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Jinyue Yu , Yan Zhang , Jonathan Wells , Zhuang Wei , Mona Bajaj-Elliott , Dennis Sandris Nielsen , Mary Fewtrell *

Posted Date: 7 March 2024

doi: 10.20944/preprints202403.0413.v1

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A Stress Reduction Intervention for Lactating Mothers Alters Maternal Gut, Breast Milk, and Infant Gut Microbiomes: Data from a Randomized Controlled Trial

Jinyue Yu [1], Yan Zhang [2], Jonathan CK Wells [1], Zhuang Wei [3], Mona Bajaj-Elliott [4], Dennis Sandris Nielsen [5] and Mary Fewtrell [1]*

[1] Childhood Nutrition Research Group, Population, Policy & Practice Department, UCL Great Ormond Street Institute of Child Health, London, UK.
[2] Microbiota Division, Department of Gastroenterology and Hepatology, The First Medical Center, Chinese PLA General Hospital, Beijing, China.
[3] Department of Child Healthcare, Beijing Children's Hospital，Capital Medical University，National Center for Children's Health, China.
[4] Infection, Immunity & Inflammation Department, UCL Great Ormond Street Institute of Child Health, London, UK.
[5] Department of Food Science, University of Copenhagen, Denmark
* Correspondence: Mary Fewtrell, m.fewtrell@ucl.ac.uk

Abstract: Background: This study explored maternal gut, breast milk and infant gut microbiomes as possi-ble mediators of the observed effects of a relaxation intervention which reduced maternal stress and promoted infant weight gain. Methods: A randomized controlled trial was conducted in healthy Chinese primiparous mother-infant pairs. Mothers were randomly assigned to interven-tion group (IG, listening to relaxation meditation) or control group (CG). Outcomes were differ-ences in microbiome composition and diversity in maternal gut, breast milk and infant gut at 1- and 8-weeks between IG and CG, assessed by 16S rRNA gene amplicon sequencing of fecal and breastmilk samples. Results: 38 mother-infant pairs were included in this analysis (IG=19, CG=19). Overall microbiome community structure in the maternal gut was significantly different between IG and CG at 1-week and the difference was more significant at 8 weeks (Bray-Curtis distance R2=0.04 vs. R2=0.13). Post-intervention, the α-diversity was significantly lower in IG breast milk (observed features: CG=295 vs. IG=255, p=0.032); the Bifidobacterium genera presented higher relative abundance. In parallel, significantly higher α-diversity was observed in IG infant gut (observed features: CG=73 vs. IG=113, p<0.001). Conclusions: The microbiome might mediate observed relaxation intervention effects via gut-brain axis and entero-mammary pathways; but confirmation is required.

Keywords: breastfeeding; gut microbiome; maternal stress; infant weight; mother-infant signaling

1. Introduction

Stress has an influence on the structure of the microbiota community in the gastrointestinal (GI) tract [1,2], potentially through the 'gut-brain axis' [3,4]. During lactation, the GI tract microbiome of mothers affected by stress could influence the milk microbiome via entero-mammary trafficking, which refers to the movement of microbiota from the maternal gut to the mammary gland [5–7]. Moreover, human breast milk contains bacteria including *Lactic acid bacteria* and *Bifidobacteria* [8] and several studies suggest that breast milk microbes influence the infant gut microbiome [9–11]. An emerging paradigm indicates that maternal psychological status could be associated with alterations in infant gut microbiome diversity [12] (eFigure 1, supplemental file).

The gut of the newborn infant is rapidly colonized by numerous microbes. The early life microbiome is known to influence infant development, and gut microbiome imbalances have been

 doi:10.20944/preprints202403.0413.v1

linked with an increased risk of certain autoimmune diseases [13]. The development of the neonatal gut microbiome can be influenced by multiple factors, including delivery mode (hence differential exposure to the maternal microbiome), feeding method, antibiotic intake in early life, stress and genetic factors [14,15]. Furthermore, the microbe and human milk oligosaccharide (HMO) content of human breast milk have a profound influence on early gut colonization of the infant [15–17].

We have shown in three randomized controlled trials (RCT) that a simple relaxation intervention significantly reduces psychosocial stress in breastfeeding women, whilst improving infant weight gain [18–20]. The present study analyzed data from one of these RCTs and investigated the role of the microbiome as a potential mediator of the observed intervention effects on weight gain in late preterm and early term infants (born at 34^{+0} to 37^{+6} weeks of gestation), a group that is relatively neglected compared to infants born at term or before 34 weeks. We used 16S rRNA gene sequencing to analyze the microbiome in the maternal gut (fecal), breast milk and infant gut (fecal) samples.

2. Materials and Methods

Details of the trial design and main outcomes have been published [18,21]. All mothers completed the 8-week data collection before COVID-19 lockdown in Beijing.

2.1. Study Design and Participants

Healthy primiparous mothers with a singleton infant born between 34^{+0}-37^{+6} weeks gestation who aimed to exclusively breastfeed (EBF) for at least two months were recruited 3-5 days after birth. Recruitment was conducted in community clinics affiliated to Beijing Children's Hospital in Beijing, China. Data collection was conducted through two home visits around 1-week and 8-weeks postpartum. To ensure consistency of procedures at each study center, all research assistants and nurses involved in the study attended training courses prior to the start of recruitment. Standard operating procedures for the study were printed and posted at each center. To control for the known effect of delivery mode on the infant microbiota, the present study only analyzed data from a subset of participants who delivered vaginally and provided maternal fecal, breast milk, and infant fecal samples at baseline (1-week) and 8 weeks home visits. The study was approved by the Research Ethics Committee of University College London (ID: 12681/002) and the Department of Child Health, Beijing Children's Hospital (ID: 2018-167).

2.2. Randomization, Procedures, and Intervention

After obtaining written informed consent, participants were randomly assigned to either intervention group (IG, listening to relaxation meditation at least once a day with standard postpartum care from local clinics) or control group (CG, standard postpartum care from local clinics). The randomization sequence was computer generated by an independent researcher, and stratified by gestational age (34-35 versus 36-37 weeks), delivery method (vaginal *versus* caesarean) and recruitment location. Assignments were stored in sealed, opaque envelopes at Beijing Children's Hospital. Participants were not told about the randomization until the end of the study; they were aware that the aim of the study is to investigate factors that could optimising breastfeeding outcomes. Nurses who collected the samples were aware of the groups; however, they had no particular interest or investment in the result of this study. The research technicians at Novogene who performed the 16s rRNA sequencing were blinded to the randomization status of the subjects.

2.3. Outcomes and Measures

Outcomes of the present study were differences in microbiome composition and diversity in maternal gut, breast milk and infant gut at 1- and 8-weeks between IG and CG, assessed by 16S rRNA gene amplicon sequencing of fecal and breastmilk samples.

Baseline characteristics of the mother-infant pairs were obtained using demographic questionnaires. The breast milk and maternal fecal samples were collected by mothers following the method in the published study protocol [18], mothers were asked to clean their areolar skin before

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collecting milk samples. Infant samples were collected by the nurse from infant diapers during home visits. All samples were collected into sterile tubes and stored at -80^{0}C at the laboratory of Beijing Children's Hospital. Samples for inclusion in the microbiome analysis were transported to the laboratory of Novogene Technology Inc. (Beijing, China) where the DNA extraction, library preparation, and the 16S rRNA gene amplicon sequencing was performed using standard procedures (eMethods in Supplemental file).

2.4. Statistical Analysis

Statistical analyses were conducted using R (version 4.12), and SPSS (version 26.0). We compared baseline characteristics, maternal stress and infant weight gain data between the 38 selected mother-infant pairs and the remainder of the 96 mother-infant pairs not involved in this analysis. We compared the diversity differences in maternal fecal, breast milk, and infant fecal samples between IG and CG at 1-and 8-weeks. The differences in α-diversity between IG and CG were examined by Wilcoxon rank-sum test using observed features. Differences in β-diversity between IG and CG were examined using Bray-Curtis dissimilarity metrics presented on a principal coordinates analysis plot (PCoA) and with differences between groups determined by ANOSIM. The relative abundance of the top 15 most abundant genera in all samples were examined and statistical differences between IG and CG samples were compared using the Wilcoxon rank-sum test and FDR adjusted p-value.

To determine the abundance of specific bacteria and potential associations with maternal stress and infant weight gain, Spearman-rank correlation was used, and results were presented in heatmaps. A standard p-value of < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Study Population and Baseline Characteristics

The original trial was conducted from October 2018 to October 2020. Of the 96 mother-infant pairs enrolled in the original trial, 38 eligible pairs were included in this secondary analysis (Figure 1, IG=19, CG=19); those 38 eligible pairs were recruited from February 2019 to January 2020. All mothers followed the traditional Chinese postpartum confinement practice during the data collection. There were no significant differences in baseline characteristics between those who were included in the microbiome analysis *versus* those that were not (eTable 1 in supplemental file). All 228 samples (38 maternal fecal, breast milk, and infant fecal samples at 1-and 8-weeks) were analyzed (Rarefaction curves are shown in eFigure 2). Distinct bacterial communities were observed in maternal gut, breast milk, and infant gut (eFigure 3 in supplemental file). Demographic characteristics of the participants are outlined in Table 1; there were no significant differences in participant characteristics between IG and CG at baseline.

Table 1. Baseline characteristics of the study participants.

Characteristics	**Total** N=38	**Control** N=19	**Intervention** N=19
	Mean (SD)		
Maternal age (yr)	31 (3)	30 (1.6)	31 (3.8)
Maternal education (yr)	16.0 (1)	15.5 (0.9)	16.4 (1.6)
Maternal BMI (kg/m^2)	23.3 (3)	22.6 (1.9)	24.0 (3.7)
Infant weight (kg)[a]	2.70 (0.3)	2.69 (0.3)	2.71 (0.4)
Infant length (cm)[a]	47.7(2)	47.5(2)	48.0(2)
	N (%)		
Gestational week			
34	1 (3)	0 (0)	1 (5.3)
35	4 (11)	2 (10.5)	2 (10.5)

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36	17 (45)	8 (42.1)	9 (47.4)
37	16 (42)	9 (47.4)	7 (36.8)
		N (%)	
Infant sex			
Male	16 (42)	7 (36.8)	9 (47.4)
Female	22 (58)	12 (63.2)	10 (52.6)
EBF			
1 week [b]	38 (100)	19 (100)	19(100)
8 weeks [c]	20 (52.6)	9 (57.9)	11 (47.4)
Use of antibiotics [d]			
During hospital stay	3 (7.9)	2 (10.5)	1 (5.3)
During 1-8 weeks	0	0	0

Notes: SD=standard deviation. N=number. BMI=body mass index. EBF=exclusively breastfeed, self-reported by mothers with definition provided on the questionnaire. [a] Weight and length were measured using standard anthropometry assessment at 1-week home visit. [b] Infant could receive expressed breast milk or formula initially but had to be EBF at 1 week enrolment. [C] Apart from those infants who were exclusively breastfed at 8 weeks, the rest of infants were mostly breastfed (breast milk >70% of the feeding). [d] Three mothers took antibiotics during hospital stay due to vaginal incision. Two mothers in control group took Cephalosporins and Amoxicillin respectively; one mother in intervention group took Cephalosporins. All mothers reported no medicine intake during the study period.

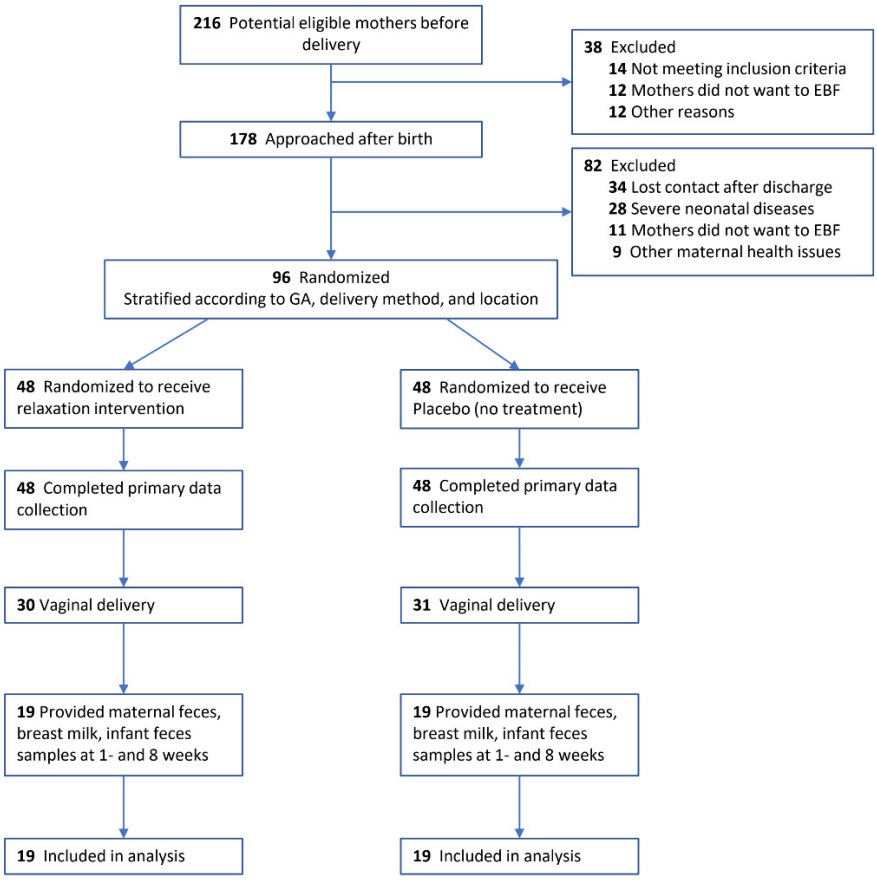

Figure 1. CONSORT Flowchart of the Randomized Controlled Trial. Notes: GA=gestational age, EBF=exclusive breastfeeding.

3.2. Microbiome Composition and Diversity in the Maternal Gut

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The number of observed bacterial taxa (observed features) in the maternal gut was not significantly different between IG and CG at both 1 and 8 weeks (Figure 2A & eTable 2).; moreover, the number of observed features did not significantly change between 1 and 8 weeks in either IG or CG. The overall composition of maternal gut microbiome (β-diversity) was significantly different between IG and CG at 1-week (Figure 2B, Bray-Curtis distance, R^2=0.04, p=0.026) as determined by Bray-Curtis dissimilarity metrics. However, the separation between IG and CG was stronger at 8 weeks (Figure 2C, Bray-Curtis distance, R^2=0.13, p=0.001).

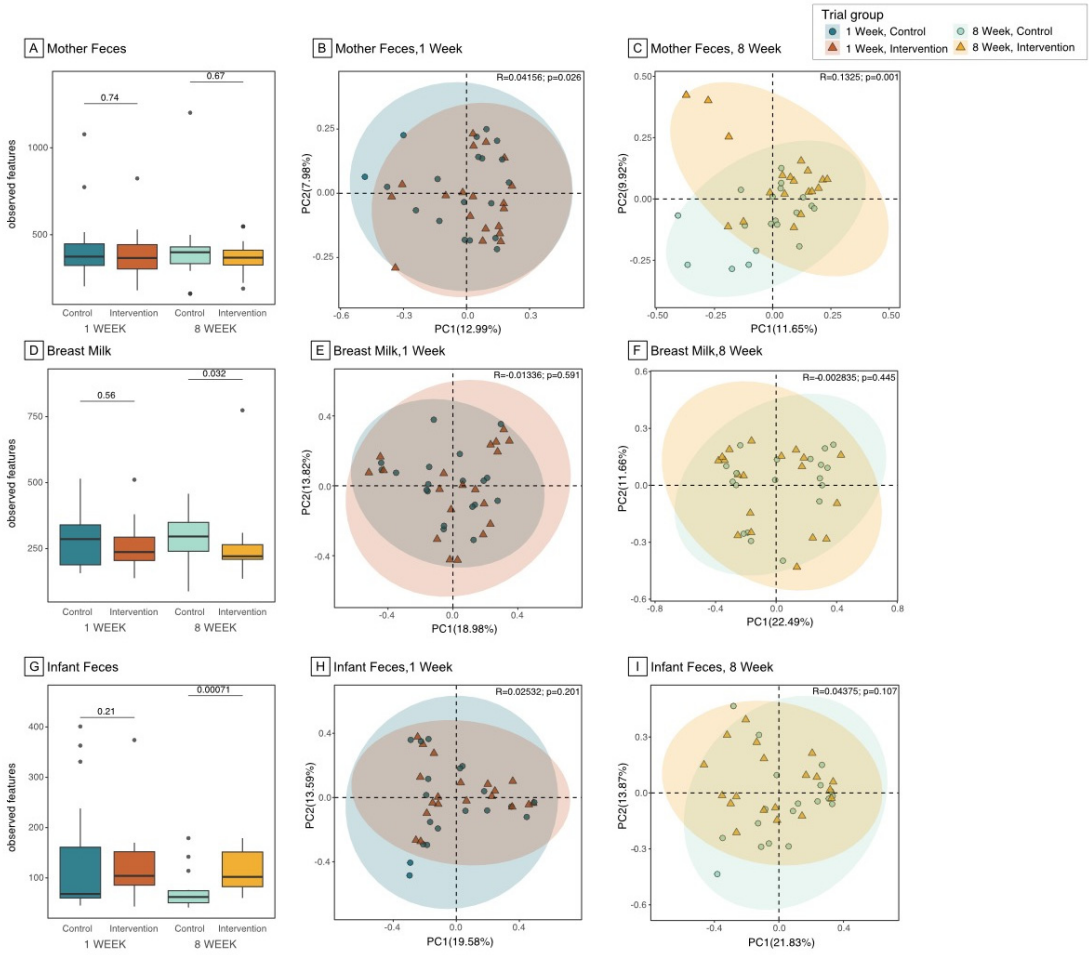

Figure 2. Microbiome Diversity in Maternal Gut, Breast Milk, and Infant Gut. Notes: Gut microbiome diversity was assessed by analyzing microbiome composition in fecal samples and breast milk. Differences in maternal gut between intervention and control group at 1 and 8 weeks were shown in A (α-diversity, within sample diversity), B and C (β-diversity, between sample diversity); differences in breast milk were shown in, D(α-diversity), E and F (β-diversity); differences in infant gut were shown in G (α-diversity), H and I (β-diversity). Difference in α-diversity was assessed by Wilcoxon rank-sum test based on observed features. Differences in β-diversity was presented using principal coordinates analysis plot (PCoA) based on Bray-Curtis distance matrix.

3.3. Microbiome Composition and Diversity in Breast Milk

The number of observed bacterial features in breast milk was not significantly different between IG and CG at 1-week, but it was significantly lower in IG relative to CG at 8 weeks (Figure 2D & eTable 2, observed features 295 vs. 255, p=0.032). However, the difference lost significance after adjusting for 1-week baseline value (coefficient B= 37.5, 95%CI -42, 117, p=0.3). The overall bacterial composition was not significantly different between IG and CG at 1- or 8 weeks as determined by Bray-Curtis dissimilarity metrics (Figure 2E and 2F).

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3.4. Microbiome Composition and Diversity in Infant Gut

Whilst no significant group differences were observed in infant gut microbiome at 1-week, the IG infant presented significantly higher evenness (eTable 2 & eFigure 4, Shannon index 1.94 vs. 2.27, p=0.015) and significantly higher number of observed bacterial features at 8 weeks (Figure 2G, observed features 73 vs. 113, p<0.001); interestingly, the difference was still significant after adjusting for 1-week baseline value (coefficient B=40.8, 95%CI 15.7, 65.9, p=0.002). The overall composition as determined by Bray-Curtis dissimilarity metrics was not significantly different between IG and CG at 1 (Figure 2H) or 8 weeks (Figure 2I).

3.5. Differences in Microbiome Composition in Maternal Gut, Breast Milk and Infant Gut between Groups

The top 15 most abundant bacteria in maternal gut, breast milk, and infant gut at 8 weeks after intervention were assessed, with group differences between IG and CG presented in Figure 3 and baseline group comparison presented in eFigure 5 (supplemental file). The relative abundance of some genera showed significant group differences at 8-weeks with no group difference at baseline, such as lower *Veillonella* and higher *Faecalibacterium* in the IG maternal gut at 8 weeks. In breast milk, on the other hand, *Veillonella* was significantly higher in IG at both baseline and 8 weeks. *Veillonella* was one of the common gut-associated obligate anaerobic genera shared between maternal gut, breast milk and infant gut, whilst *Faecalibacterium* was commonly shared between maternal gut and breast milk. Two other gut-associated anaerobic genera, *Bifidobacterium* and *Blautia*, both had higher relative abundance in the IG infant gut at 8 weeks, but this was only significant for *Blautia*. Moreover, although not significant, the relative abundance of *Bifidobacterium* in breast milk was lower in IG at baseline (eFigure 5 and eFigure 6 in supplemental file) but higher at 8 weeks after intervention (Figure 3). The relative abundance of two putative pathogens, *Enterococcus* and *Acinetobacter*, was also significantly lower in IG at 8 weeks.

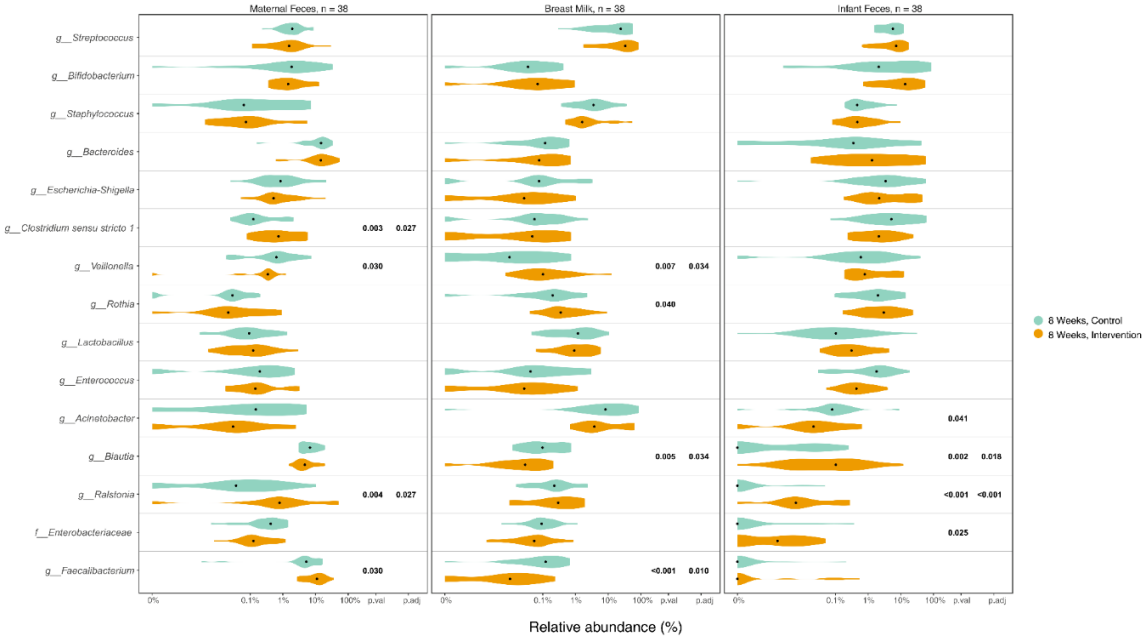

Figure 3. Relative abundance of the Top 15 bacteria among Maternal Feces, Breast Milk, and Infant Feces Samples. Notes: Relative abundance of the top 15 most abundant genera in maternal feces, breast milk, and infant feces were examined and statistical differences between intervention and control group were compared using the Wilcoxon rank-sum test and FDR adjusted p-value.

Notably, as a member of *Proteobacteria* phylum, *Ralstonia* has commonly been identified in breast milk, but rarely in the gut [10,22]. However, the present study observed a significantly higher relative abundance of *Ralstonia* in IG maternal and infant gut at both baseline and 8 weeks.

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3.6. Correlation between the Top 15 Microbial and Maternal Stress/Infant Weight

We examined whether the infant gut microbiome 8 weeks was correlated with infant weight at 8 weeks and weight gain from 1 to 8 weeks, as well as with maternal stress at 8 weeks; the top 15 bacteria were included in the analyses. Higher abundance of *Ralstonia* in infant gut was significantly correlated with infant weight gain from 1 to 8 weeks (r=0.38, p=0.017) and higher absolute infant weight at 8 weeks (r= 0.33 p=0.04). No significant correlation was identified between any of the 15 bacteria and maternal stress (Figure 4).

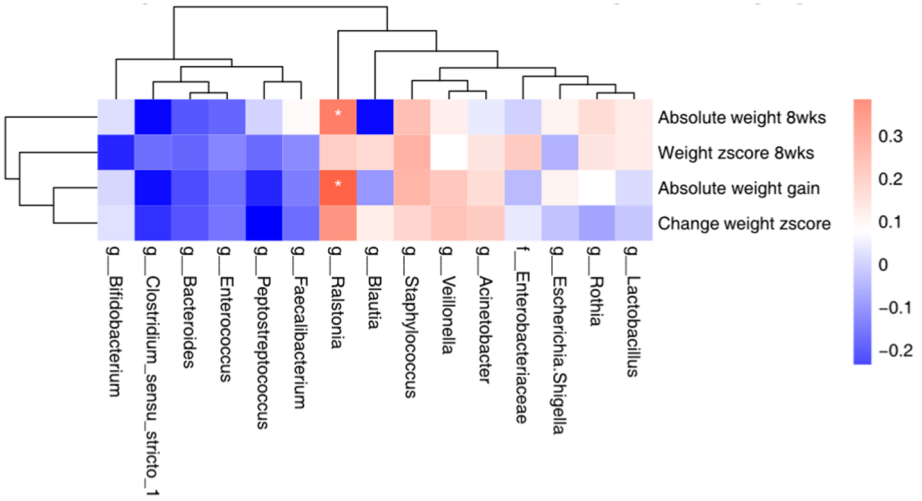

Figure 4. Top 15 bacteria in infant feces samples and its correlation with infant weight at 8 weeks and weight gain from 1 to 8 weeks. Notes: wks= weeks, weight z-score was calculated based on 21st intergrowth study preterm newborn database.

3.7. Unintended Effects

No unintended effects were reported by mothers included in this study.

4. Discussion

Using an experimental approach, we found that a simple relaxation intervention which reduced maternal stress and increased infant weight gain led to changes in the microbiome composition and diversity in the maternal gut, breast milk and infant gut. This is consistent with the hypothesis that the microbiome might be a mediator of the observed relaxation intervention effects via gut-brain and entero-mammary pathways. Key support for this finding comes from the differences in the microbial communities when comparing IG and CG of maternal gut, breast milk, and infant gut microbiomes.

Compared to baseline, the overall microbial community structures in maternal gut showed greater separation between IG and CG at 8 weeks, suggesting that the microbiome community structure was altered after the intervention. Nevertheless, the number of observed taxa in the maternal gut did not significantly differ between IG and CG and did not change significantly from 1- to 8 weeks, underlining that the microbiome richness in the adult gut may be relatively stable. Comparatively, the observed features and evenness of the microbiome in infant gut were significantly higher in IG than CG at 8 weeks. This may be regarded as beneficial, since studies have suggested that higher infant gut α-diversity reflects a more mature, adult-like community [23,24]. A study conducted in Mexican school-aged children showed a significant difference in microbiome composition among children in normal-weight, undernourished and obese groups ($p < 0.01$), with the normal-weight group showing greater α-diversity than undernourished and obese groups [25]. However, it should be mentioned that the interpretation of α-diversity is complex and can depend on various factors and research context. These finding merits further investigation with larger sample size.

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As previously hypothesized, the changes in infant gut microbiome could be related to the maternal gut microbiome though breast feeding. However, whilst the α-diversity was significantly higher in IG infant feces, it was significantly lower in IG breast milk. On the one hand, a lower microbial diversity in breast milk could potentially be more consistent in its effects on infant health [26], since in a lower-diversity community, there might be stronger microbial competition. This competition could potentially inhibit the growth of harmful or pathogenic microorganisms, contributing to a healthier microbial balance in the infant's gut [27]. On the other hand, whilst breastfeeding plays a key role in infant intestinal colonization [28], the infant gut microbiome does not share the composition and community structure seen in breast milk. Breast milk promotes a balanced microbiota development for the newborn, owing to its high content of unique oligosaccharides. These HMOs are the third most abundant solid component in breast milk after lactose and lipids, and can promote intestinal colonization in the infant gut [29,30]. Therefore, the content of HMOs in breast milk is an important factor in determining the microbiota diversity and composition in the infant gut. We did not measure the HMO content of breast milk in our trial. However, it is possible that HMOs were more abundant in the breast milk of IG mothers following the intervention, since previous evidence reported more abundant HMOs in mothers with good mental health compared to those who were distressed [31,32].

Bifidobacterium are predominant in the gut microbiota of infants, and they are considered to be important for infant health and development [8,33,34]. *Bifidobacterium* in breast milk has been reported to activate immunoglobulin A (IgA)-producing plasma cells in the neonatal gut [35] and could control inflammation through mucosal host-microbe crosstalk [36]. The present study showed a lower relative abundance of *Bifidobacterium* in IG breast milk at baseline but higher abundance after the relaxation intervention, suggesting the intervention may have increased the relative abundance of *Bifidobacterium* in IG breast milk. However, the increase in *Bifidobacterium* in infant gut following the intervention was not as obvious as that in breast milk. This finding is in agreement with studies showing that *Bifidobacterium* colonizes the infant gut rapidly within the first few months [11,34], although the CG infants showed a significantly lower baseline abundance than IG, it increased in both groups and no significant group difference was observed at 8 weeks. Overall, we suggest that the relaxation intervention contributed to an increase of *Bifidobacterium* in breast milk but had less impact on its colonization in infant gut.

Modulation of the *Blautia* genera is worth noting. Although its role in infant gut homeostasis remains less known, studies in adult patients undergoing allogeneic hematopoietic stem cell transplantation for leukemia highlight a positive association of *Blautia* with reduced rate of gut Graft *versus* Host Disease (GvHD) post-transplantation [37]. Future studies investigating potential crosstalk between beneficial bacteria that colonize the infant gut including *Bifidobacterium*, *Lactobacillus* and *Blautia* genera are warranted.

The published main outcomes from our trial included greater infant weight gain (mean difference in z-score=0.51, 95%CI: 0.2, 0.9), and lower maternal stress (mean difference in Perceived Stress Scale=2.7, 95%CI: 0.8, 4.5) in the IG at 8 weeks [18]. The correlation analysis in the present study further identified a significant association between higher abundance of infant gut *Ralstonia* and greater infant weight at 8 weeks. *Ralstonia* has commonly been observed in breast milk, whilst in the human gut, *Ralstonia* has mostly been reported as an opportunistic pathogen causing nosocomial infections in immunocompromised patients. In the present study, *Ralstonia* was observed rarely in the CG yet a significantly higher relative abundance of *Ralstonia* was observed in IG maternal and infant gut at both baseline and 8 weeks, with a positive correlation between *Ralstonia* and infant weight gain and the absolute weight value at 8 weeks. Again, these finding merits further investigation, since *Ralstonia* can be a common contaminant of DNA extraction kits or PCR reagents, which may lead to its erroneous appearance in microbiota or metagenomic datasets [38]. However, it is less plausible that only IG gut samples would show contamination, since all samples were coded before being sent for analysis and the research assistants were unable to distinguish between the groups.

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Our study has several limitations. For reasons of privacy, most samples were collected by mothers without supervision, and although clear instructions were provided in advance, this might have led to contamination of the collected samples. Additionally, we did not collect samples from the mouth of mother or infant, maternal areolar skin or vagina, all of which could contribute additional bacteria to the infant gut microbiome. We also did not collect maternal dietary data which could have influenced microbiome composition. However, all mothers were randomly assigned into relaxation or control groups with no difference in baseline characteristics between groups; moreover, mothers were following the traditional postpartum confinement practice with similar diet and lifestyle, thus reducing concerns of bias. The analyses are somewhat limited by the relatively small sample size. However, the study sample was characterized by a high degree of homogeneity, as all mothers were primiparous Chinese women following vaginal delivery at 34-37 weeks, which increased the power to detect significant difference between IG and CG, and reduced potential bias. Furthermore, it should be noted that although we randomly assigned mothers with no significant differences observed in baseline demographics, the baseline β-diversity was significantly different between IG and CG mothers' gut. Compared to baseline status, our results showed a stronger difference in gut microbiome diversity after the intervention, which may imply potential effects of the intervention, However, this should be treated with caution and needs to be further confirmed in larger trials.

This is the first study to test the hypothesis that the microbiome could act as a signal between mother and infant during breastfeeding using an experimental approach. By altering maternal psychological status using the relaxation intervention, we could evaluate the causal impact of maternal stress on maternal gut, breastmilk microbiome and subsequent consequences on infant gut microbiome and health. Consistent with our hypothesis, we found significant differences in microbiome composition and diversity between groups. Together with observed differences in the enrichment of specific genera and correlations between biomarkers and clinical outcomes, which are best considered as hypothesis-generating, these findings can inform the design of future studies, including larger trials in different populations, ideally with maternal dietary data and the collection of the additional biological samples mentioned above; and the application of metagenomic sequencing and metabolomics.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, CONSORT checklist; eFigure 1; eTable 1; eFigure 2; eFigure 3; eTable 2; eFigure 4; eFigure 5; eFigure 6.

Author Contributions: JY contributed to manuscript generating and statistical analysis; YZ contributed to microbiome analysis and preparation of the supplemental documents; MF contributed to revision of the manuscript, JY and ZW conducted the original trial, J.C.W, M.B.E, and D.S.N provided valuable comments and contributed to refining the manuscript. All authors read and approved the final manuscript.

Funding: The original research was conducted as part of a PhD and expenses were covered from the research funds held by Professor Fewtrell in The Childhood Nutrition Research Group. All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Research Ethics Committee of University College London (ID: 12681/002) and the Department of Child Health, Beijing Children's Hospital (ID: 2018-167). .

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability statement: All raw sequencing data associated with this study have been uploaded to the Sequence Read Archive (SRA) under citation accession PRJNA1000236. Data described in the manuscript, code book, and analytic code will be made available upon request.

Acknowledgments: We would like to thank Novogene Technology Inc. (Beijing, China) for conducting the 16S rRNA sequencing. We also thank Xueling, Li, Yufeng An, Wenming, Cui, Changeshuan Yang and all research nurses from Beijing Children's Hospital for assistance during data collection.

Conflicts of Interest: Professor Mary Fewtrell receives an unrestricted donation for research on infant nutrition from Philips (Amsterdam, NL). The remaining authors declare no other conflicts.

 doi:10.20944/preprints202403.0413.v1

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