) test followed by Dunnett's *post-hoc* test and Kruskall Wallis non-parametric equivalent test. *T*-test for related samples was performed to compare microbial community composition between breastfeeding and adulthood. The correlation between *Firmicutes*/*Bacteroidetes* index with glucose levels and spatial learning was performed using the Spearman correlation coefficient. A level of probability of $p < 0.05$ was set as statistically significant. Sample sizes can be found in the figure legends, where n represents the number of animals used for each analysis.

RESULTS

Maternal High-Sweeteners Intake During Gestation and Lactation Influences the Birth Weight and Body Weight Gain of Male Pups

Figure 2 shows the weight gain of male pups throughout the experimental period. Statistically significant decrease in birth weight were found between HS-C (5.18 ± 0.27 g; $p < 0.001$) and Ho-C (5.46 ± 0.15; $p < 0.01$) groups compared with the Control-C group (6.41 ± 0.11 g) (**Figure 3A**). Likewise, body weight gain during the 3 weeks of the breastfeeding period was significantly lower in the HS-C (25.77 ± 0.52 g) and Ho-C (20.37 ± 0.42 g) groups compared with the Control-C group ($p < 0.001$) (**Figure 3B**).

In addition, after the breastfeeding period, male offspring of HS-C and Ho-C groups continued to gain less weight compared with the Control-C group, showing significant results from week 4 (Ho-C group) and 5 (HS-C group) to week 26 of life (**Figure 2**). Therefore, postweaning weight gain (3rd to 26th week) was lower in the HS-C group (298.38 ± 10.34 g) and Ho-C group (291.34 ± 10.20 g) compared with the Control-C group ($p < 0.001$), whereas no significant differences were found when Control-C (377.72 ± 8.71 g) and HSt-C (346.21 ± 32.03) groups were compared ($p = 0.38$) (**Figure 3C**).

Otherwise, we found a significant increase in the fasting glucose levels of male offspring rats in the HS-C (82.60 ± 3.40 mg/dl) and HSt-C (81.60 ± 2.63 mg/dl) groups when compared with the Control-C (67.20 ± 2.47 mg/dl; $p < 0.01$) (**Figure 3D**).

The Effect of Maternal High-Sweeteners Intake During Gestation and Lactation on Learning and Memory Performance in Adult Male Offspring Rats

For the analysis of learning and spatial memory, training and short- and long-term tests were carried out in the Barnes Maze

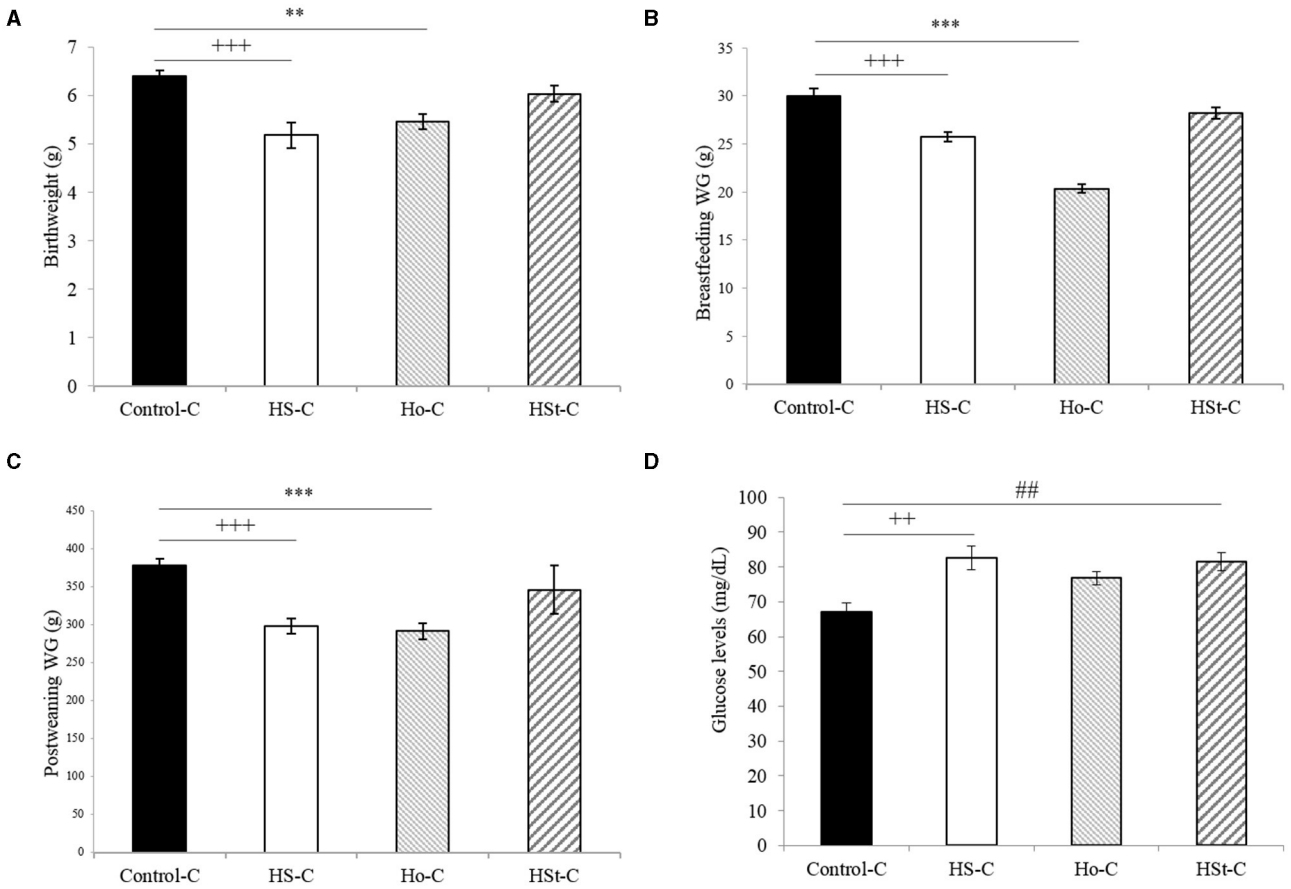

FIGURE 3 | Effect of maternal high-sweeteners diet on birthweight (g) **(A)**, breastfeeding weight gain (g) **(B)**, postweaning weight gain (g) **(C)**, and blood glucose levels (mg/dl) **(D)** in male offspring rats. All the results are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test *post-hoc*. Control-C ($n = 10$), HS-C ($n = 11$), Ho-C ($n = 8$), HSt-C ($n = 5$). $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ (Control-C vs HS-C); ** $p < 0.01$, *** $p < 0.001$ (Control-C vs Ho-C); ## $p < 0.01$ (Control-C vs. HSt-C).

platform (**Figure 4**). We found that the Control-C group by day 1 of training spent an average of 30.28 ± 4.25 s to find the escape hole on the platform. On the other hand, when evaluating the average time to find the escape hole per groups, it was observed that the HS-C group (72.28 ± 7.35 s) displays defective performance to find the exit from the platform when compared with the Control-C group ($p < 0.05$). Similar results were found between the Control-C group and HSt-C group (67.65 ± 8.52 s). Nevertheless, although the average time of the Ho-C group (46.72 ± 7.37 s) was longer than the Control-C group, no significant differences were observed between groups. Following the training schedule, no significative differences were observed between the HS-C, HSt-C, and Ho-C groups and Control-C group (**Figure 4A**).

Likewise, when analyzing the error scale during training days, the Control-C group preserves the error scale throughout the training schedule (**Figure 4C**). Of note, as we found in the latency analysis, a significant difference in the scale of error at the first day of training ($p < 0.05$) was found between the HS-C (2.95 ± 0.09) and HSt-C (2.65 ± 0.35) groups compared with the Control-C group (1.45 ± 0.09).

Once the training was completed, the male pups of the HS-C, HSt-C, and Ho-C and Control-C groups performed the short-term (1 day after training) and long-term (1 week after the short-term test) tests. We found no significant differences between groups (**Figures 4B,D**).

Maternal High-Sweeteners Intake During Gestation and Lactation Modifies Gut Microbial Profile

Gut microbiota composition varied between the HS-C, Ho-C, and HSt-C groups when compared with Control-C group. Therefore, we generated relative abundance (%) values for each animal and present the mean ± SEM per group. In addition, although samples were evaluated from breastfeeding and adulthood periods, changes in phylum, order, family, and genus levels were analyzed only in adult male rats.

At the phylum level, maternal high-sweeteners diets decreased *Bacteroidetes* and *Cyanobacteria,* while *Elusimicrobia* and *Firmicutes* increased (**Figure 5**). Thus, maternal high-sucrose diet during gestation and lactation significantly decreased the relative

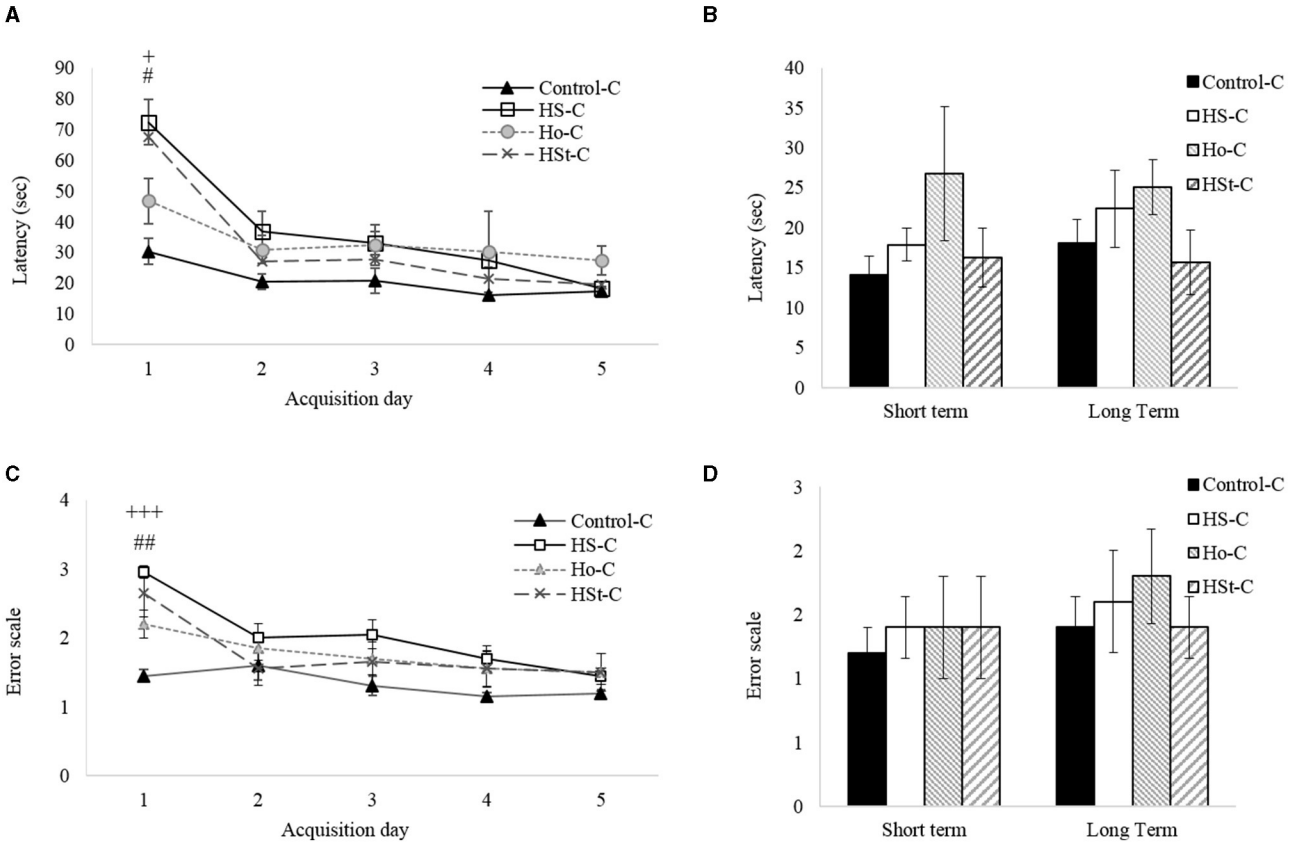

FIGURE 4 | Barnes maze test. Effect of maternal high-sweeteners diet on latency (s) **(A, B),** and error scale **(C, D)** in male offspring rats. All the results are expressed as the mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test post-hoc. Control-C ($n = 5$), HS-C ($n = 5$), Ho-C ($n = 5$), HSt-C ($n = 5$). $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.001$ (Control-C vs HS-C); # $p < 0.05$, ## $p < 0.01$ (Control-C vs HSt-C).

abundance of *Bacteroidetes* ($p < 0.001$) and *Cyanobacteria* ($p < 0.05$) (**Figures 5A,B**). Conversely, HS-C group significantly increased the relative abundance of *Elusimicrobia* ($p < 0.01$) and *Firmicutes* ($p < 0.05$) (**Figures 5C,D**). According to previous results, in male offspring rats from dams fed high-stevia diet, *Bacteroidetes* and *Cyanobacteria* decreased, while *Elusimicrobia* and *Firmicutes* increased compared with the Control-C group. However, only significant results were obtained in *Bacteroidetes* and *Firmicutes* phylum ($p < 0.05$). Interestingly, Ho-C group did not present significant differences compared with the Control-C group at the phylum level (**Figure 5**).

At the order level, maternal high-sucrose diet significantly decreased the relative abundance of *Bacteroidales* (Phylum: *Bacteroidetes*) ($p < 0.01$) and increased *Clostridiales* (Phylum: *Firmicutes*) ($p < 0.01$) when compared with Control-C group (**Figures 6A,C**). Also, an increase in the relative abundance of *Lactobacillales* (Phylum: *Firmicutes*) was found in the HSt-C group compared with the Control-C group ($p < 0.01$) (**Figure 6B**). At the family level, a significant increase in the *Elusimicrobiaceae* (Phylum: *Elusimicrobia*) ($p < 0.001$), *Ruminococcaceae* (Phylum: *Firmicutes*; Order: *Clostridiales*) ($p < 0.01$), and *Enterobacteriaceae* (Phylum: *Proteobacteria*) ($p = 0.057$) was found only in the HS-C group compared with the Control-C group (**Figures 6D–F**).

Finally, at the genus level, the abundance of *Elusimicrobium* (Phylum: *Elusimicrobia*; Family: *Elusimicrobiaceae*) was significantly increased in the HS-C group compared with the Control-C group ($p < 0.05$) (**Figure 6G**). In contrast, the relative abundance of *Lactobacillus* (Phylum: *Firmicutes*; Order: *Lactobacillales*) and *Clostridium* (Phylum: *Firmicutes*; Order: *Clostridiales*) was significantly higher in the HSt-C group compared with the Control-C group ($p < 0.01$ and $p < 0.05$, respectively) (**Figures 6H,I**).

Impact of Maternal High-Sweeteners Diet on the Bacterial Diversity of Male Pups During Breastfeeding and the Reshaping After 23 Weeks Fed Control Diet

In **Figure 7**, the Shannon index by groups is observed when analyzing fecal samples during breastfeeding and in adulthood of male pup rats. Results show that during breastfeeding, the Control-C group (5.89 ± 0.07) exhibits greater α-diversity compared with the HS-C (5.05 ± 0.20) and HSt-C (5.16 ± 0.46)

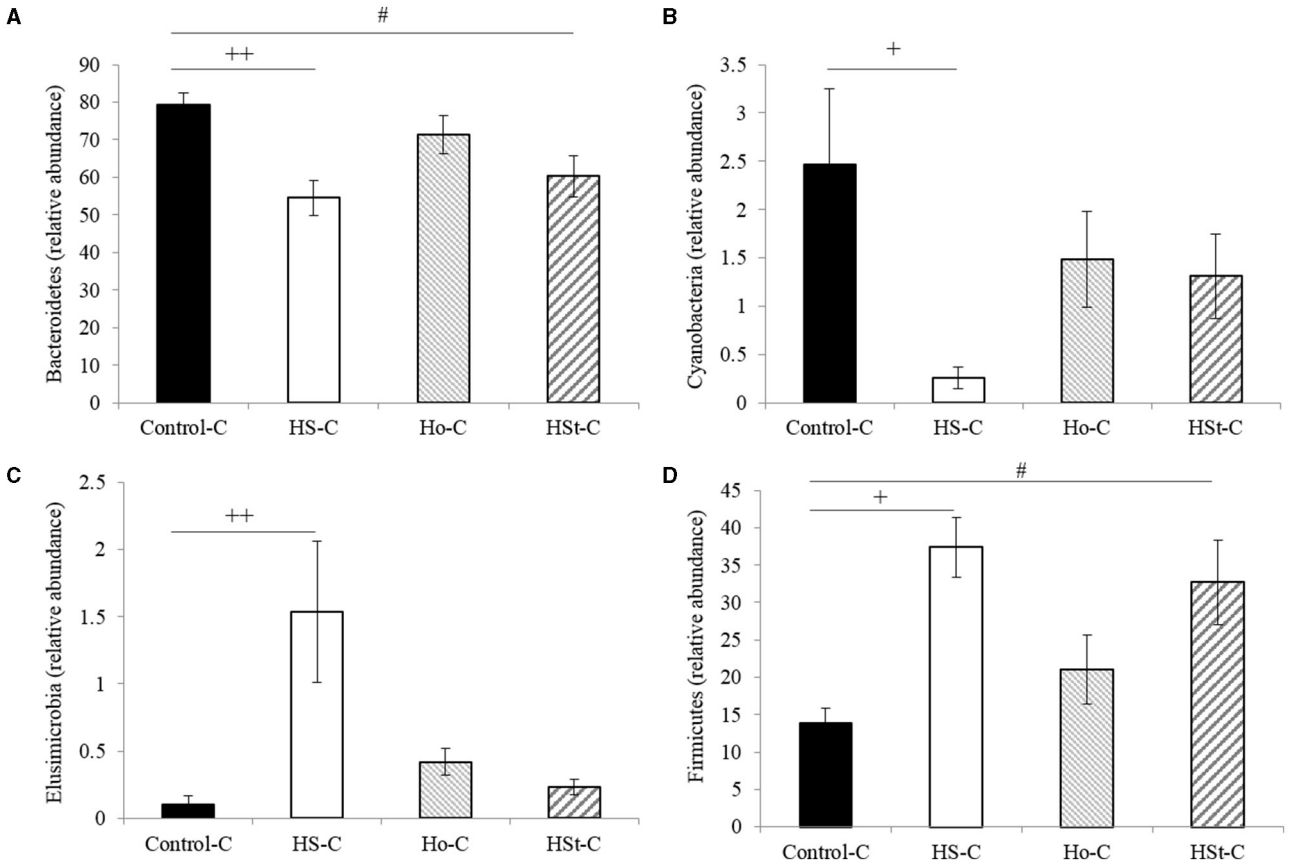

FIGURE 5 | Relative abundance of bacterial phyla of adult male rats. All the results are expressed as relative abundance (%) of sequences and presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test post-hoc or Kruskall Wallis non-parametric equivalent test. Control-C ($n = 5$), HS-C ($n = 5$), Ho-C ($n = 4$), HSt-C ($n = 5$). $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ (Control-C vs HS-C); $^{\#}$ $p < 0.05$ (Control-C vs HSt-C).

groups ($p < 0.01$). However, unexpectedly, no difference was observed in the Ho-C group compared with Control-C group (5.46 ± 0.08).

Nevertheless, no significant changes in the α-diversity of adulthood were identified between the HS-C, HSt-C, and Ho-C groups when compared with Control-C group. However, significant changes were observed when comparing the Shannon index between breastfeeding and adulthood periods per groups. This indicates that both the HS-C group ($p = 0.017$) and the Ho-C group ($p = 0.036$) reshape the gut microbiota after 23 wk fed control diet. However, it is noteworthy that in the HSt-C group, no significant differences were observed during breastfeeding and adulthood in the Shannon index ($p = 0.11$) (**Figure 7**).

Moreover, the Firmicutes/Bacteroidetes index was calculated (**Figure 8**). Thus, no significant differences were found between groups in the breastfeeding period. However, a significant difference was observed between Control-C group (0.18 ± 0.03) and HS-C group (0.73 ± 0.13) in adulthood ($p < 0.05$). This change indicates a considerable 4.05-fold increase in the HS-C group.

Modulatory Effect of Maternal High-Sweeteners Diet on Firmicutes/Bacteroidetes Index Contributes to the Cognitive Dysfunction in Adult Male Rats

Significant and positive correlations were found between *Firmicutes/Bacteroidetes* (F/B) index calculated in adult male rats fed control diet, and glucose levels (mg/dl) ($r = 0.679$; $p < 0.001$) and latency (s) in Barnes maze ($r = 0.619$; $p < 0.001$) (**Figure 9**).

DISCUSSION

Epidemiological and experimental studies have shown a relationship between maternal environment during the perinatal period and the risk of developing chronic diseases in adult offspring (26). Reports using animal models documented the impact of the maternal diet on the susceptibility to developing metabolic disorders, such as obesity, in adult offspring (27–30). Therefore, in a recent review, Ribaroff et al. analyze the effects of the maternal high-fat diet on offspring health, confirming effects on adiposity and final body weight, whereas no changes

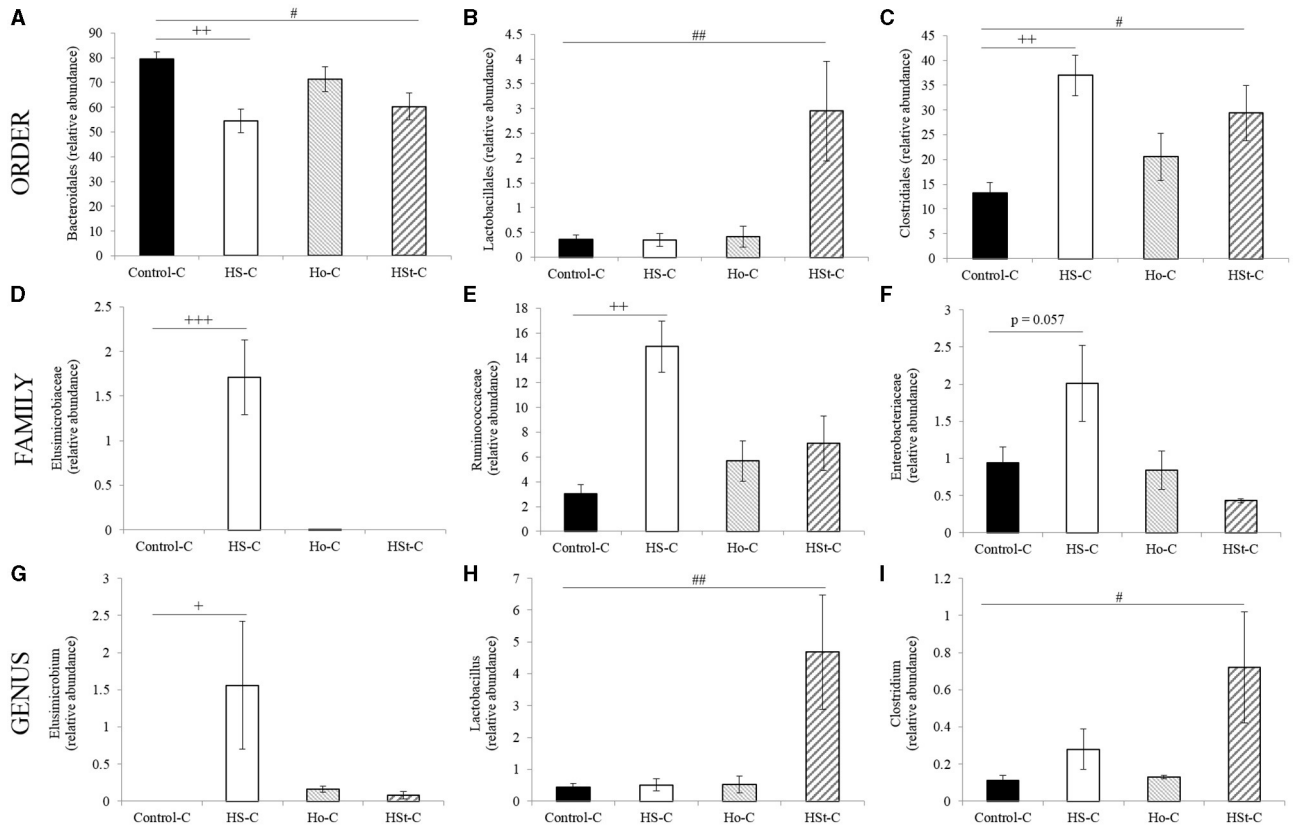

FIGURE 6 | Relative abundance of gut bacterial taxa at the different level **(A–C)** at the order level; **(D–F)** at the family level; **(G–I)** at the genus level of adult male rats. All the results are expressed as relative abundance (%) of sequences and presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test post-hoc or Kruskall Wallis non-parametric equivalent test. Control-C ($n = 5$), HS-C ($n = 5$), Ho-C ($n = 4$), HSt-C ($n = 5$). $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ (Control-C vs. HS-C); $^{\#}$ $p < 0.05$ (Control-C vs. HSt-C).

were found in the pups' birthweight (31). Otherwise, maternal high-sucrose diet has been related to higher birthweight in male offspring (32). However, in addition to the effects on metabolic disorders in the offspring, maternal exposure to high caloric diets favors behavioral changes such as depression (33), anxiety (18), as well as learning and memory (34). Furthermore, some studies report that high-sucrose or high-fructose corn syrup diets can impact learning and memory processes, regardless of the obesity development (35). These changes might potentially be associated with the effect of the maternal diet on offspring gut microbiome (24). Based on this proposal, our study analyzed the shifts on gut microbiota of male offspring rats associated to maternal high-sweeteners diets during gestation and lactation and the impact on different metabolic parameters and cognitive dysfunction.

In recent years, the role of gut microbiota as responsible for the relationship between high-sucrose diet and glucose intolerance has been highlighted (36). In contrast, a study performed with dyslipidemic rats reported that supplementation with honey from Mimosa quadrivalvis L. increased glucose tolerance (19). Honey is a caloric sweetener (3.4 kcal/g) composed mainly of glucose and fructose, and it is also a source of flavonoids and phenolic acids, and its composition depends on the type of flower used by bees (17). Conversely, in recent years, the effect of non-caloric sweeteners on the regulation of glucose levels has also been evaluated. The results to date are controversial, finding that some artificial sweeteners induce glucose intolerance (37). This may be due to the type of non-caloric sweetener consumed, its absorption and transport in the small and large intestines, as well as shifts in gut microbiota (38). For example, in a crossover trial with healthy subjects, after the consumption of 1 g of stevia diluted in 300 ml of water, no significant changes were observed in postprandial glucose levels (12).

Thus, in our study, we found high glucose levels in HS-C and HSt-C groups compared with the Control-C group; in addition, a correlation between glucose levels and the F/B index was found. The F/B index has been widely used as a marker of obesity; however, there are contradictory data when associating the F/B index with a health status (39). Different studies have defined dysregulations in Firmicutes and Bacteroidetes phyla associated with metabolic changes such as obesity (21). However, these changes have also been associated with cognitive impairments (40). Surprisingly, in our study, the changes found in *Firmicutes*

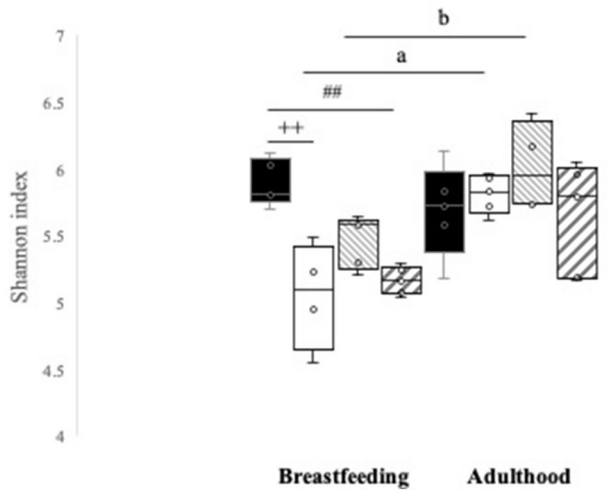

FIGURE 7 | Maternal high-sweeteners diet modifies the gut microbiota diversity by Shannon index. All the results are expressed as relative abundance (%) of sequences and presented as mean ± SEM. Control-C, HS-C, Ho-C, HSt-C. Statistical analyses were performed using one-way ANOVA and Dunnett's test *post-hoc* or Kruskall Wallis non-parametric equivalent test. Differences of means per group between the two periods were analyzed by *T*-test for related samples. Control-C ($n = 5$), HS-C ($n = 5$), Ho-C ($n = 4$), HSt-C ($n = 5$). $^{++}$ $p < 0.01$ (Control-C vs. HS-C); $^{\#\#}$ $p < 0.01$ (Control-C vs. HSt-C); ^{a} $p < 0.05$ (HS-C); ^{b} $p < 0.05$ (Ho-C).

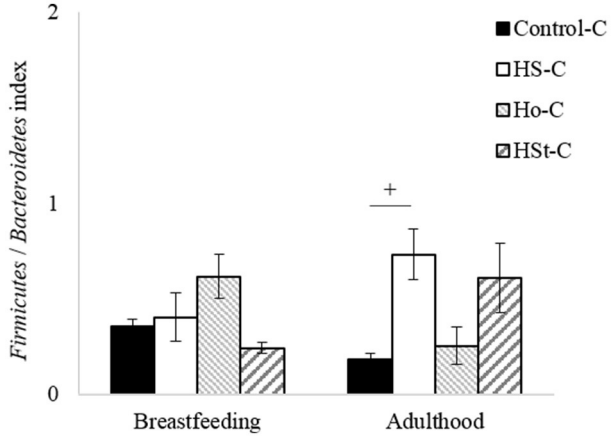

FIGURE 8 | *Firmicutes*/*Bacteroidetes* index of male pups at breastfeeding and adulthood periods. All the results are expressed as relative abundance (%) of sequences and presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA and Dunnett's test *post-hoc* or Kruskall Wallis non-parametric equivalent test. Control-C ($n = 5$), HS-C ($n = 5$), Ho-C ($n = 4$), HSt-C ($n = 5$). $^{+}$ $p < 0.05$ (Control-C vs. HS-C).

and *Bacteroidetes* phyla were in adulthood regardless of body weight gain.

Likewise, in addition to the correlation between F/B index with glucose levels, a positive association was also found between F/B index with the escape latency, an indicator of spatial memory. On this context, the offspring of the HS-C and HSt-C groups showed the longest time to find the escape hole during the Barnes maze test. Several studies have reported that high-sucrose diet is related not only to glucose intolerance but also to cognitive defects (14, 18, 41). Our study provides evidence that maternal high-sucrose diet promotes a shift on gut microbiota of adult rats, which correlates to changes in glucose levels and memory loss. Likewise, in recent years, the use of non-caloric sweeteners such as steviol glycosides has been preferred to "decrease" caloric intake (12). However, in our study, maternal high-stevia diet programmed the offspring by altering glucose levels and inducing memory loss. Thus, although the male pups from dams fed high sucrose and steviol glycosides diets did not show increase in body weight, we propose that HS-C and HSt-C groups presented shifts on gut microbiota that may be associated with high glucose levels and cognitive deficits.

Experimental evidence have documented that selective dietary ingredients modulate the gut microbiota, as well as its relationship with neurocognitive dysfunction (40). For instance, at the order level, high-sucrose diet increases *Clostridiales* (Phylum: *Firmicutes*) and decreases *Bacteroidales* (Phylum: *Bacteroidetes*), which were correlated with a cognitive deficit (1). Similar results were found in our study in HS-C group compared with the Control-C group. These results show an increase in bacterial communities that have been associated with learning and memory defects. In addition, in our study, we also found an increased abundance of *Clostridiales* and a decrease in *Bacteroidales* in adult male rats from dams fed high-stevia diet. Unlike the high-sucrose diet, to date, the relationship between *Stevia rebaudiana* consumption and changes in gut microbiota is not entirely clear. A report identified that administered water with 2.5% steviol glycosides to male wistar rats leads to lower α-diversity, in contrast to other sweeteners such as sucrose or honey (42). Furthermore, it has been reported that *Bacteroides* are, at the genus level, the group of bacteria that hydrolyze stevioside and rebaudioside A to steviol. However, the use of steviol glycosides as a substrate for *Clostridium* and *Lactobacilli* was not found (38). In this regard, in our study, we found that only adult male offspring of dams fed high-stevia diet significantly increase the relative abundance of *Lactobacillus* and *Lactobacillales* at the genus and order levels, respectively.

In contrast, other studies have reported that the *Lactobacilli* genus uses steviol glycosides very poorly (43). Furthermore, in another study, the authors reported that in the presence of stevia sweeteners stevioside and rebaudioside A, the growth of the *Lactobacillus reuteri* species is inhibited (44). *Lactobacillus* have been reported to facilitate the transport of short chain fatty acids (45), which seem to be associated with cognitive performance (2). Nevertheless, although our study did not find an increase in *Lactobacillales* in the Ho-C group, other authors have reported an increase in *Lactobacillus* spp. in dyslipidaemic rats supplemented with honey (19). The difference between these reported data may be due to the fructans content in honey or Stevia rebaudiana. In fact, fructans enhance the growth of *Lactobacilli* and *Bifidobacteria*, key bacteria in gut health (38). However, although no difference of *Lactobacillus* (Order: *Lactobacillales*) was found in Ho-C group, maternal high-honey

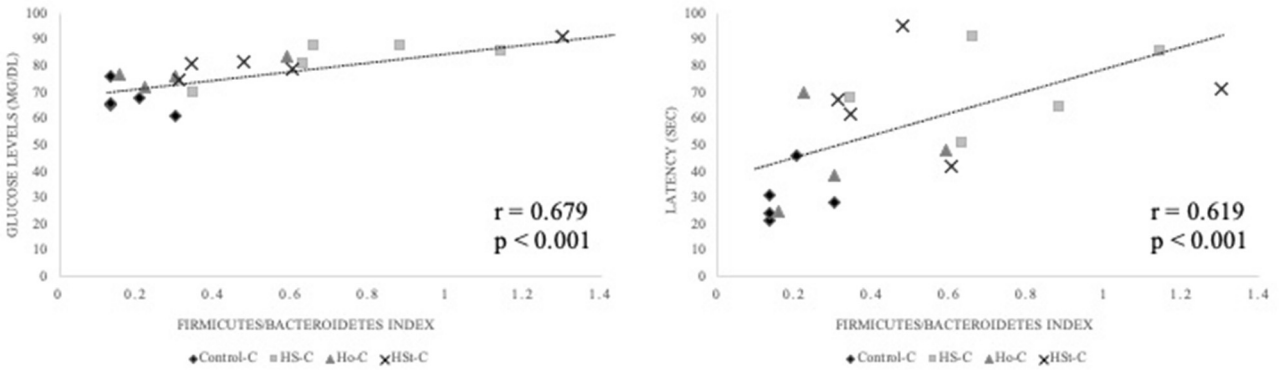

FIGURE 9 | Correlations between the F/B index with glucose levels (mg/dl) and latency (s) in Barnes maze of adult male offspring rats. Spearman's rank correlations were conducted taking into consideration Control-C ($n = 5$), HS-C ($n = 4$), Ho-C ($n = 4$), and HSt-C ($n = 5$). Results were considered statistically significant when $p < 0.05$.

diet programmed the male offspring to show greater bacterial diversity than HS-C and HSt-C groups.

Experimental research have highlighted the shifts in bacterial diversity, caused by diet, and its relationship with health (3, 42). As an example, exposure to western or cafeteria diets affects bacterial diversity leading to diet-related diseases (46). Bacteria adapt to environment and can dynamically interact with each other and the host, contributing to the host's health (47). Therefore, when evaluating the reshaping fecal gut microbiota in male adult offspring fed control diet for 23 weeks, it was observed that the Ho-C group increased bacterial diversity significantly. Likewise, HS-C group also restored the bacterial diversity by significantly increasing the Shannon index in adulthood compared to breastfeeding period. However, in the HSt-C group, no significant changes were found in Shannon index between both periods. This suggests that in addition to the loss of microbial diversity due to the maternal diet, there is a progressive loss due to the programming of stevia diet early in life, related to the breastfeeding period. The foregoing may be related to the effects found in spatial memory of HSt-C group regardless of the increase of *Lactobacillales*.

Finally, although there is few evidence on the phylum *Elusimicrobia*, an association between the genus *Elusimicrobium* and the decrease in blood glucose levels in diabetic rats has been reported (48). In this regard, although there is no evidence of the relationship with cognitive defects, our results are similar in the increase in blood glucose levels in the HS-C group and the positive correlation with the relative abundance of *Elusimicrobiaceae* family. In addition, it was also found that the HS-C group increases relative abundance of *Enterobacteriaceae*, which has been reported to be associated with gut and brain inflammation (35). Although no links between *Elusimicrobiaceae* and *Enterobacteriaceae* and memory defects have been found, there is evidence that both hyperglycemia and inflammation may be related to brain-related diseases (34, 49). Therefore, one of the mechanisms involved in the development of cognitive diseases may be the bacteria presence such as *Enterobacteriaceae* and *Elusimicrobiaceae* that regulate inflammatory and glycemic process in the host.

Thus, in this study, the downstream *Bacteroidales* and the upregulation of *Clostridiales* abundance at the order level were a key pathway for the cognitive dysfunction. In addition, reshaping gut microbiota is possible in adulthood, but bacterial diversity increased only in HS-C and Ho-C groups, highlighting the long-term effect of maternal high-stevia diet on the gut microbiota of the offspring.

It is noteworthy that our study reports interesting data on the effect of maternal high-sweeteners diet on the bacterial abundance and diversity of adult male offspring rats. Furthermore, these significant changes in male pups' microbiota may be responsible for the effects on learning and memory processes. This suggests that shifts on gut microbiota through the maternal diet exposure may be the mechanism involved in the development of cognitive problems in adult offspring. On the other hand, although tests were also carried out on female pups and the results have already been published previously (15), the microbiota analysis was only performed on male pups. However, both female and male offspring were found to have effects on long-term learning and memory.

In summary, the pups of dams fed high-sucrose and stevia diets induced hyperglycemia and experience defective memory performance in adult male offspring rats, regardless of weight gain. One of the mechanisms involved in these effects may be changes in the microbial diversity of male offspring caused by the maternal diet.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Dryad repository with accession number 10.5061/dryad.7sqv9s4th.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Bioethics Committee of the Faculty of Public Health and Nutrition (CE 2/2019-13).

AUTHOR CONTRIBUTIONS

AdlG: conceptualization. BR-D, AM-T, BC-Z, and DM-Y-C: investigation. MC-T and MS-T: methodology. AdlG, NT, and AC-M: supervision. AdlG and AC-M: visualization. AdlG, BR-D, AM-T, MC-T, BC-Z, DM-Y-C, MS-T, NT, and AC-M: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Effects of Complementary Feeding With Different Protein-Rich Foods on Infant Growth and Gut Health: Study Protocol

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Background: An urgent need exists for evidence-based dietary guidance early in life, particularly regarding protein intake. However, a significant knowledge gap exists in the effects of protein-rich foods on growth and development during early complementary feeding.

Methods: This is a randomized controlled trial of infant growth and gut health (primary outcomes). We directly compare the effects of dietary patterns with common protein-rich foods (meat, dairy, plant) on infant growth trajectories and gut microbiota development (monthly assessments) during early complementary feeding in both breast- and formula-fed infants. Five-month-old infants (up to $n = 300$) are randomized to a meat-, dairy-, plant-based complementary diet or a reference group (standard of care) from 5 to 12 months of age, with a 24-month follow-up assessment. Infants are matched for sex, mode of delivery and mode of feeding using stratified randomization. Growth assessments include length, weight, head circumference and body composition. Gut microbiota assessments include both 16S rRNA profiling and metagenomics sequencing. The primary analyses will evaluate the longitudinal effects of the different diets on both anthropometric measures and gut microbiota. The secondary analysis will evaluate the potential associations between gut microbiota and infant growth.

Discussion: Findings are expected to have significant scientific and health implications for identifying beneficial gut microbial changes and dietary patterns and for informing dietary interventions to prevent the risk of overweight and later obesity, and promote optimal health.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT05012930.

Keywords: growth, gut microbiota, protein, complementary feeding, infant

INTRODUCTION

Undesired growth patterns during infancy, namely rapid weight gain or excessive weight gain relative to length, are strongly associated with childhood obesity (1, 2). Overweight and obese children have an increased risk of becoming overweight and obese adults and could experience an earlier onset of chronic diseases such as type 2 diabetes and CVD (3). Given the current obesity rates in U.S. children and adolescents (4), identifying modifiable risk factors underpinning excessive weight and adiposity gain early in life is urgently needed.

Early complementary feeding represents the progressive introduction of solid foods between 5 and 12 months of age as infants no longer rely solely on breastmilk or formula. Growth trajectories and shifts in the composition of the gut microbiota during this critical period have the potential to program long-term body weight, body composition, and disease risk, with diet exhibiting significant influence. Among the macronutrients, protein has gained great interest over the years. Multiple observational and randomized controlled trials (5–7) have evaluated protein content in liquid diet (i.e., infant formula) and most studies observed a lower-protein content in infant formula led to less weight gain and lower weight-for-length Z scores (WLZ). These findings at least partially contributed to the recommendations of reducing protein intake during complementary feeding (8). However, a significant knowledge gap exists regarding the effects of different protein-rich foods on growth during early complementary feeding.

Research of protein-rich complementary foods on growth and risk of overweight is quite limited. Several observational studies focused on the long-term effect of early protein intake, but results were inconsistent. One study in Iceland found that the intake of animal protein at 12 months (meat and dairy combined), but not plant-based protein, was associated with higher BMI at age 6. Importantly, dairy protein was also associated with higher IGF-1 (9), which is associated with a higher risk of obesity early in life (10). A larger study in the Netherlands had similar findings, and the association between animal protein and BMI did not differ between meat and dairy (11). However, another study from Germany found that dairy intake at 12 months was associated with BMI at age 7, while meat or plant was not (12). A 2019 Cochrane review on animal-source foods for growth and development in infants and young children rated the quality of the current evidence as very low overall and no firm conclusions can be drawn (13). Likewise, a 2019 systematic review by the B-24 committee concluded that there is insufficient evidence of an association between protein intake and incidence of overweight or obesity early in life (14).

Another health indicator that might link diet and growth is the gut microbiota. The role of gut microbiota in human health, including obesity risks, has been examined primarily in adults and animal models (15, 16). Emerging research suggests that early-in-life colonization plays a critical role in the establishment and maturation of gut microbiota, and disruption of the optimal microbial succession may result in long-term health impairments (17). Although gut microbiota are greatly influenced by diet, very few studies have addressed the effects of complementary foods on infant gut microbiota. Several observational studies (18) showed significant shifts of both diversity (19) and community structure of the gut microbiota during weaning and dependent on types of complementary foods consumed (20). One animal study (21) compared meat-, dairy- and plant-protein extracts and found *Ruminococcaceae* was one of the characteristic bacteria in rats fed with meat proteins. A previous study from our group in 6–9 month-old infants showed that compared with a low-protein, cereal-based complementary diet (9% energy from protein), a high-protein, meat-based diet (17% energy from protein) increased the abundance of short-chain fatty acids (SCFA)-producing *Lachnospiraceae* (22). This project will directly assess the development of gut microbiota during the transition from liquid diets (breastmilk or formula) to complementary feeding, and in response to different protein-rich foods.

Emerging research has shown that gut microbiota can directly regulate growth. One animal study (23) found that gut microbiota drives growth during the juvenile period. A landmark cohort study (24) identified bacterial species whose proportional representation defined a healthy and mature gut microbiota during the first year of life in Malawian infants. Specifically, deviation from the normal gut microbiota, such as low diversity and absence of certain species, resulted in "immature" gut microbiota and growth impairment (24). Furthermore, transplanting gut microbiota from stunted infants to germ-free mice also transmitted impaired growth phenotypes, and adding back the two major growth-discriminatory species (*Ruminococcus gnavus, Clostridium symbiosum*) ameliorated growth impairment in mice (24). *Ruminococcus* and other short-chain fatty acids (SCFA) producing strains may increase the gut SCFA content, which, in animal models, could directly promote bone growth (25). Two recent cohort studies found that disrupted maturation of the gut microbiota, as indicated by low diversity, is associated with growth failure in preterm infants (26) and slower growth in weight in Malawian infants (27). However, a recent cohort study (28) found that the abundances of SCFA producing families *Lachnospiraceae* and *Ruminococcaceae* at 4 months were associated with a higher risk of overweight at 12 months. Current findings, although limited and primarily in animal and cohort studies, suggest that gut microbiota could impact growth and risk of overweight. The project presented here will directly assess the relation of gut microbiota and infant growth longitudinally and its potential mediating effect.

Objectives and Hypotheses

Goal

The goal of this randomized controlled feeding trial is to establish how infant diet with different protein-rich foods affects growth trajectories and gut microbiota development during the early complementary feeding phase.

Aims

The objective of this study is to determine the impact of different types of common protein-rich foods on infant growth (Aim 1) and gut microbiota (Aim 2). We also seek to identify the role of the gut microbiota in infant growth (weight, length, head circumference, adiposity), specifically, whether the gut

microbiota is a mediator linking protein-rich foods and infant growth (Aim 3).

Hypotheses

Primary: Growth trajectories (weight, length and body composition) and gut microbiota (diversity and composition) during early complementary feeding will differ by types of protein-rich foods consumed. Specifically, meat- and plant-based diets will have a lower weight-for-length Z score and adiposity, more age-appropriate and diverse gut microbiota than dairy and the reference groups. Secondary: A more mature, diverse microbiota will be positively associated with infant growth and that diet-induced infant growth trajectories will be mediated by the gut microbiota.

METHODS

Study Design

This is a randomized controlled feeding trial with four groups. After screening, eligible participants and their caregiver(s) will visit the Clinical & Translational Research Center (CTRC) at Children's Hospital Colorado (CHCO) to complete the baseline visit (**Figure 1**) at 5 months of age. After obtaining informed consent, participants will be assigned and randomized to one of the four study diets: (1) a meat-protein-predominant diet group (Meat); (2) a dairy-protein-predominant diet group (Dairy); (3) a plant-protein-predominant diet group (Plant); or (4) a reference group without intervention (Reference).

The baseline visit includes consenting to study participation, a questionnaire of infant feeding and family health history, family demographics, parental weight and height, gestational weight gain, parity, maternal smoking, and other variables that could affect outcomes. Blood, urine and stool samples are collected. Urine samples are used for body composition and total energy expenditure assessments using the doubly labeled water method with procedures designed for infants. A fasting breastmilk sample is also collected at baseline. When the intervention ends at 12 months of age, participants come to Children's Hospital Colorado CTRC to complete the end of the intervention visit. During the intervention (5–12 months), monthly home visits are conducted to (1) deliver study foods to Meat, Dairy and Plant and grocery vouchers to Reference, and infant formula if formula-fed or mixed feeding; (2) complete a health and dietary questionnaire; (3) obtain length and weight measurements; (4) collect a stool sample; (5) collect 3-day diet record and the food tracking log; (6) collect a breastmilk sample if breastfeeding.

Timeline and Milestones (in Months, Commencing March 1, 2021)

0–5 Study preparations: Manuals of operation, IRB approval, Children's Hospital Colorado Research Institute Approval and Clinicaltrials.gov registration.

6–48 Enrollment of 260–300 eligible participants and complete all baseline visits.

21 Completion of 50% participants in each group complete.

54 All clinical procedures complete.

60 Laboratory analyses of blood, urine and stool analyses, data analyses complete.

Participants

Participants are being recruited in the metro Denver area from households with 3–4 month-old infants via direct mailing by Colorado Department of Public Health & Environment (CDPHE) which has access to the birth registry. Up to 300 infants will be recruited (75/group) to have at least 240 completers (60/group), based on a conservative estimate of 20% attrition.

Inclusion Criteria

Full term (gestational age equal or over 37 weeks); generally healthy without conditions that would affect protein metabolism or growth; no previous complementary food exposure; no prior exposure of antibiotics during delivery or after birth; able to consume study foods; No known food allergies.

Exclusion Criteria

Pre-term or small-for-gestational age infants; Infants having conditions that would affect normal growth; Infants having had complementary foods prior to the start of the study; not willing to feed the complementary foods provided; antibiotics exposure during delivery or from birth to 5 months of life; multiple births.

Ethical Approval

Ethical approval for this clinical trial was obtained through the Colorado Multiple Institutional Review Board (COMIRB) (COMIRB 20-2232). COMIRB conducts the initial and annual reviews of the trial, monitoring enrollment and retention, protocol deviations, and any reported adverse effects as a result of the study. All participants are provided with a copy of the signed informed consent. This protocol is registered at ClinicalTrials.gov NCT05012930.

Study Procedures

Enrollment

Participant enrollment begins before 5 months of age. Interested participants are screened by phone or email; if eligible, an in-person baseline visit is scheduled and informed consent is obtained at the baseline visit. Enrollment of eligible infants take place over the first 4 years of the study. As part of the informed consent process, participants are notified that they may withdraw at any time and of the actions that may deem the infant ineligible for the study. At time of enrollment, each participant is assigned a unique study-ID (MINT-001 through MINT-300) for subsequent data collection.

Randomization

Upon enrollment, participants are randomized into one of four groups, differentiated by source of protein-rich foods they consume (meat, dairy, plant or control group). A stratified random sampling design is used where the strata are defined by mode of feeding (breastfed or formula-fed), sex (male or female) and mode of delivery (cesarean or vaginal delivery). The four feeding groups are randomized within each strata in groups of four so each diet (Meat, Dairy, Plant, Reference) occurs exactly once per four enrollments. Given our rolling

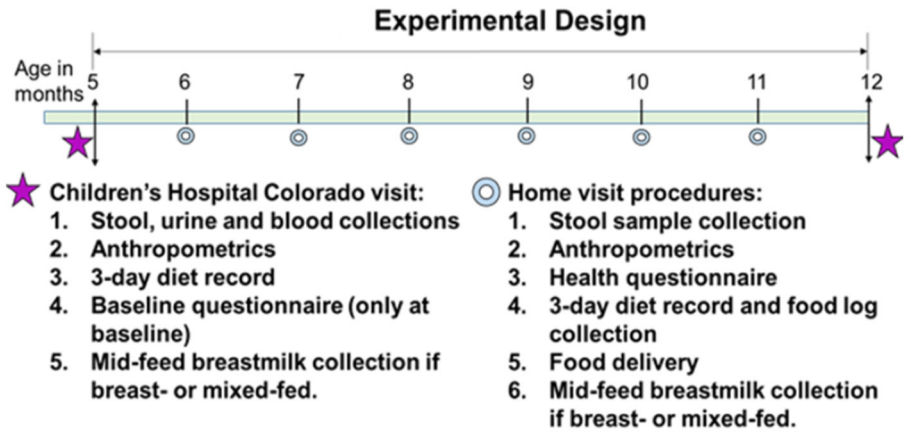

FIGURE 1 | Experimental design.

recruitment strategy over 4 years, this ensures that the initial randomization of group within each strata is close to balanced and that there is little to no relationship with the order of recruitment. A stratified randomization design controls for possible confounding variables and results in an increase in power.

Baseline Data to Be Collected Includes

Demographic Information

Study participants are given a questionnaire at the baseline visit to report both the infant and parental ethnicity, race, gender, high-risk behaviors such as smoking tobacco or other recreational drugs, education level, and gross family income.

Infant and Paternal Health History

Another questionnaire is given to the caregiver to discuss birth details such as mode of delivery and infant weight and length. Caregivers are asked to disclose the family health history of the following: obesity, cardiovascular disease, type 2 diabetes mellitus, and allergies in immediate family members. Infant feeding habits for the previous month are also assessed in addition to any illness, medication, supplements, or antibiotic use. This infant health questionnaire is to be repeated at each subsequent monthly home visit.

Anthropometric Measurements

Pediatric nurses at the CTRC, who are blinded to the treatment groups, obtain three measurements of weight in kilograms, three measurements of length in centimeters, and three measurements of head circumference in centimeters. Study coordinators calculate the average measurement as the baseline anthropometrics for the participant. Parental self-reported height and weight measurements are also to be obtained at this visit.

Dietary Intake

A weighed 3-day diet record is collected from caregivers before the intervention begins. A kitchen scale and calibration weight, plus the diet record with instructions, are provided to caregivers. For breastfed infants who do not use bottles, testing weighing using Seca^{TM} 757 infant scale is conducted before and after each feed during the 3 days when the 3-day diet record is filled out. Registered dietitians from the research team train parents/caregivers to fill out the diet record.

Biospecimen Samples

At the baseline visit, pediatric nurses at the CTRC draw blood from all able participants via venipuncture (3–5 ml). Samples are centrifuged, and serum is stored at −80°C until analyzed. Individual aliquots will be used to measure ICF-1, ICFBP3, insulin, blood lipids (triglycerides, HDL, LDL and total cholesterol), alpha 1-acid glycoprotein (AGP), high sensitivity C-reactive protein (CRP), and quantitative amino acids. After the baseline visit at the Children's Hospital Colorado CTRC, urine samples (one pre-dose and seven post-dose) are collected by the caregiver to assess total energy expenditure and two-compartment body composition using the doubly labeled water technique (29). Urine samples are collected by placing cotton balls in the diaper and after soaked with urine, cotton balls will be placed in the barrel of a 20 ml sterile syringe and urine will be expressed into a sterile plastic tube. A stool sample is also collected from participants' homes for gut microbiota analysis. Stool samples are collected by placing a diaper liner in the infant's diaper, which allows urine to pass and stool to stay. Caregivers are advised to collect the soiled diaper liner and keep it in the home freezer before the study coordinator retrieves it. Lastly, a fasting breastmilk sample is collected from caregivers who are breastfeeding. Macronutrient and energy profiles of breastmilk are assessed via mid-infrared spectroscopy (Human Milk Analyzer, Miris, Uppsala, Sweden). Detailed instructions on how to collect urine, stool and breastmilk samples, and sample collection kits are provided to caregivers.

Dietary Intervention

Participants in the three intervention groups are asked to avoid protein-rich foods from other assigned groups (30) and consume protein only from their assigned group. All complementary

foods, including infant cereal, low-protein fruits and vegetable purees, and designated protein-rich foods are provided to the three intervention groups. For formula-fed infants, the same brand cow-milk based infant formula is provided. The most recent NHANES report showed that the median protein intake of US infants 6–11 months (31) is 10% total energy or 2.5 g/kg/d. This quantity is used as the targeted total protein intake. Caregivers of infants in the reference/control group are given compensation to purchase complementary foods. Based on weight recorded at the visit and the reported formula/breastmilk intake, a recommended amount of meat- dairy- or plant-based food will be provided to caregivers to approximate a total protein intake of 2.5 g/kg/d. This estimation is updated every month. A sample diet plan is in **Table 1**, based on a formula-fed infant with a 60th percentile weight-for-age at 9 months.

Follow-Up Procedures: Home Visits

Monthly home visits at ages 6, 7, 8, 9, 10, and 11 months of age (±7 days) are conducted for each participant by one or two study coordinators. At each home visit, coordinators collect the following: stool sample (pre-collected by parents within 48 h before the visit), anthropometric measures (weight, length, head circumference), health questionnaire, 3-day diet record, food intake log, and breastmilk collection if the participant consumes breastmilk. The 9-month diet record is a weighed 3-day diet record. Caregivers also conduct test weighing again at 9 months if the participants consume breastmilk. Study coordinators also deliver study food (intervention groups) or food voucher (reference group) at the visit. Growth parameters are plotted on the infant's growth chart and Z-scores are calculated.

Follow-Up Procedures: 12-Month and 24-Month Visits

At 12 months of age (±7 days), the study participant returns to the Children's Hospital CTRC to complete their end of intervention visit. The visit includes the same procedures and sample collections as the baseline visit. The dietary intervention ends at 12 months. At the 12-month visit, caregivers are asked whether they want to consent to a 24-month follow-up visit. If so, caregivers sign another consent for the 24-month follow-up. When the participant turns 24 months, 1 year after the intervention ends, they come back to the Children's Hospital CTRC and repeat the procedures at baseline and 12 months, except for body composition and total energy expenditure assessment.

Primary Outcomes

1) *Infant growth*

Pediatric nurses at the Children's Hospital Colorado CTRC (at baseline, 12 and 24 months) and study coordinators (at monthly home visits) obtain a series of infant anthropometric measurements. These measurements including weight (Seca^{TM} 337 infant scale), length (obtained in recumbent position using an infant stadiometer), and head circumference-for-age (Seca infant head circumference measuring tape). Growth Z scores are calculated using length and weight based on WHO/CDC standards (32).

2) *Body composition and total energy expenditure at 5 and 12 months of age*

At baseline and 12 months, a pre-dose urine sample is collected to document basal enrichment of ^{2}H and ^{18}O, then 0.1 g/kg body weight 99% enriched $^{2}H_2O$ and 0.3 g/kg 10% enriched $H_2^{18}O$ are orally administered. Caregivers are asked to collect one daily sample for 7 days post-dosing. Urine samples are analyzed for ^{18}O and ^{2}H enrichment by Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS, Los Gatos Research Inc., Mountain View CA) (33). Isotope dilution space will be calculated via the standard equation of pre-dose and zero-time intercept enrichment via back-extrapolation, which is validated in infants (34). Total body water will be calculated from dilution space × 1.04 to account for water exchange in non-aqueous tissues. Finally, total fat-free mass will be obtained from total body water ÷ age/sex-specific hydration factor (i.e., 79% for 6-month-old infants). CO_2 production, obtained from the fractional turnover rates of ^{2}H and ^{18}O (35), is converted to total energy expenditure via Weir equation (36).

3) *Gut microbiota*

Caregivers will obtain a fresh stool sample each month of participation (5–12 months of age) and one at 24 months. 16S rRNA gene amplicon sequencing (37–39) will be performed on all samples to profile fecal bacterial communities. Following sequence QA/QC (37–39), 16S rRNA gene sequences are classified using the SINA/Silva classifier.

Shotgun metagenomic sequencing (40) will be performed on baseline and 12-month samples to determine the functional genomic capacity of fecal microbiota and broaden taxonomic profiles to include archaea, bacteriophage, DNA viruses, and microbial eukaryotes. Following sequence QA/QC, ***[1–4]*** sequences will be co-assembled with MEGAHIT ***[5]***, open reading frames predicted (Prodigal ***[6]***), and annotated using DIAMOND ***[7]*** to query the NCBI non-redundant database. Bacterial species and strains are inferred using MIDAS ***[8]***.

All sequences and associated metadata will be deposited in the NCBI sequence read archive, following the MIMARKS standard.

Secondary Outcomes

1) *Dietary intake*

Three-day diet records are analyzed by the CTRC Nutrition Core using the NDSR software (Minneapolis, MN) by a trained dietitian. The Food and Nutrient Database in NDSR contains over 18,000 foods, including brand-name infant foods used in the proposed study. Outputs include total energy, carbohydrate (fiber, fructose, glucose, sucrose, starch, etc.), fat (cholesterol, different kinds of fatty acids), protein (meat, dairy, plant, breakdown of amino acids), vitamins and minerals. Macronutrient and energy profiles of breastmilk samples are assessed via mid-infrared spectroscopy (Human Milk Analyzer, Miris, Uppsala, Sweden).

2) *Blood biomarkers*

IGF-1, IGFBP3, insulin, blood lipids, alpha 1-acid glycoprotein (AGP), high sensitivity C reactive protein (hsCRP) and quantitative amino acids will be measured at the University of Colorado Anschutz Medical Campus CTRC Core lab (30).

TABLE 1 | Examples of diet plans for the three intervention groups[a].

	Food item	Protein	Calorie
Total	Total calorie needs		~650 kcal/d
	Total protein (2.5 g/kg/d)	21 g/d	84 kcal/d
Formula (not restricted)	Formula 18 ounces	9 g/d	400 kcal/d
Dairy group[b]	1 Yogurt (Yobaby®)	4 g/d	80 kcal/d
	Cheese (shredded) (Horizon®)	6.5 g/d	80 kcal/d
	Others (e.g., 1 Beech Nut® zucchini and banana blend; or one servicing of fortified rice cereal)	1 g/d	
Meat group[b,c]	1.5 jar of ham and gravy (Gerber®)	10 g/d	80 kcal/d
	Others (e.g., 1 Beech Nut® zucchini and banana blend; or one servicing of fortified rice cereal)	1 g/d	
Plant group[d]	1.5 vegetable pouch (e.g., Ella's kitchen® four bean feast)	5 g/d	90 kcal/d
	1.5 vegetable pouch (e.g., Earth's best® spinach lentil)	5 g/d	50 kcal/d
	Others (e.g., 1 Beech Nut® zucchini and banana blend; or one servicing of fortified rice cereal)	1 g/d	
Reference group	No restriction (observational group and will follow standard of care)		

[a] *These estimates are based on an exclusively formula-fed 9-month-old female with a 8.5 kg body weight which is ~60th percentile weight-for-age.*
[b] *Both Dairy and Meat groups are advised to avoid plant foods of relatively high protein contents (a list will be provided). Cheese will be shredded before providing to the infant.*
[c] *The Meat group is allowed to have fish. However, commercial fish-based infant foods are very rare and if parents choose to feed home-made fish to the participants, they will record the time, type and amount.*
[d] *Both dairy- and plant-based complementary foods have low iron content. Participants in all intervention groups are advised to consume one serving of iron-fortified cereal per day to meet their iron needs.*

Power Calculation and Sample Size Justification

We hypothesize that both the gut microbiota and infant growth trajectories are dependent on types of protein-rich foods consumed. We will recruit up to 75 infants/arm and use a conservative 20% attrition which results in 60/group (total completers $n = 240$). Sample size and power justification are from the longitudinal models with feeding group as the predictor and either gut microbiota (i.e., diversity and *Ruminococcus* abundance) and growth (i.e., linear growth parameter LAZ, overweight parameter WLZ and growth velocity using the WHO 0–24 months standards) as the outcomes. We completed power simulations (1,000 replicates) using a linear mixed-effects model (LME) with random effects by subject. For the simulations, we used parameter estimates (e.g., fixed and random effects) from our randomized pilot study on the gut microbiota and infant growth trajectories after receiving meat- or dairy-based complementary diet (30). We simulated a variety of scenarios assuming that Plant had microbiota and growth outcomes that were either worse than Dairy, between Dairy and Meat, or the same as Meat. *To be conservative, we estimated power after increasing the within-group variability by 50%.* The power is above 80% in all simulated scenarios (80–90%) where the effect size was simulated to be at least 50% of that observed in our pilot study (30). The power of most simulation scenarios was below 80% when the effect size was 25% of that observed in the pilot study (30).

Statistical Approach

Aim 1: Using ANOVA for continuous variables and chi-squared test for categorical variables, we will assess whether matching and randomness held by testing whether potential confounders (e.g., feeding mode, maternal height, maternal BMI, education, sex, race/ethnicity, and smoking during pregnancy) differ between groups. Variables with a $p < 0.05$ and those that have a strong association with the outcome of interest are included in future models. To model the relationship between diet, time, and diet by time interaction and LAZ and WLZ, we use LME model with random effects by subject. This structure explicitly models multiple time points per infant and can adjust for changes of cofounders during the intervention (e.g., a participant changed from breastfeeding to formula feeding at 9 months). Given a significant diet by time interaction, we will test pairwise differences in growth trajectories using Tukey's multiple comparisons.

Aim 2: We model the effects of diet on the gut microbiota at the end of the intervention and longitudinally, incorporating likely covariates and potential cofounders. 16S amplicon sequencing and metagenomic sequencing will each generate tables of annotated sequence counts. Both datasets will be used to model the diet's impact on microbiota taxonomic composition and functional capacity (i.e., genes and pathways identified through shotgun metagenomics) over the course of the intervention. To evaluate differences in overall taxonomic/functional community composition (i.e., beta-diversity), we use the Microbiota Regression based Kernel Association Test (MiRKAT). MiRKAT models the microbiota using phylogenetic kernels to account for differences in microbial profiles (16S or metagenomics count data) (41). As different measures of dissimilarity are optimal under different conditions, optimal-MiRKAT allows multiple dissimilarity metrics to be tested simultaneously. To model microbial taxonomic and functional profiles over time, we use two methods recently proposed (42). First, we use a bi-exponential distribution to summarize microbiota diversity curves over time. To make inferences, we will incorporate the distribution into a hierarchical model with a random intercept to account for multiple measurements for each person. This approach will model changes in individual taxa, both the rare and common, over time. Additionally, we will evaluate longitudinal

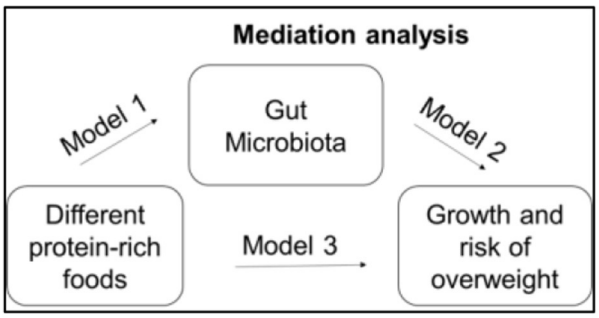

FIGURE 2 | Mediation analysis.

changes in common alpha-diversity metrics (e.g., richness, evenness, Shannon diversity, and effective species [e.g., Hill's number (42)]). Finally, we use the control group to build a gut microbiota maturation model via a random forest approach as previously described (24) and compare the intervention groups to this model.

Aim 3: We use LME on growth Z scores and the microbiota over time with random effects by subject, and include group as a covariate. We also complete secondary analyses stratified by group to identify relationships between Z scores and microbiota that differ by group. We will use microbiota diversity measures and relative abundances of individual taxa and genes/pathways to model diversity and composition of the microbiota. We also include necessary confounders, e.g., change from breastfeeding to formula. To evaluate whether taxonomic and/or functional features of the microbiota mediate the relationship between diet and growth, we use a method and R package called MedTest (43). As in classic mediation analysis, MedTest evaluates three models (44). In reference to **Figure 2**, the first model estimates the direct path between protein-rich foods and the gut microbiota and implements as part of Aim 1 using MiRKAT. The second model estimates the path between protein-rich foods and infant growth and will be implemented using a standard linear model in R. The third model estimates the path between protein-rich foods and growth controlling for the path between gut microbiota and growth and will be implemented using MiRKAT. Using these three models, MedTest tests for significances of the mediation effect of the gut microbiota. Since it is unknown which microbiota features may mediate the relationship, MedTest implements multiple microbiota distances: unweighted (45), weighted (46), and generalized UniFrac (47) as well as Jaccard and Bray-Curtis distances. To ensure a well-controlled type-I error rate, MedTest uses a modified permutation procedure to arrive at an omnibus test over all distances.

DISCUSSION

Innovation and Significance

We focus on a critical developmental phase that has not been well-studied and propose a novel concept that during early complementary feeding, types of common protein-rich foods will differentially impact infant growth and risk of overweight and that gut microbiota will mediate this impact. To our knowledge, this is the first RCT that directly compared meat, dairy and plant consumptions on infant growth and gut microbiota. We also propose to use non-invasive body composition assessment with doubly labeled water, which also provides a reliable estimation of energy expenditure, a crucial component to assess the risk of overweight. Furthermore, we propose a plausible mechanism that protein-rich foods from different sources could impact infant growth trajectories by modulating the gut microbiota. Overall, this project is innovative because it is a substantively different concept and approach. It will greatly support implementing effective dietary interventions to prevent undesirable infant growth patterns and long-term negative health impact.

Compliance Monitoring

Although we will use controlled feeding, we are aware that compliance with the dietary regime is a major challenge. Building on extensive experience, we will utilize the following strategies to optimize and monitor compliance: (1) A weekly multiple-choice log to record food intake based on the monthly recommendations (**Table 1**) as described in our pilot study (30). (2) Caregivers will be asked to return unconsumed foods/formula at the monthly home visit. (3) Weighed 3-day diet record will be collected at 5, 9, and 12 months; (4) Blood urea nitrogen (BUN), measured at 5 and 12 months is a crude marker of total protein intake, and values of BUN will reflect the total amount of protein consumed for comparisons between groups and over time. We also understand the study procedures may be burdensome to some caregivers. Our team has successfully conducted a number of infant trials with similar or more extensive procedures and low drop-out rate (30, 39, 48, 49). Our experienced team members are dedicated to fully support caregivers and will be available by phone/email and in person, and will make extra home visits if needed.

Risks

The study protocol is expected to have minimal risk because there are no invasive procedures except the three blood draws at 5, 12, and 24 months of age, which are necessary to answer the research question. The 12-month blood draw will include analyses (Pb and Hb) routinely obtained for well-child surveillance; results will be provided to the primary care provider to avoid a second clinical blood draw. There is an unlikely risk of food allergies. During screening, the caregiver is asked if the infant has any food restrictions or allergies, including milk/dairy protein allergies (i.e., on a special formula). If the answer is yes, the infant is not eligible to participate. If the participant has no food restrictions or allergies, he/she is unlikely to be allergic to the complementary foods provided because most of the study foods are not considered highly allergenic foods. At the baseline visit, the study coordinator explains the potential risk of allergy development when starting complementary feeding and caregivers are given a list of common signs of food allergies. Food allergy is closely monitored by monthly health questionnaires. If

repeated concerns are expressed, the participant will be removed from the study and advised to consult the primary care physician.

AUTHOR CONTRIBUTIONS

MT conceived of the trial. NK, EM, DF, and AH supported the design and development of the trial. KM, LB, and KD contributed to finalizing the manuscript. All authors contributed to the article and approved the submitted version.

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Exclusive Breastfeeding and Factors Influencing Its Abandonment During the 1st Month Postpartum Among Women From Semi-rural Communities in Southeast Mexico

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Introduction: In this study we describe breastfeeding practices among women from semi-rural communities in southeast Mexico, and explore which factors, modifiable or not, are associated with such practices.

Materials and Methods: This was a formative cross-sectional study that included 143 mothers with infants 4–6 months old, from semi-rural communities in Tabasco, Mexico. We collected data on two categories of factors: (1) women's sociodemographic characteristics, and (2) maternal / infant factors. We first analyzed the frequency of various breastfeeding practices. Then, we classified participants into the up to 1 month of exclusive breastfeeding group (≤1 m-EBF) and the beyond 1 month EBF group (>1 m-EBF), if they practiced EBF for less or more than 1 month, respectively. We compared the two categories of factors between groups and then, using logistic regression models, explored which factors were associated with practicing >1 m-EBF.

Results: By the end of the 1st month postpartum, 51.7% of participants had abandoned EBF, introduced milk formula (35%), other food (9.1%), non-nutritive liquids (7.7%), or had stopped breastfeeding completely. In the next months, EBF practice fell sharply and mixed feeding grew importantly.

Logistic regression models showed that women were more likely to be in the >1 m-EBF group if they lived with the baby's father, had complications during pregnancy, delivered vaginally and attended a health center at least three times postpartum. To the contrary, women were less likely to be practice >1 m-EBF if they gave infants other liquids during their hospital stay; experienced pain or discomfort in breasts/nipples, or used a pacifier after hospitalization; had larger bodies (i.e., higher BMI); and believed that you should give the infant powdered milk or some other food when the baby is not full.

Conclusion: Many factors associated with abandoning EBF, particularly in the early postpartum period, are modifiable and can be altered through timely interventions that

include giving correct information and ensuring its comprehension; assertive personal counseling and accompaniment must be provided to mothers; and reinforcement during the early postpartum at health facilities and other settings.

Keywords: exclusive breastfeeding (EBF), breastfeeding, infant feeding, Mexico, Tabasco (Mexico), food insecurity, social determinants of health, breastfeeding beliefs

INTRODUCTION

Breastfeeding confers life-long benefits to the infant, such as increased likelihood of survival, better health, development, and cognitive achievements (1), which in time contribute to the society's human capital and sustainable development (2). To warrant these benefits, exclusive breastfeeding (EBF) is recommended by the World Health Organization and UNICEF as the optimal way to feed infants for the first 6 months, which means that no other foods or liquids, including water, are provided to them during that period.

In Mexico, data from the latest nationwide surveys show an increase in the prevalence of EBF among infants younger than 6 months, from 13.0 to 20.7% between 2009 and 2018 (3). However, these figures are still below 44%, the global rate of EBF and far from the global target goal of 70%, proposed by the World Health Assembly to be reached by 2030 (4).

International agencies have identified the type of actions that are needed to enable women to breastfeed adequately for an appropriate duration, while initiatives and programs have been proposed to achieve their execution (4). Some of those actions have been undertaken in Mexico by the government, civil society and academia (5). For example, several hospitals offering maternity services have been nominated as "baby friendly;" some of the provisions of the Code of Marketing of Breastmilk Substitutes are contemplated in the Mexican legislation; and a nationwide breastfeeding training program for health service providers was developed. However, there has not been a formal assessment of the effectiveness of these interventions, and some have no national coverage, adequate funding or legislative backing (6).

Moreover, breastfeeding practices may be influenced by many factors of diverse nature, ranging from sociocultural and economic characteristics, to family or social support networks, availability of health services, and mother's attitudes, beliefs or even exposure to breastmilk substitute advertisements (7, 8). Considering such diversity of factors influencing breastfeeding practices, in this study we designed a formative research to (1) describe breastfeeding practices among women from semi-rural communities in Tabasco, southeast Mexico; and (2) to explore which factors condition such practices.

MATERIALS AND METHODS

Study Setting and Design

This was a formative, cross-sectional study carried out in Tabasco, a coastal southeastern state in Mexico, characterized by a hot and humid climate and a large presence of rainforests and water bodies (wetlands and rivers) (**Figure 1**). At the time the study took place (2016), 50.9% of Tabasco's population lived in poverty, of which 11.8% was extreme; these figures have increased since then to 53.6 and 12.3% (9).

The study took place in Centro, one of Tabasco's 17 municipalities/health jurisdictions, and included women who received prenatal care at the Health Center with Expanded Services (CESSA, initials in Spanish), located in an urban town called Villa Luis Gil Pérez, or at one of the 17 first level public health units (FLPHU) affiliated to CESSA. The FLPHU are smaller health centers located in rural or semi-rural villages (**Figure 1**). Most of the CESSA and FLPHU users are people with the most basic governmental social security (Seguro Popular) and often among low socioeconomic levels.

The study's protocol was approved by the Research and Ethics Committees of the National Institute of Perinatology in Mexico City (212250-3310-11406-03-16) and authorized by the local health authorities at Centro Health Jurisdiction in Tabasco. Data was collected from March to June 2016.

We included women and their babies if they (1) lived within the geographical limits of Villa Luis Gil Pérez, (2) received prenatal care at CESSA or one of the 17 health units, (3) had a single and clinically healthy pregnancy, (4) had not been hospitalized for any condition that could be a barrier for breastfeeding initiation; (5) babies were between 4 and 6 months old at the time of the study, and (6) accepted to participate and signed an informed consent.

We identified potential participants from a census of women who carried out their prenatal control in Villa Luis Gil Pérez's CESSA. We invited these women to participate in the study by telephone and / or by home visits. Data was collected between March and July 2016.

Sample Size

The sample size was calculated to estimate the proportion of women that would be breastfeeding exclusively in our study population. We considered a precision of 5%, a confidence level of 95%, and the prevalence of exclusive breastfeeding in children under 6 months available at the time: 14.4% nationwide and 15.5% for the southern states of the country (10). With these parameters, the sample size initially considered was 190–200 women. However, due to the much lower exclusive breastfeeding prevalence we found in the community during the course of the study, the aimed sample size was modified to 150 women.

We evaluated selection bias by comparing the basic sociodemographic data from included and not included women. This data was collected by applying a general characteristics questionnaire when inviting women to participate (**Supplementary Material** Section A).

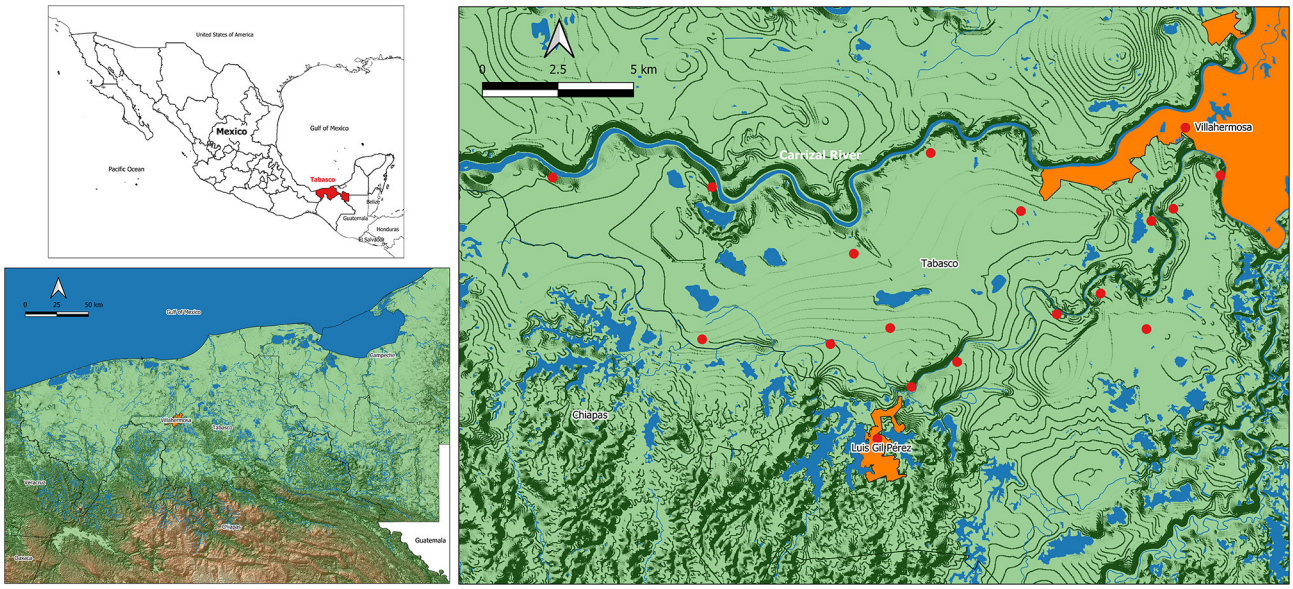

FIGURE 1 | Geographical location of the area of study. Tabasco, in southeast Mexico (top left), is a coastal plain with hot, humid climate and a large presence of wetlands and rivers (bottom left). Participant women received their prenatal medical care in one of the 17 rural first level public health centers (right, red dots) managed from Villa Luis Gil Pérez (orange area to the south), a town located a few kilometers southwest of the state's capital city, Villahermosa (top right corner).

Study Variables and Data Analysis

For this study, we included a series of variables related to the participant's characteristics, experiences, and thoughts. Data for constructing these variables was obtained from an *ad-hoc* questionnaire that we applied to participants during the study appointment (**Supplementary Material**). Data were either used as reported, collapsed and/or developed into categories as a means of data reduction.

Since infants 4–6 months old were included in our study, it was not possible to use the status quo "exclusive breastfeeding under 6 months" indicator proposed by the WHO which considers infant feeding current practices (i.e., previous day). Moreover, we think the WHO status quo EBF indicator has one major limitation: if the mother is only asked what her child ate the day before, it is possible that in previous days the infant ate or drank something other than breast milk. This food would not be registered and therefore lead to an overestimation of exclusive breastfeeding figures.

Therefore, in order to be able to describe the moment when EBF was stopped, we asked participant women their infant's age in months, the first time they received non-nutritive liquids (water, tea, juice, or both), formula milk, and/or solid foods. From these data, we constructed the outcome variable "breastfeeding practices," composed of four categories: (1) exclusive breastfeeding (EBF, breastfeeding with no other food or drink, not even water); (2) predominant breastfeeding (mainly breast milk but with other liquids, such as water and water-based drinks or fruit juice); (3) mixed feeding (formula milk, liquids and/or solid foods in addition to breast milk); and (4) no breastfeeding (having stopped breastfeeding completely). Constructing the outcome variable in this way allowed us to describe not merely current breastfeeding practices but how they changed over the 1st months of infants' lives.

Then, to explore which factors condition EBF, we first classified participants in two groups according to the duration of breastfeeding: the early abandonment of exclusive breastfeeding group ($\leq$1 m-EBF) included women who practiced EBF for $<$1 month, while the beyond 1 month EBF group ($>$1 m-EBF) included those who practiced EBF for more than 1 month. We selected 1-month as a cut off point for creating groups because very early EBF desertion was common in this population. We then compared women in the $\leq$1 m-EBF - EBF with those in the $>$1 m-EBF in terms of two groups of factors: (1) sociodemographic characteristics, (2) maternal and infant factors.

Sociodemographic Characteristics

These factors included: participant's age (years), whether she lived with the baby's father (yes/no), if it was important for the baby's father that she breastfed (agree/disagree), occupation (housewife/work outside home), household type (monoparental/nuclear/extended), schooling (years), whether she had governmental social security (Seguro Popular/IMSS/ISSSTE) or was beneficiary of any government support program (yes/no), household welfare level and household food security.

We estimated household welfare levels using the AMAI rule 8X7, a tool developed by the AMAI (in Spanish, Mexican Association of Market Intelligence and Public Opinion Agencies). It consists of eight items and classifies households in seven socioeconomic levels according to the head of the household's ability to satisfy their members' needs (11). Since we

observed that women in the higher levels tended to abandon EBF earlier, we collapsed the seven resulting levels into two broad categories according to income distribution: a higher, privileged segment which dedicates a greater proportion of their spending to education, entertainment, communication, saving and automobile acquisition (scores A/B, C+, C, and C-), and a lower, underprivileged group which spends mainly on food and drinks, transport and personal care (scores D+, D, and E) (11).

We evaluated food security using the Latin American and Caribbean Scale of Food Security (ELCSA), which consists of 15 questions with "yes" or "no" answers. It classifies households in four categories (food security, mild food insecurity, moderate food insecurity and severe food insecurity) according to the women's opinion and experience regarding their difficulty to access food as a result of lack of money or other resources.

Maternal/Infant Factors

Maternal and infant factors comprised a wide array of variables related to women's reproductive history, last pregnancy, birth and hospitalization; and also early postpartum factors, breastfeeding experiences and beliefs.

Regarding the participant's reproductive history, we asked the number of previous liveborn children. With regard to the participant's previous child, factors included: age of the previous child at the time of the study (years), length of exclusively breastfeeding her previous child (months), and whether she had been satisfied with her previous experience (yes/no).

Participant's last pregnancy factors referred to the baby they were currently breastfeeding, and included: whether it was a planned pregnancy (yes/no), the moment of her first prenatal care visit to FLHU (gestational weeks), number of prenatal care visits to FLHU, received information about EBF until 6 months old (yes/no), developed gestational diabetes or hypertensive disorder of pregnancy (yes/no).

Birth factors included: place of delivery (third level hospital, other public hospitals, private clinic, home), mode of delivery (vaginal/cesarean section), whether the birth was attended by medical staff (yes/no), hospitalization length (hours), baby's sex, gestational age at birth (weeks), if the baby was premature (yes/no), weight at birth (kg), and length at birth (cm).

Hospitalization factors included: initiated breastfeeding within the 1st hour (yes/no), roomed-in with baby (yes/no), problems with breastfeeding during hospitalization (yes/no, cause), offered liquid other than breast milk during hospitalization (yes/no), and used pacifier or bottle nipple during hospitalization (yes/no).

Early postpartum factors included: number of visits to FLHU for infant follow-up, received breastfeeding information/support during postpartum visits to FLHU (yes/no).

Breastfeeding factors included: duration of exclusive breastfeeding (weeks), reasons to stop exclusive breastfeeding. Additionally, we explored participants' thoughts and beliefs about breastfeeding and formula milk. We asked participants if they agreed with the following statements (yes/no): "*I am convinced that giving only breast milk until the baby is 6 months old, without giving any other food, is the best for her/him,*" "*When you finish breastfeeding, you are always sure that your baby under 6 months is full,*" "*Formula milk is an important food to accompany breast milk before 6 months,*" "*When the baby is not full, you should give her/him powdered milk or some other food, even if she/he is <6 months old.*"

Data Analysis

We performed exploratory data analysis in all variables as well as normality tests in continuous variables to analyze whether they had normal distributions. For evaluating selection bias, we compared sociodemographic, pregnancy, birth and hospitalization variables from included and not included women using Student's *t*-test or Mann-Whitney *U*-test depending on variable distribution.

For the statistical analyses, first, we performed a bivariate analysis to establish the association between the outcome variables and each independent variable. We used Chi squared, Student's-*t* or Mann-Whitneys's *U*-test depending on the type and distribution of the potentially influencing factor. Independent variables that were associated with the outcome variable ($p \leq 0.10$) were included in backward stepwise logistic regression models. We checked the uptake of variables for collinearity and accepted correlations > 0.35, tolerance > 0.79 and variance inflation factor (VIF) < 1.27.

In order to explore which factors were associated with the duration of EBF, we conducted two logistic regression models; in both of them the predicted probability was for being in the >1 m-EBF group.

In model 1 we explored sociodemographic factors, which included: lives with the baby's father, occupation, and household food security. In model 2 we included maternal / infant factors that showed association with the outcome variable (>1 m-EBF) in the bivariate analyses. In this model we included sociodemographic factors as confounding variables in order to minimize or eliminate possible residual confounding.

Finally, to propose a conceptual model that describes the association of studied factors with EBF, we performed a bivariate analysis among the independent factors, using Chi squared, Student's-*t* or Mann-Whitney's *U*-test depending on variable type and distribution.

RESULTS

In this study, we aimed (1) to describe breastfeeding practices among women from semi-rural communities in southeast Mexico; and (2) to explore which factors, modifiable or not, condition such practices.

Study Sample

We invited a total of 200 women to participate, 57 (28.5%) of which did not meet the inclusion criteria: 24 had not received their prenatal care at CESSA, 22 had babies older than 6 months, eight had had complications during birth and three did not accept to participate. Therefore, 143 women were included in the final sample.

We compared our final sample to the group of women not included in the study. There were no differences in relation to most sociodemographic characteristics evaluated,

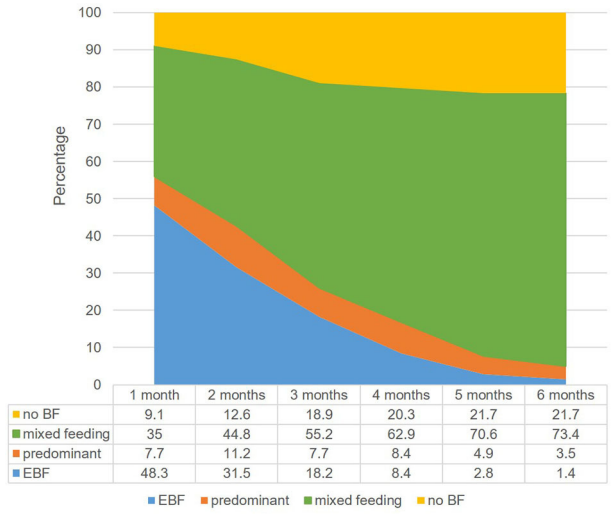

FIGURE 2 | Breastfeeding practice trends. Numbers in the figure's table represent percentages.

except that more included women had the type of governmental social security whose beneficiaries are people without formal employment (Seguro Popular) (97.9%, $n = 140$ vs. 87.7%, $n = 50$; $p < 0.01$). A higher proportion of women in the not included group were beneficiaries of IMSS/ISSSTE, governmental social security for people with formal employment. This difference was anticipated since the latter women were expected to have their prenatal care in their designated clinics, unlike those with Seguro Popular who would be attended at CESSA or FLHU units.

A higher proportion of not included women experienced complications during pregnancy (31.6%, $n = 18$ vs. 15.4%, $n = 22$; $p = 0.01$); gave birth through C-section (43.9%, $n = 25$ vs. 21.0%, $n = 30$; $p < 0.01$) and were hospitalized after giving birth (28.1%, $n = 16$ vs. 8.5%, $n = 12$; $p < 0.01$). Hospital stay in hours was different (median 24, p25–p75 24–75 vs. 24, 17–39; $p = 0.01$). These differences also reflect selection criteria since we did not include women who have had conditions that could be a barrier for breastfeeding initiation. Women with such barriers would be expected to have gestational complications, deliver by cesarean section and/or had longer hospital stay.

Breastfeeding Practices

Figure 2 shows that by the end of the 1st month of life, half of the women (51.7%, $n = 74$) in the study had abandoned EBF, some (7.7%, $n = 11$) had introduced non-nutritive liquids; most (35%, $n = 50$) had introduced milk formula or other food (9.1%, $n = 13$) and others had stopped breastfeeding completely. As months went by, EBF practice fell sharply and mixed feeding grew importantly.

Since EBF was abandoned by an important proportion of women as early as the 1st month of life, we wanted to find out which factors might be associated with this early abandonment.

Sociodemographic Characteristics

Table 1 shows the sociodemographic characteristics of the study population, comparing the ≤1 m-EBF and >1 m-EBF groups. Significantly more women in the >1 m-EBF were living with their baby's father, were housewives, and lived in households in the lower welfare level or with some level of household food insecurity.

As expected, there was an important correlation between the household welfare and level and food security ($r = -0.36$). More women classified in the high level of household welfare lived in a food secure household (44.7%, $n = 17$ vs. 15.2%, $n = 16$); there was a similar proportion of mildly insecure households between the two categories of welfare (47.7%, $n = 18$ vs. 47.6%, $n = 50$) and a lower proportion of moderately (7.9%, $n = 3$ vs. 21.9%, $n = 23$) and severely insecure (0%, $n = 0$ vs. 15.2%, $n = 16$) ($p < 0.01$). Another correlation was present between the variables "Lives with the baby's father" and "*It is important for the baby's father that you breastfeed*" ($r = -0.42$, $p < 0.01$), as more women who lived with the baby's father said he was interested in BF (91.9%, $n = 113$ vs. 50%, $n = 10$; $p < 0.01$); therefore, we did not include the variables household welfare level and "*It is important for the baby's father that you breastfeed*" in the following logistic regression analysis.

In a model adjusted by the significantly different variables in bivariate analysis, women living with the baby's father and with severe household food insecurity, were more likely to breastfeed beyond the 1st month (model 1 in **Table 4**).

In order to propose a conceptual model about how sociodemographic characteristics influence EBF, we further analyzed the possible associations between them (**Figure 3**). For example: only 10% ($n = 10$) of women living with the baby's father worked outside home, compared to 30% ($n = 6$) of those not living with him ($p = 0.01$) ($r = -0.24$, $p = 0.01$). There was no difference in the proportion of the women who worked outside their home or lived with the baby's father between the food security categories.

Maternal/Infant Factors

In **Table 2** we show information about the women's reproductive history and last pregnancy, including previous breastfeeding experience. Significantly more women in the >1 m-EBF had at least one previous liveborn baby. Also, more women in this group were diagnosed with GDM or a hypertensive disorder of pregnancy. In contrast, more women in the ≤1 m-EBF had never breastfeed or had stopped EBF before 1 month with their previous child, delivered by a cesarean section and had a higher BMI at the time of the study visit.

Regarding prenatal care, most women attended at least five prenatal visits to the health care service during pregnancy, around half of them went for the first time during the first 8 weeks of gestation. During these visits 59.4% ($n = 85$) received information about the importance and benefits of breastfeeding; 45.4% ($n = 65$) were counseled to exclusively breastfeed till their baby was 6 months; 8.4% ($n = 12$) were told to practice on-demand breastfeeding and 45.4% ($n = 65$) were taught how to

TABLE 1 | Sociodemographic characteristics.

	Total	≤1 m-EBF (*n* = 63)	>1 m-EBF (*n* = 80)	*p*
Age				
Years	23.0 (19.0 - 27.0)	22.0 (19.0 - 27.0)	23.0 (20.0 - 27.7)	0.45
Lives with the baby's father				
Yes	123 (86%)	49 (77.8%)	74 (92.5%)	**0.01**
It is important for the baby's father that you breastfeed				
Agree	123 (86%)	50 (79.4%)	73 (91.3%)	**0.05**
Occupation				
Housewife (vs. work outside home)	127 (88.8%)	52 (82.0%)	75 (93.8%)	**0.03**
Household type				
Monoparental	4 (2.8%)	3 (4.8%)	1 (1.3%)	0.10
Nuclear	79 (55.2%)	29 (46%)	50 (62.5%)	
Extended	60 (42%)	31 (49.2%)	29 (36.3%)	
Schooling				
Years	9.0 (8.0 - 12.0)	9.0 (8.0 - 12.0)	9.0 (8.2 - 12.0)	0.68
Governmental Social Security				
Yes	140 (97.9%)	62 (98.4%)	78 (97.6%)	0.74
Household welfare level				
Lower level	105 (73.4%)	41 (65.1%)	64 (80.0%)	**0.04**
Household food security				
Secure	33 (23.1%)	19 (31.1%)	14 (17.1%)	**0.04**
Mild insecurity	68 (47.6%)	29 (46.0%)	39 (48.8%)	
Moderate insecurity	26 (18.2%)	11 (17.5%)	15 (18.8%)	
Severe insecurity	16 (11.2%)	3 (4.8%)	13 (16.3%)	

Data shows number of cases (%) or median (p25–p75). We compared continuous variables between groups using Student's t-test or Mann-Whitney's U-test, and categorical variables using Chi squared test.
Bold values indicate statistically significant differences (p < 0.05) between study groups.

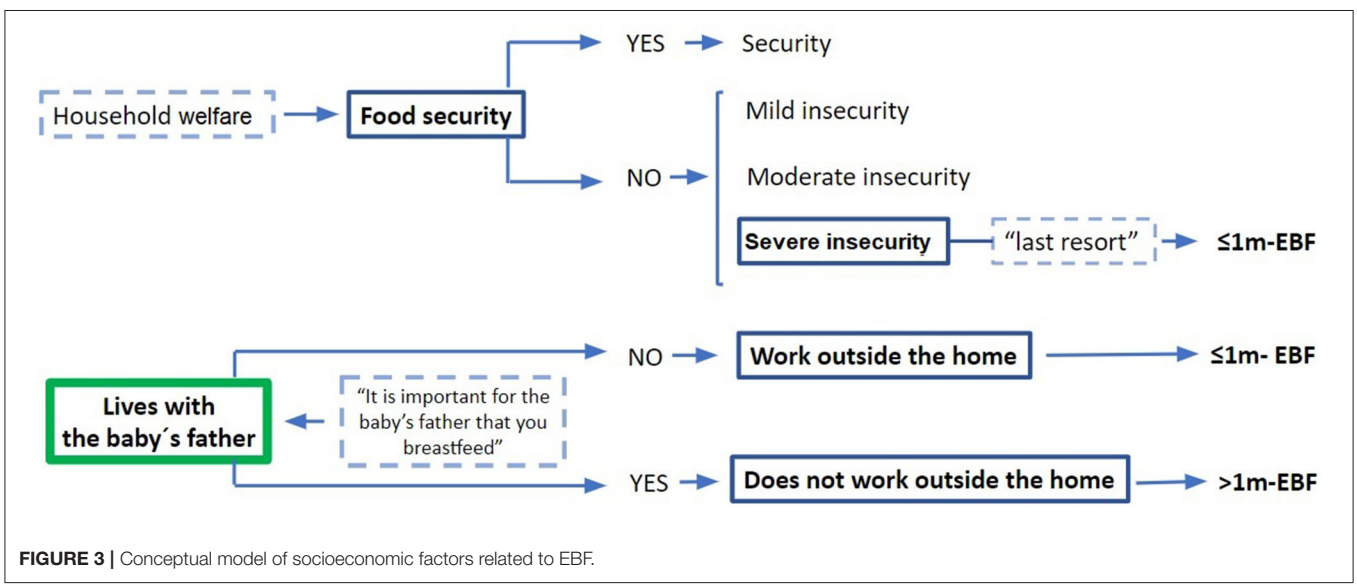

FIGURE 3 | Conceptual model of socioeconomic factors related to EBF.

position the infant on the breast. Neither prenatal care attendance nor the various types of received information were statistically different between the ≤1 m-EBF and >1 m-EBF groups.

None of the participants reported smoking and only 2.1% ($n = 3$) drank alcohol at the time of the study visit.

Regarding pregnancy resolution, most women (85.3%, $n = 122$) gave birth at the High Speciality Regional Hospital for

TABLE 2 | Reproductive history and last pregnancy factors.

	Total	≤1 m-EBF ($n = 63$)	>1 m-EBF ($n = 80$)	*p*
Reproductive history				
Has at least one previous liveborn baby	86 (60.1%)	31 (49.2%)	55 (68.8%)	**0.02**
Previous baby				
Any breastfeeding	72 (83.7%)	24 (77.4%)	48 (87.3%)	0.23
No previous BF or early EBF termination	98 (68.5%)	51 (81.0%)	47 (58.8%)	**< 0.01**
Overall BF duration (months)	8.50 (1.75 - 15.5)	7.0 (1.0 - 14.0)	12 (4.0 - 18.0)	0.19
Satisfied with BF experience (yes)	22 (25.6%)	9 (29.0%)	13 (23.6%)	0.58
Current pregnancy				
Planned pregnancy (yes)	65 (45.5%)	30 (47.6%)	35 (43.8%)	0.64
First prenatal care visit before 8 wk gestation	69 (48.3%)	27 (42.9%)	42 (52.5%)	0.25
At least five prenatal care visits	111 (77.6%)	47 (74.6%)	64 (80.0%)	0.44
Received information about EBF until 6 mo	65 (45.5%)	32 (50.8%)	33 (41.3%)	0.25
Pregnancy complications (GDM, HDP)	22 (15.4%)	5 (7.9%)	17 (21.3%)	**0.03**
Delivery mode (cesarean section)	30 (21%)	19 (31.1%)	11 (13.4%)	**0.01**
Maternal BMI	26.42 ± 5.49	27.61± 6.03	25.49 ± 4.88	**0.02**

GDM, gestational diabetes mellitus; HDP, hypertensive disorder of pregnancy. Data shows number of cases (%), mean ± s.d. or median (p25–p75). We compared continuous variables between groups using Student's t-test or Mann-Whitney's U-test, and categorical variables using Chi squared test.
Bold values indicate statistically significant differences ($p < 0.05$) between study groups.

TABLE 3 | Newborn characteristics.

	Total	≤1 m-EBF ($n = 63$)	>1 m-EBF ($n = 80$)	*p*
Girls ($n = 143$)	76 (53.1%)	34 (54.0%)	42 (52.5%)	0.86
Gestational age (weeks) ($n = 140$)	40 (38 - 42)	41 (39 - 42)	40 (38 - 42)	**0.03**
Premature ($n = 140$)	5 (3.6%)	1 (1.6%)	4 (5.1%)	0.38
Weight at birth (kg) ($n = 140$)	3.10 (2.81 - 3.50)	3.12 (2.90 - 3.47)	3.10 (2.80 - 3.50)	0.52
Length at birth (cm) ($n = 80$)	50.0 (48.0 - 52.0)	50.5 (49.0 - 52.0)	49.0 (48.0 - 51.0)	0.07

Some answers were not included because they were not plausible or the woman did not remember. Data shows number of cases (%) or median (p25–p75). We compared continuous variables between groups using Student's t-test or Mann-Whitney's U-test, and categorical variables using Chi squared test.
Bold values indicate statistically significant differences ($p < 0.05$) between study groups.

Women in Villahermosa City; a minority gave birth at another public hospital (4.9%, $n = 7$), or a hospital ran by the Mexican Institute of Social Security (5.6%, $n = 8$), or a private clinic (2.1%, $n = 3$) or at home (2.1%, $n = 3$). Likewise, most births were attended by a health professional (97.9%, $n = 140$), and required short hospital stays (median 24 h, p25–p75 17.50–39.00). There was no difference between ≤1 m-EBF and >1 m-EBF groups regarding birthplace ($p = 0.58$), birth attendants ($p = 0.42$), and hospitalization length ($p = 0.32$).

Table 3 shows the comparison of newborn characteristics between ≤1 m-EBF and >1 m-EBF groups. Regarding infant sex, gestational age, prematurity, weight and length at birth, only gestational age at birth was different between study groups.

Figure 4 shows breastfeeding related factors during participants' hospitalization. Most women initiated breastfeeding within the 1st hour after giving birth, were roomed in with their babies and perceived sufficient milk production; there was no difference between groups on these variables. In contrast, significantly more women in the ≤1 m-EBF group experienced pain or discomfort on their breasts and/or nipples, gave their newborn a liquid food other than her own milk and used a nipple or pacifier during hospital stay.

There was an important correlation between the variable "gave liquid other than her own milk" and variables "used a nipple or pacifier" ($r = -0.49$, $p < 0.01$) and "had pain or discomfort in breasts/nipples" ($r = 0.35$, $p < 0.01$); therefore, we did not include the last two variables in the logistic regression model. Also, the variable length-at-birth was not included due to the large number of missing data.

Between birth and the study visit, more women in the >1 m-EBF had attended the CESSA or FLHU at least three times (87.5%, $n = 70$ vs. 76.2%, $n = 48$; $p = 0.07$), primarily to receive vaccination for their baby (95.8%, $n = 137$). Around a fifth of the women in our sample recalled receiving breastfeeding information/support during their postpartum visits to the health facilities (≤1 m-EBF 17.5%, $n = 11$ vs. >1 m-EBF 21.2%, $n = 17$; no difference between groups). When the women who received

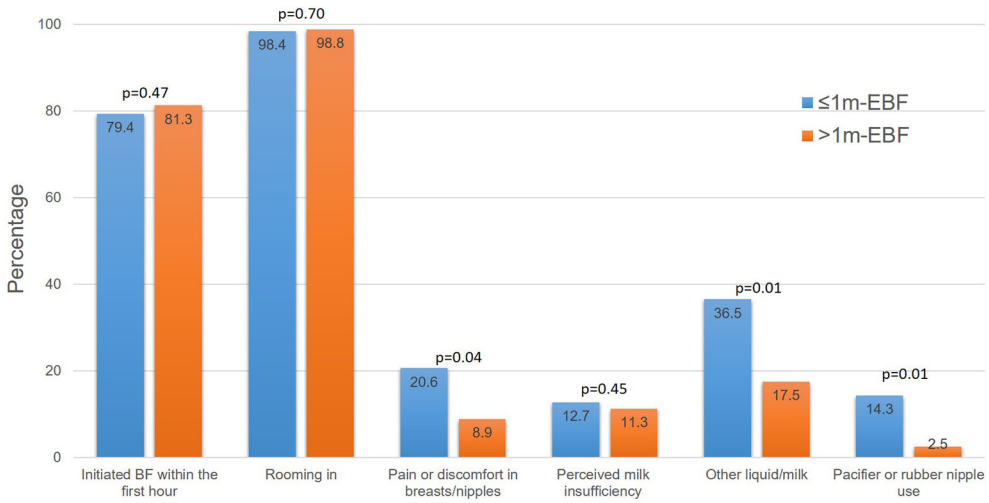

FIGURE 4 | Breastfeeding practices during hospitalization. Groups were compared using the chi-square test.

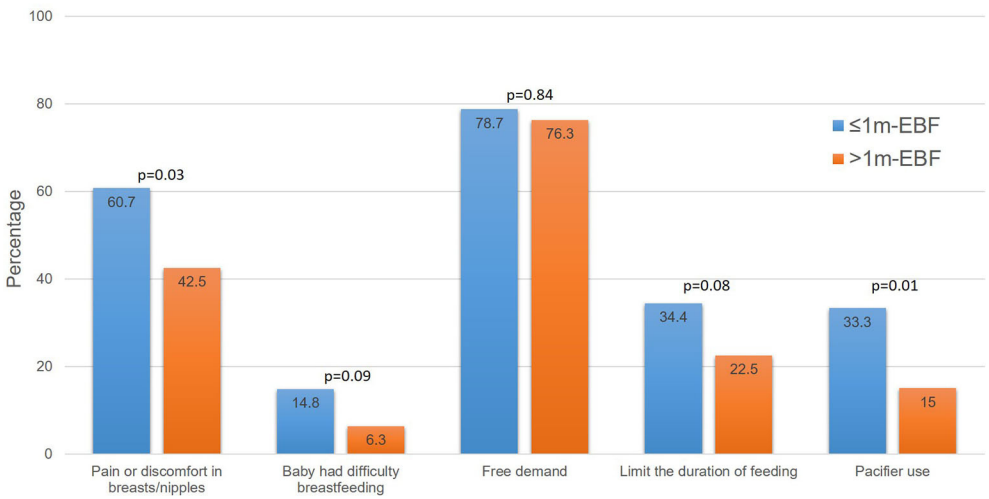

FIGURE 5 | Breastfeeding during early postpartum. Groups were compared using the chi-square test.

information, was asked about the type of information they had received, more women in the >1 m-EBF (53.5%, $n = 15$ vs. 21.4%, $n = 6$; $p = 0.06$) recalled correct information such as the importance of EBF until the baby turned 6 months or breast massages to alleviate breast discomfort. However, women also remembered erroneous information such as to offer formula milk if the baby remained hungry after a feed, or to give clean drinking water if the baby was thirsty.

More than half of our study population (61.5%, $n = 88$) also attended other health facilities besides CESSA or FLHU; most of them went to a private physician (45.5%, $n = 40$) or a pharmacy (52.3%, $n = 46$), mainly because of child's sickness. At these alternative facilities, 23.1% of participants ($n = 33$) received information about breastfeeding; unfortunately, we didn't ask what kind of information they received. Study groups were not different considering postpartum visits to health facilities or received information.

Figure 5 shows breastfeeding practices after leaving the hospital. Most of the study participants practiced on demand breastfeeding. More women in the ≤1 m-EBF group limited the time they let their infant suck at their breast, reported their baby had difficulties to latch and breastfeed correctly, experienced breast and/or nipple pain, and gave a pacifier to their infants.

More women in the L-EB group stopped breastfeeding due to an illness (18.8%, $n = 15$ vs. 7.9%, $n = 5$; $p = 0.06$). The ailments they presented were common infections, like cold, vaginal or tooth infections (6.3%, $n = 9$); mosquito transmitted viral diseases such as dengue or chikungunya (4.2%, $n = 6$), herpes zoster (not on the mammary gland, 0.7%, $n = 1$), anemia (0.7%, $n = 1$) or colitis (0.7%, $n = 1$). None of these illnesses

TABLE 4 | Logistic regression models for factors associated with L-EBF.

	Model 1 Sociodemographic characteristics		Model 2 Global model	
	OR (95% CI)	***p***	**OR (95% CI)**	***p***
Household food security				
Secure	1	**0.03**		
Marginally insecure	2.08 (0.88 - 4.96)	0.09		
Moderately insecure	2.45 (0.82 - 7.30)	0.10		
Severely insecure	9.93 (2.09 - 47.26)	**0.01**		
Lived with the baby's father (yes)	4.93 (1.58, 15.37)	**<0.01**	3.83 (1.09, 13.37)	**0.03**
GDM or HDP (yes)			6.32 (1.41, 28.27)	**0.02**
No previous BF experience or EBF < 1 mo			0.35 (0.12, 1.02)	0.05
Maternal BMI at the time of study			0.91 (0.84, 0.98)	**0.02**
Received other liquid in the hospital (yes)			0.32 (0.11, 0.92)	**0.03**
Vaginal delivery			3.21 (1.03, 9.93)	**0.04**
Attended health center at least three times postpartum (yes)			3.24 (1.06, 9.89)	**0.04**
Had pain or discomfort in breasts/nipples after hospital discharge (yes)			0.31 (0.12, 0.80)	**0.01**
Limits the duration of the feed (yes)			0.37 (0.13, 1.01)	0.05
Pacifier use after hospital discharge			0.31 (0.10, 0.94)	**0.04**
"*When the baby is not full, you should give her/him powdered milk or some other food, even if she/he is <6 months old*" (agree)			0.22 (0.0.08, 0.55)	**<0.01**

Model 1. Sociodemographic characteristics: variables not included in the model: occupation (stay-at-home mother or work away from home).
Model 2. Global model variables not included in the final model: household food security, occupation, gestational age and baby had difficulty breastfeeding.
In both models, the predicted probability is for being in the L-EBF group.
Bold values indicate statistically significant differences ($p < 0.05$) between study groups.

contraindicated breastfeeding. It is interesting to note that most of the participants who stopped, did resume breastfeeding upon recovery (15%, $n = 12$ vs. 3.8%, $n = 3$; $p < 0.01$).

Regarding the participants' thoughts about formula milk and breastfeeding, more women in the ≤1 m-EBF group agreed with the phrases "*Formula milk is an important food to accompany breast milk before 6 months*" (66.7%, $n = 42$ vs. 37.5%, $n = 30$; $p < 0.01$) and "*When the baby is not full, you should give her/him powdered milk or some other food, even if she/he is <6 months old*" (66.7%, $n = 42$ vs. 42.5%, $n = 34$; $p = 0.02$). There was no difference between groups in the proportion of women who agreed with the phrases "*When you finish breastfeeding, you are always sure that your baby under 6 months is full*" (58.7%, $n = 37$ vs. 70.0%, $n = 56$; $p = 0.37$) and "*I am convinced that giving only breast milk until the baby is 6 months old, without giving any other food, is the best for her/him*" (60.3%, $n = 38$ vs. 72.5%, $n = 58$; $p = 0.30$).

There was an important correlation between the variable "*When the baby is not full, you should give her/him powdered milk or some other food, even if she/he is <6 months old*" and variables "*Formula milk is an important food to accompany breast milk before 6 months*" ($r = 0.413$, $p < 0.01$). Therefore, we did not include the last variable in the second logistic regression model.

To explore the interaction of those factors most strongly associated with >1 m-EBF, we performed a second logistic regression model including maternal/infant factors that resulted statistically significant in the bivariate analyses and adjusted for sociodemographic variables as confounders (model 2 in **Table 4**). When such interaction was considered, household food security was no longer significantly associated with >1 m-EBF. Overall, women who lived with the baby's father, had complications during pregnancy, delivered vaginally or attended a health center at least three times postpartum were more likely to practice >1 m-EBF. Conversely, women with larger bodies (i.e., higher

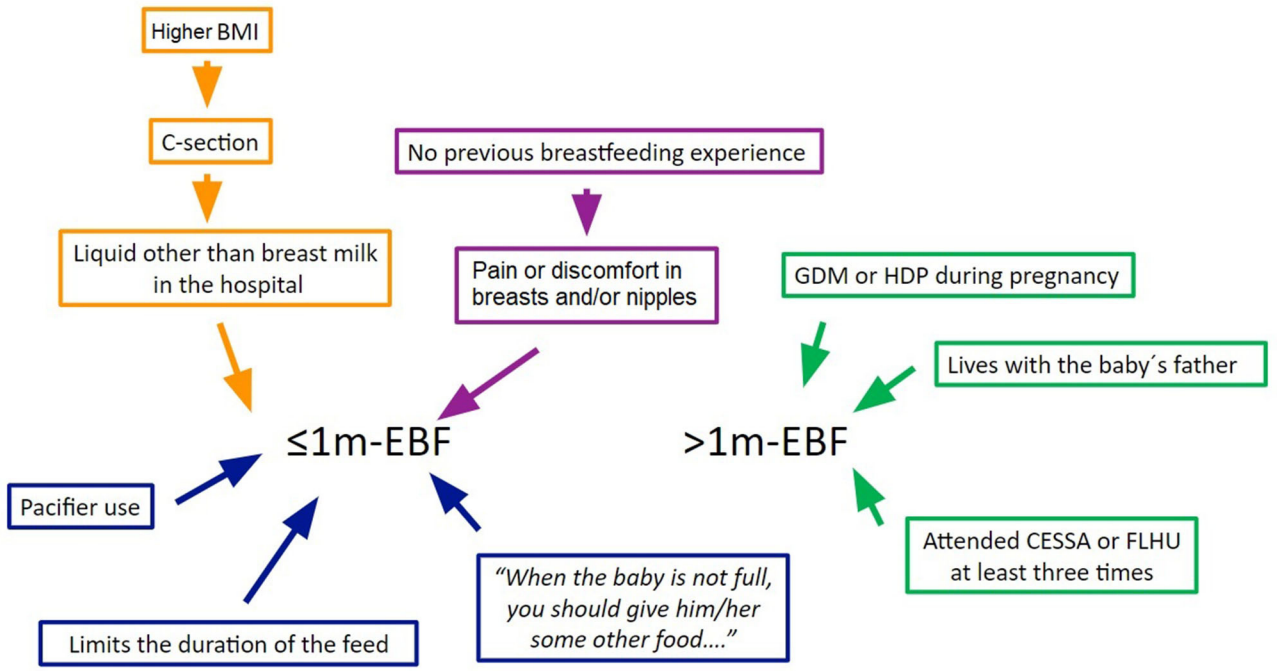

FIGURE 6 | Conceptual model of maternal / infant factors related to EBF. ≤1 m-EBF = up to 1 month exclusive breastfeeding; >1 m-EBF = beyond 1 month exclusive breastfeeding; GDM, gestational diabetes mellitus; HDP, hypertensive disorder of pregnancy.

BMI); who gave other liquids during their hospital stay; who had pain in their breasts or used a pacifier after hospitalization; and who believed that you should give the infant powdered milk or some other food when the baby is not full, were less likely to practice EBF beyond 1 month. Having no previous BF experience and limiting the duration of the feed marginally reduced the likelihood of >1 m-EBF.

Lastly, in order to illustrate how the variables in our global model may influence >1 m-EBF, and propose a conceptual model, we analyzed the association between them (**Figure 6**). We found that less than half (20.4%, $n = 23$) of the infants delivered vaginally received a liquid different to breast milk as opposed to infants delivered by C-section (46.7%, $n = 14$) ($p = <0.001$) ($r = 0.24$, $p < 0.01$). Compared to women with no previous BF experience (59.4%, $n = 57$), fewer women who had breastfed before (31.1%, $n = 14$) suffered pain or discomfort in breasts and nipples ($p < 0.01$) ($r = -0.26$, $p < 0.01$). More women who agreed that "When the baby is not full, you should give … other food …" gave her infant a pacifier (30.3%, $n = 23$) as opposed to those who did not agree (14.9%, $n = 10$) ($p = 0.02$) ($r = -0.18$, $p = 0.04$). Women who delivered by C-section had higher BMI than those delivering vaginally (28.57 ± 5.34 vs. 25.85 ± 5.42, $p = 0.01$). Similarly, women that gave their infants liquids different to breast milk during hospitalization had higher BMI than those who did not (28.58 ± 6.39 vs. 25.67 ± 4.96, $p < 0.01$). Finally, women with no previous BF experience had lower BMI (25.15 ± 6.14 vs. 27.26 ± 4.88, $p = 0.02$); however this association was not taken into account in the conceptual model since many women in the inexperienced group were primiparas and parity is associated with weight retention and increased BMI (12).

DISCUSSION

The results of our work show that very early abandonment of EBF during the 1st month postpartum is a common practice in the community; by the end of the 1st month, half of the women had stopped practicing it. A previous study held in the Mexican states of Puebla and Chihuahua, using the WHO status quo EBF indicator, also documented that by the end of the 1st month, 55.2% of women had stopped EBF their infants (13). However, this trend may not be clearly appreciated from the national data obtained in surveys, since the gross indicator of "exclusive breastfeeding under 6 months" which is suggested by the World health Organization (WHO) and UNICEF aggregates all infants 0–5 months to calculate the percentage who were exclusively breastfed the day previous to the survey.

It is very relevant that such a large proportion of women in these communities decide to stop EBF from such an early stage, so our findings contribute to understanding which factors are associated with this inadequate practice in order to design and target breastfeeding interventions at the community and individual levels.

In our study there were four sociodemographic factors associated with exclusive breastfeeding beyond the 1st month of life: the mother "living with the infant's father," living in a household with low welfare level or with food insecurity,

and the mother staying at home (instead of going out to work). However, when the first logistic regression was performed, occupation was dismissed from the model, "living with the baby's father" and living in a severely insecure household increased the odds to breastfeed beyond the 1st month. In the global logistic regression model, the only sociodemographic variable that explained EBF beyond 1 month was "living with the baby's father." The conceptual model (**Figure 3**) suggests pathways in which these factors influence EBF duration.

Regarding the association between EBF duration and household food insecurity, it has been documented that households with the lowest welfare and incomes, which also tend to have members with the lowest rates of education and access to goods and services, are often more likely to be food insecure (14–16). These households would particularly benefit from a longer duration of EBF in two ways: firstly, because of its protective role on the nutritional, physical and emotional health of both infant and mother; secondly, to avoid expenses that the family would have to make to face an illness: visits to the health services, medicines, as well as the indirect costs caused by the absenteeism of the parents to care for the sick child. It would also protect the household's economy by avoiding the purchase of breast milk substitutes (15, 17, 18).

In our study, severe food insecurity was an independent predictor of EBF practice beyond the 1st month of life, even when other sociodemographic factors were taken into account. The "positive" effect of food insecurity on breastfeeding indicators, such as BF initiation and duration, has been observed before (19, 20). In particular, the influence of extreme food insecurity on EBF was documented through qualitative data in a study held in Haiti (21, 22). Some mothers continued BF when they could not afford to buy other foods for their infants, i.e., formula milk or other complementary foods. These women continued with EBF not by decision but as a "last resort." forced by not having money to give them something else.

However, when food insecurity was not severe, we found no association with EBF beyond the 1st month postpartum. This may be the result of the coincidence of positive and negative effects of food insecurity on EBF that nullified any observation. Previous studies have documented some beliefs associated with food insecurity that may stimulate a woman's decision to stop EBF or any type of BF. For example, when there is not enough food for the woman to eat, her concern is that her milk would be insufficient and of low quality (with not enough nutrients) to adequately nourish her infant (21, 23). Another concern is that negative emotions induced by food insecurity such as stress, could either pass to the infant during breastfeeding, and affect his/her appetite or mood; or directly reduce the woman's milk production, becoming insufficient for her baby (23).

Regarding maternal occupation, work outside home has been linked to the reduction of EBF (21, 24, 25). In Mexico, women working in the formal sector have paid maternity leave for 12 weeks in total, divided in two 6 weeks periods, before and after birth. And with prior authorization, up to 4 of the 6 weeks off before delivery can be transferred to the postpartum period. This means that they can spend between 1.5 and 2.5 months postpartum at home (26); however this time is still not long enough to protect breastfeeding (3). Women working in the informal sector, that is, women who are self-employed or have a non-salary contractual arrangement, are not entitled to a paid maternity leave (27). Most women in our study belonged to this informal economic sector (93.7%) and therefore lacked income security during lactation. These women belong to the most socioeconomically disadvantaged sectors, and possibly face the need to return to work early (21). Concomitantly, women in these circumstances may experience unfavorable work conditions that have been found to interfere with EBF breastfeeding. For example, lack of job flexibility, long commute time to work, lack of support, no guaranteed scheduled breaks or a suitable facility to breastfeed or extract and store milk (15, 28, 29).

In our study, we found that living with the infant's father increased the probability of EBF beyond the 1st month of life. This positive influence of a partner or spouse to BF practices has also been observed in previous studies (13, 30). The role of the father might be related to the practice of EBF, in at least in two ways: first, the woman's perception about her partner's attitudes and beliefs about BF may influence her own attitudes and decisions about their child's feeding. Second, by the emotional and instrumental support he provides to the BF woman. For example, giving praise and encouraging compliments and by making the mother feel comfortable and giving practical assistance in household chores (31, 32). Although we did not look at the kind of support the fathers might have given to the women in our study, one interesting observation was that "living with the baby's father" was the only significant sociodemographic variable in the global model. This suggests that the father's presence in the household may be protective of the more severe form of food insecurity and possibly reduce, or at least postpone, the woman's need to go back to work (33), in turn having a positive effect on EBF.

We were able to identify several maternal and infant factors associated with the duration of EBF, from non-modifiable pregnancy and hospitalization conditions beyond women's control, to modifiable practices and beliefs. Based on the associations between these factors, we were able to propose a conceptual model to describe their interactions (**Figure 6**).

In our study population, women whose last pregnancy was complicated by GDM or a hypertensive disorder were more likely to practice EBF beyond 1 month. This was an unexpected result since other studies have shown that women with GDM are less likely to practice EBF or, if they do, it is usually for shorter periods than other mothers (34).

However, this result could suggest that, due to their condition, those women may have received special information, either during their prenatal care or postpartum visits, on the benefits of EBF on diabetes and hypertension. Indeed, BF has been shown to improve the glycemic status of women with previous GDM through lowered rates of impaired glucose tolerance and lower fasting plasma glucose (35). Such women have a lower risk of developing type 2 DM than those who had GDM but did not breastfeed (36, 37). Similarly, BF is associated with a lower risk of maternal hypertension, especially when practiced for more than 1 month (38), and exerts a protective effect against

migraine attacks, a condition closely related to hypertensive disorders (39).

Although we asked participants whether they received information during their prenatal care about the benefits of BF, we did not delve into the details of such information and therefore cannot know if EBF was mentioned as a protective factor against DM or hypertensive disorders. However, since Mexican law requires health professionals to closely monitor women with such complications during and after pregnancy (40), it is likely that participants may have received related information during their prenatal care.

Regarding the mothers' previous experience with EBF, this variable was significantly associated with EBF beyond 1 month. Maternal previous experience has also been shown to influence both the intention and the actual practice of BF in other populations (41–45). However, in our study, maternal previous experience was marginally associated with >1 m-EBF when the interaction of other early postpartum and breastfeeding factors was taken into account in the logistic regression models. Our results in this regard may suggest that while previous experience may predispose the mother toward a positive intention to adequately BF, it may be outweighed by the challenges she endures in her current BF practice; we will discuss this further ahead.

It is interesting to notice that the information about EBF that women received during their prenatal care was not associated with the duration of EBF. Considering this in conjunction with the fact that living with the baby's father and his interest in BF were indeed related with >1 m-EBF, it may seem that the attitudes and information provided by the mother's close social group may be more influential in her BF practice than what health professionals may tell her. In this regard, Humphreys et al. reported that low-income women in the southwest USA were less influenced in their infant feeding decisions by health professionals' attitudes than by the attitudes and beliefs of members of their social support networks, including family members, the baby's father, and lactation consultants (44). Further studies about the structure and dynamics of the social networks in particular contexts may provide new insights into the women's influences and beliefs, as well as allowing for the design of interventions that target not only the mother but also key members of her social group.

Women whose infants received liquids other than breastmilk during their hospital stay were also less likely to practice >1 m-EBF. While studies usually focus on the introduction of liquids during the 1st months of infants' lives, few report on this practice during the neonatal hospital stay. The only other study we have found reporting this practice was carried out in Istanbul, Turkey, where researchers found a similar negative association between introducing formula milk during the hospital stay and duration of EBF (46).

Providing neonates with liquids other than their mother's breast milk during hospital stay, including formula milk or breastmilk from a milk bank, is only indicated when either infant or mother courses through a medical condition that limits their capacity to breastfeed. These include infant metabolic diseases, extreme prematurity, maternal HIV/hepatitis/herpes infections or substance abuse, or undergoing an emergency medical procedure (47). Nevertheless, a quarter of the infants in our study were given liquids other than breastmilk during their newborn hospital stay, although none of the participants or their babies had any condition that limited BF and maternal/infant hospitalization was a non-inclusion criteria. Therefore, there was no clinical reason for giving infants liquids other than breastmilk during their neonatal hospital stay.

In our study the most commonly given liquid during hospital stay was formula milk (81%), followed by milk from the bank (11%) and sweetened water (dextrose solution, 8%). The inadequate practice of giving newborns formula milk in hospitals has also been documented in other parts of Mexico, both urban and rural (13), as well as in other countries (48–50). Giving newborns dextrose solution while in the hospital has been described as a common practice in Mexican rural communities since the late 1980's (51). Unfortunately, we did not ask the mothers the reasons why their infants were given liquids. It would be important to document the prevalence of such practices and their justifications not only among mothers but also among medical and nursing staff as well, particularly in hospitals that may not comply entirely with international guidelines such as WHO's Baby-friendly Hospital Initiative.

Women with larger bodies (i.e., higher BMI) at the time of the study visit were less likely to practice EBF beyond 1 month, a finding in accord with previous reports (52, 53). Since higher maternal BMI or higher fat mass percentage have been associated with shorter duration of EBF and delayed lactogenesis (53), physical, hormonal or socio-cultural factors are commonly proposed to explain such correlations (54). Physical concerns are usually about women with large breasts having difficulty adopting an adequate BF posture (55). Hormonally, women with large bodies have a lower prolactin response to suckling in the 1st days postpartum, but by day 7 their response is not different from those with smaller bodies, and serum progesterone diminishes equally in all of them (56). Finally, women with large bodies/breasts may have socially related concerns or body image problems that could limit their intention to BF (57).

But none of these interpretations for the association between shorter duration of BF and maternal BMI (breast size, adiposity, body image) holds true only for women with large bodies. Indeed, women who fall into the "normal" BMI category and have large breasts may face the same challenges with BF due to posture difficulties or delayed lactogenesis than women with larger bodies. Similarly, women who may be classified as having an adequate or even low BMI but who have body image problems may also choose not to BF or do so for shorter periods. However, in none of these cases would the women's body size (BMI) would be considered a barrier nor would they be encouraged to lose weight or diminish their weight gain, which is the traditional approach for women with larger bodies who are classified as overweight/obese based on BMI.

Furthermore, body size has also been shown to be related with socioeconomic conditions, both at the individual and country levels. In some social settings, larger bodies may reflect wealth or higher social status as body size is differentially related

with various dimensions of individual socioeconomic level (e.g., income, education) as well as with country's level of development (58). Also, providing infants with milk substitutes (e.g., formula milk) is associated with wealth and may be considered a desired social trait (59).

When such a complex interrelation of bio-psycho-social factors related to body size is taken into account, it becomes clear that maternal body size (i.e., BMI) in itself may not be the true barrier to EBF. Therefore, in order to promote EBF, the rather simplistic focus on maternal BMI and weight reduction would not be an adequate approach and may only be reflecting weight stigma (60). Research is needed in order to distinguish, for example, between obesity and large breasts as separate challenges for breastfeeding (61). Maternal body size should not become a barrier for practicing EBF, when women with large bodies are provided with adequate, compassionate, non-biased counseling and accompanying, especially in the 1st days postpartum (55, 61, 62).

Similar to our results, several studies have documented that delivery mode influences breastfeeding rates (63). For example, a study held in China documented that infants delivered by C-section had lower breastfeeding rates from 1 to 6 months and were more likely to receive formula milk (64). However, when other variables concerning early feeding difficulties were considered, such as delayed initiation and weak suction consequence of anesthesia, the effect of C-section was attenuated or disappeared. Authors concluded that C-section *per se* was not a negative factor, but rather the difficulties that arise after the procedure. It should be noted that such difficulties are susceptible to breastfeeding counseling in order to reverse their negative effect on BF.

With regard to early postpartum, breastfeeding factors and beliefs, it is striking that, despite the fact that most of the women in our study did return to CESSA, FLHU, or other health facilities more than once during early postpartum, few reported receiving information and support regarding breastfeeding and some recalled receiving incorrect information. This has already been documented in a previous study in low-resource urban and rural populations from the Mexican states of Queretaro and Oaxaca (65). Indeed, participants who attended health facilities three or more times during early postpartum were more likely to practice >1 m-EBF. This could be interpreted in different ways that unfortunately we were not able to explore further due to our study design: women may have received more information and/or counseling during such visits; or they may comprise a highly motivated subgroup; or they may have easier access to health centers (e.g., living closer).

Another important thing to note is the fact that many women also attend private clinics and pharmacies when their child is ill, where they too receive infant feeding information. It has been documented both in Mexico and other countries that the promotion of breastmilk substitutes is a common practice in such facilities (8, 66, 67). For example, in a study in the Mexican states of Puebla and Chihuahua, authors documented that, when attending public and private health facilities, in 48.4% and 40.7% of cases, respectively, mothers of children younger than 24 months were recommended to give them a breast milk substitute. Researchers also found advertisements, discounted prices, promotional items, and even free samples for these foods in pharmacies (8).

After leaving the hospital, most participants in our study practiced on demand breastfeeding. However, more women in the ≤1 m-EBF group reported limiting the duration of the feeding, having latching difficulties, experiencing breast or nipple pain, and using a pacifier. These practices have been reported to reduce the duration of EBF (68, 69). Additionally, while maternal/infant illness has also been reported as a barrier for EBF (70, 71), it is important to note that the majority of participants in our study who stopped BF due to some illness, resumed the practice upon recovery.

After considering all the analyzed factors and their relationships, we were able to propose a conceptual model that illustrates their association with the duration of exclusive breastfeeding. The model (**Figure 6**) distinguishes between non-modifiable factors, which are represented in the upper part, that comprise situations mostly out of the woman's control. However, other factors result from the mother's immediate practices and beliefs; these are represented in the lower half of the conceptual model. Such factors would be central to the design of community-tailored interventions since they can be modified through appropriate information and counseling. It should be noted that women who present the non-modifiable factors should also receive close monitoring and counseling in order to overcome the intrinsic difficulties that would come with presenting those factors.

As stated above, the correct type and amount of information must be provided at prenatal care facilities to mothers and members of their inner social network alike, while ensuring its adequate comprehension and integration. During hospitalization and the 1st days/weeks after delivery, assertive personal counseling and accompaniment must be provided to mothers, irrespective of their individual conditions (e.g., previous BF experience, body size). And both information and counseling should be reinforced during the early postpartum, whether at health facilities or other settings. The higher aim is to translate this knowledge and awareness into more effective interventions that, when tailored to specific socio-cultural contexts, prove effective for increasing the duration of EBF.

Study Limitations and Strengths

There are some limitations to our study. Since we did not use the WHO EBF indicator which asks about feeding practices the day prior to the interview and because our study has a cross-sectional design, several limitations arise: (1) caution must be used when comparing our information to others obtained using the WHO EBF indicator; (2) we cannot make causal claims about the relationship of the studied factors and EBF; and (3) we must consider a possible recall bias, specifically about the precise moment in which participants stopped EBF. However, regarding the latter, estimates of breastfeeding duration by maternal recall have been found to be reliable and valid during the first 3 years of the child's life (72).

It must also be kept in mind that we defined in our study >1 m-EBF as breastfeeding > 1 month postpartum, but this

was defined in dependence upon our study population's practices and by no means can be considered long breastfeeding practice according to international recommendations.

Furthermore, due to the study design and inclusion criteria, our sample of participants had low risk of abandoning BF and was quite homogeneous in their sociodemographic characteristics. Thus, our results cannot be generalized to populations living in other socioeconomic settings or circumstances. However, it can give an idea of what happens with similar populations, which are of low-income, semi-rural and beneficiaries to the most basic government social security.

A strength of our study is that the sample showed a very similar prevalence of food insecurity as that reported for México. Data from the 2016 national health and nutrition survey documented that 70% of the population classified as having some degree of food insecurity, and that 29.5% classified in the categories of moderate and severe food security (14). Another similarity is the proportion of household welfare level distribution in our study, which was very similar to the percentages reported for all households in Tabasco during the year of the study (73).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research and Ethics Committees of the National Institute of Perinatology in Mexico City. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

IV-O: investigation, methodology, data curation, and writing—review and editing. RV-S: formal analysis, writing—original draft, and review and editing. EM-M: investigation, data curation, and writing—review and editing. SH: conceptualization and writing—review and editing. MF-Q: conceptualization, methodology, formal analysis, supervision, writing—original draft, and review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.826295/full#supplementary-material

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