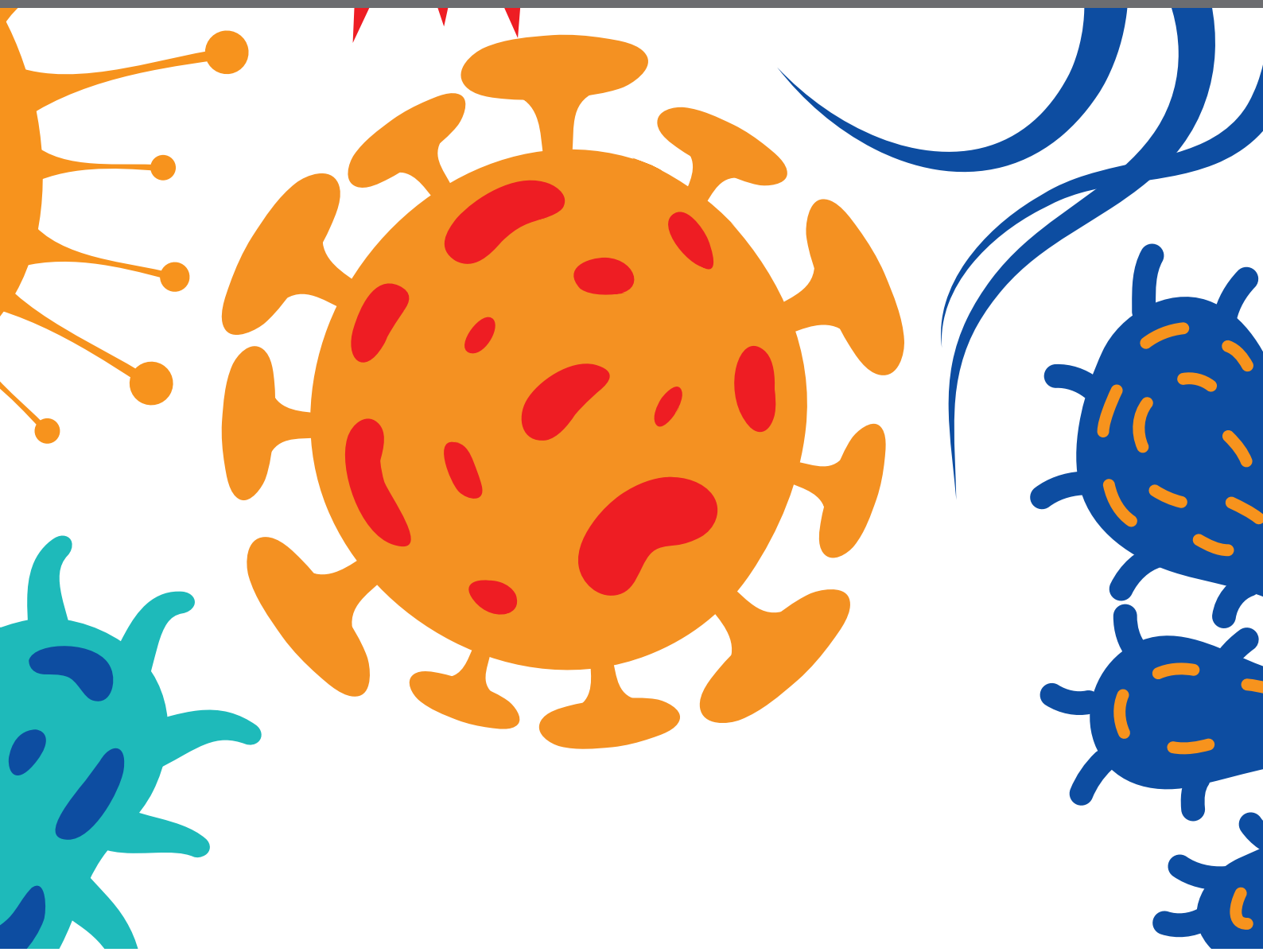
WOMEN IN MICROBIOME IN HEALTH AND DISEASE 2021

EDITED BY: Elisabetta Caselli, Gislane Lelis Vilela de Oliveira, Veeranoot Nissapatorn, Maria De Lourdes Pereira, Maayan Levy and Heather D. Bean

PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-584-7
DOI 10.3389/978-2-83250-584-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

WOMEN IN MICROBIOME IN HEALTH AND DISEASE 2021

Topic Editors:
Elisabetta Caselli, University of Ferrara, Italy
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil
Veeranoot Nissapatorn, Walailak University, Thailand
Maria De Lourdes Pereira, University of Aveiro, Portugal
Maayan Levy, University of Pennsylvania, United States
Heather D. Bean, Arizona State University, United States

Citation: Caselli, E., de Oliveira, G. L. V., Nissapatorn, V., Pereira, M. D. L., Levy, M., Bean, H. D., eds. (2022). Women in Microbiome in Health and Disease 2021. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-584-7

Table of Contents

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 11 October 2022
DOI 10.3389/fcimb.2022.1054190

OPEN ACCESS

EDITED AND REVIEWED BY
Xin Xu,
Sichuan University, China

*CORRESPONDENCE
Gislane Lelis Vilela de Oliveira
gislane.lelis@unesp.br
Veeranoot Nissapatorn
nissapat@gmail.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 26 September 2022
ACCEPTED 28 September 2022
PUBLISHED 11 October 2022

CITATION
Pereira MdL, Levy M, Nissapatorn V and de Oliveira GLV (2022) Editorial: Women in microbiome in health and disease 2021.
Front. Cell. Infect. Microbiol. 12:1054190.
doi: 10.3389/fcimb.2022.1054190

Editorial: Women in microbiome in health and disease 2021

Maria de Lourdes Pereira[1†], Maayan Levy[2†], Veeranoot Nissapatorn[3*†] and Gislane Lelis Vilela de Oliveira[4*†]

[1]Centre for Research in Ceramics and Composite Materials (CICECO) - Aveiro Institute of Materials & Department of Medical Sciences, University of Aveiro, Aveiro, Portugal, [2]Microbiology Department, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [3]School of Allied Health Sciences and World Union for Herbal Drug Discovery [WUHeDD], Walailak University, Nakhon Si Thammarat, Thailand, [4]Institute of Biosciences, Humanities and Exact Sciences (IBILCE), São Paulo State University (UNESP), Sao Jose do Rio Preto, Brazil

KEYWORDS

women in science, pregnancy, women health, microbiome, dysbiosis

Editorial on Research Topic
Women in microbiome in health and disease 2021

Currently, less than 30% of researchers worldwide are women. Long-standing biases and gender stereotypes are discouraging girls and women from moving away from science-related fields, and STEM research in particular. Science and gender equality are, however, essential to ensure sustainable development, as highlighted by UNESCO and the United Nations. To change traditional mindsets, gender equality must be promoted, stereotypes defeated, and girls and women should be encouraged to pursue STEM careers.

This Research Topic of Frontiers in Cellular and Infection Microbiology celebrates the achievements of women in science and highlights the diversity of research performed across the entire breadth of the microbiome in health and disease research, and presents advances in theory, experimental, and methodology with applications to compelling problems. A total of 14 articles authored by women were published and 97 researchers participated in this Research Topic. Figure 1 summarizes the main findings of the articles in relation to associations between microbiomes and women health.

Emerging findings from animal models and human pregnancy studies have shown that different factors affect maternal and fetal complications, including intrauterine or extrauterine infections, oral or intestinal dysbiosis, and dysregulated immune responses (Romero et al., 2014; MacIntyre et al., 2015; Piler et al., 2017; Fettweis et al., 2019; Serrano et al., 2019; Jehan et al., 2020; Pansieri et al., 2020; Kumar et al., 2021). Two mechanisms have been proposed to explain how gut microbes move into the uterus: 1) gram-negative microbes induce mediators of inflammation, such as prostaglandins that facilitate the ascent of microbes through the vagina; 2) intestinal hyperpermeability during pregnancy promotes bacterial translocation to the uterus or placenta (Edwards et al., 2017). Kumar et al. revised and discussed how pathogens can cross the placental barrier and promote undesirable outcomes in the pregnancy, in the childbirth and the newborn. Also, they

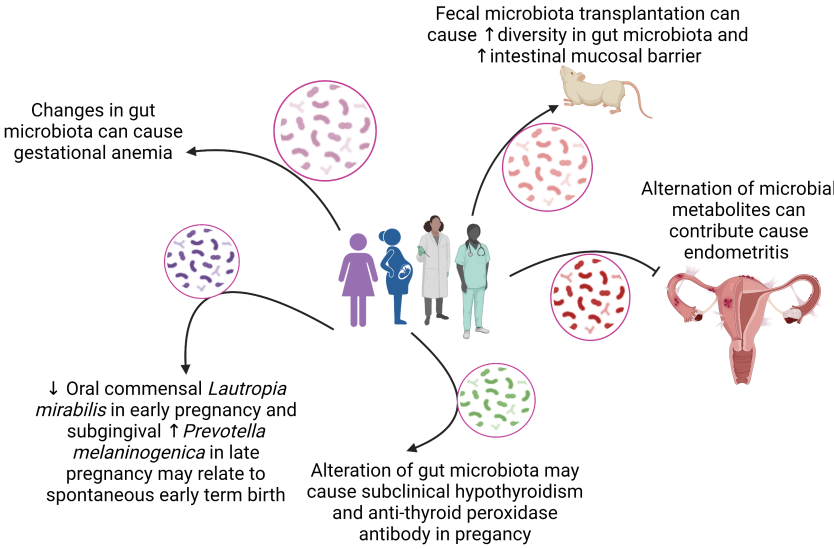


FIGURE 1
The association between microbiomes and women health.

suggest that vaginal dysbiosis can induce an abnormal immune response in pregnant women and function as predictor marker for adverse outcomes from congenital infections.

Regarding dysbiosis, some studies have suggested the connection between alterations in the diversity and function of the oral, gut and vaginal microbiota and pregnancy complications (Borgo et al., 2014; Cobb et al., 2017; Fujiwara et al., 2017; Goltsman et al., 2018). For example, oral dysbiosis with decreased *Lactobacillus* and increased *Porphyromonas gingivalis, Neisseria*, and *Treponema* may contribute to preeclampsia, miscarriage, preterm labor and low birth weight (Cobb et al., 2017; Fujiwara et al., 2017; Goltsman et al., 2018; Jang et al., 2021). Interestingly, the presence of *Fusobacterium nucleatum* in the amniotic fluid of preterm labor pregnant women indicates oral microbes translocation to the placenta (Nuriel-Ohayon et al., 2019; Amir et al., 2020). The gut microbiota of pregnant women with or without complications differs significantly, with a greater abundance of opportunistic pathogens in the first group, impacting maternal metabolism and fetal development (Koren et al., 2012; DiGiulio et al., 2015; Edwards et al., 2017; Jiang et al., 2021).

The composition of the vaginal microbiota during pregnancy changes significantly and the decrease in *Lactobacillus* in this environment is associated with preterm birth (Aagaard et al., 2012; Di Simone et al., 2020; Marangoni et al., 2021). Zakaria et al. revised several physiological changes in the intestinal, oral and vaginal microbiomes during pregnancy, relating these alterations to mother and fetal health. In addition, researchers finalized by discussing the effect of probiotics to manage the microbiome during pregnancy. Similarly, Dreisbach et al. provided us an updated overview of the impact of obesity on maternal gut microbiota and metabolism thought animal model studies. Researchers also discussed reports on probiotic applications in these settings.

The gestational anemia (GA) is associated with adverse maternal and fetal outcomes, including preterm birth, low birth weight, neonatal and maternal mortality (Guignard et al., 2021; Shi et al., 2022). In a prospective study, Wei et al. evaluated the gut microbiota in GA (n = 156) and in healthy pregnant women (n = 402). Alpha and beta diversities were calculated and researchers showed significant differences between microbial communities in the third trimester, compared to the second trimester, in addition to the differential relative abundance of *Megamonas*, *Veillonella*, and *Haemophilus* when compared to healthy controls. A microbial co-abundance group network predicted upcoming anemia in healthy pregnant women with an area under the ROC curve of 0.7738 (95%CI: 0.7171, 0.8306), suggesting the possibility of identifying women at high-risk for the GA development, and the gut microbiota as a target for therapeutic modulation, through the use of functional foods and probiotics.

In a single-center prospective observational study, Wu et al. evaluated the fecal microbiota and metabolic functions in pregnant women with subclinical hypothyroidism, with (n = 75) or without (n = 90) positivity for anti-thyroid peroxidase antibodies (TPO). The group was also subdivided according to the levothyroxine (LT4) therapy (no treatment *vs.* low-dose LT4 *vs.* high-dose LT4). Researchers did not find significant differences in microbiota richness and evenness when comparing TPO positive and TPO negative women,

however, they observe significant microbial diversity between TPO+ and TPO- in the high-dose LT4 group.

Using LEfSe analysis, authors detected a microbial signature related to the LT4 replacement, such as decreased species of *Ruminococcus* and *Bacteroides massiliensis* in low-and high-dose LT4 groups, respectively, as well as nineteen differential metabolic functions involved in lipid and amino acid metabolism discriminating TPO+ and TPO- pregnant women in the second trimester. The subclinical hypothyroidism in TPO + pregnant women was normally associated with miscarriage, preterm birth, pre-eclampsia and gestational diabetes. Since the intestinal microbiota can affect the synthesis and functions of thyroid hormones (Knezevic et al., 2020; Fenneman et al., 2020; Bargiel et al., 2021), the study suggests microbiota modulation as a therapeutic option to treat TPO+ pregnant women.

In a very interesting longitudinal descriptive study, Yang et al. evaluated the subgingival microbiota in 59 pregnant women, from 8 to 14 weeks and from 24 to 30 weeks, correlating this data with gestational age and birth outcomes. No significant differences were observed in the richness, evenness and diversity of microbiota between 8 to 14 and 24 to 30 weeks of gestation; however, in the latter group, alpha and beta diversities were different between women who had early term births and those who had delivered at term.

Interestingly, the top twenty taxa represented in the subgingival microbiota of participants throughout pregnancy include bacteria involved in the progression to periodontal disease, including *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella*, and *Campylobacter* species. The oral dysbiosis is associated with periodontitis in pregnant women, and this disease is an important risk factor for preterm births (Jang et al., 2021; Saadaoui et al., 2021). In the present study, researchers identified that a decrease in *Lautropia mirabilis* at 8 to 14 weeks and an increase in *Prevotella melaninogenica* at 24 to 30 weeks of pregnancy were both associated with spontaneous early term birth and represent an important target for future studies.

The dietary habits during intrauterine life and after birth have an important impact on the health of the newborn´s microbiota and on the establishment of the adulthood microbiota later in life (Cadenas-Sanchez et al., 2017; Singh and Mittal, 2020). The gut microbiota colonization begins in the uterus and at birth, acquiring microbes from the mother and the environment. The main factors for the microbiota composition in early life are delivery type (normal or C-section), breastfeeding, time of introduction of solid foods, antibiotic use, and hygiene conditions (Martin et al., 2016; Le Doare et al., 2018). In a cross-sectional study in Brazil, Freitas et al. examined early-life data, body mass index (BMI), and collected fecal samples from 114 women with a mean age of 28 years and a mean BMI of 24.5 kg/m^2. Beta diversity analysis of the microbiota showed two microbiota profiles, one driven by the *Blautia* genus (n = 56) and another driven by *Prevotella* (n = 58). The breastfeeding and adequate nutritional status were positively correlated with increased abundance of *Blautia, Anaerostipes*, and *Lachnoclostridium*. The exclusive breastfeeding (≥ 6 months) is associated with *Blautia*-driven profile of healthy women, showing the importance of early-life events and exclusive breastfeeding for infant gut colonization and health later in life.

The human milk oligosaccharides (HMO) are indigestible glycans that acts in the microbiota establishment and immunity maturation in the infant gut, and HMO are known to have bifidogenic effects (Bode, 2012; Wiciński et al., 2020). Kijner et al. investigated the mechanisms of HMO utilization by infant gut microbes by isolating *Bacteroides* strains from fecal samples, and testing them with the six most common HMO, 2′-fucosyllactose (2'-FL), difucosyllactose (DFL), 3′-sialyllactose (3'-SL), 6′-sialyllactose (6'-SL), lacto-N-tetraose (LNT), and lacto-N-neotetraose (LNnT). Differential RNA sequencing analysis showed that *Bacteroides dorei* presents an important glycoside hydrolase (GH) activity in break carbon bonds from HMO *in vitro*. Seventeen GH families were upregulated when cultivated with 2'-FL, 21 in DFL, 19 in 3'-SL, 23 in 6'-SL, 15 in LNT, and 18 in LNnT (log2 fold change > 1, p adj < 0.05), expanding our knowledge on the microorganisms involved in the HMO digestion, in addition to the already known *Bifidobacterium* species.

Regarding women's health, in a multicenter, randomized, blinded clinical trial, Li et al. evaluated women with mixed aerobic vaginitis with bacterial vaginosis who received an effervescent suppository (n = 39) or clindamycin (n = 41). Women aged 20 to 55 years were randomized to receive either the suppository or clindamycin, vaginal swabs were collected at three time points (V1: -2~0 days; V2: 15-17 days; V3: 40 ± 3 days), and the DNA sequenced by Accurate 16S absolute quantification. Before treatment (V1), the pathogenic species *Gardnerella vaginalis, Atopobium vaginae, Sneathia amnii*, and *Prevotella bivia* were found in both groups.

After treatment, according to Shannon and Simpson indexes, the microbiota diversity significantly decreased in V2 in both groups (p < 0.001), and slightly increased in V3 in the suppository group. The absolute abundance of *Lactobacillus* increased in the suppository group compared to untreated patients, and the genera *Gardnerella, Prevotella*, and *Atopobium* were enriched in V3 in the clindamycin and suppository groups. Authors concluded that the effervescent suppository is effective to treat women with mixed aerobic vaginitis with bacterial vaginosis by restoring the vaginal microbiota.

Recent studies have investigated the relationship between the vaginal microbiota and cervical cancer (CC) to better understand the involvement of dysbiosis in the establishment, progression, or remission of the disease (Bray et al., 2018; Baezconde-Garbanati et al., 2019; Martínez-Rodríguez et al., 2021). In an observational cross-sectional study in Mexico,

Manzanares-Leal et al. included 120 women aged between 21 to 71 years, 60 with advanced CC and 60 without the disease. Cervicovaginal swabs were collected to obtain culturable aerobic microbes, strains were evaluated by PCR-RFLP and identified by 16S sequencing. Researchers detected a specific microbiota associated with advanced stages of CC, including *Streptococcus urinalis, Escherichia coli, Bacillus safensis, Bacillus malikii, Corynebacterium jeikeium, Corynebacterium striatum*, and *Lactobacillus rhamnosus*. They concluded that there is no causal association between the aerobic vaginitis and cervicovaginal neoplasia, but proposed that vaginitis is fundamental for the progression of preneoplastic lesions to cancer (Vieira-Baptista et al., 2016; Plisko et al., 2021). Dong et al. contributed to a research article that aimed to assess the effects of acute endometritis on intestinal microbes and their metabolites. Endometritis is generally caused by bacterial infections, and accumulating evidence has shown that the occurrence of disease may be related to the gut microbiota (Plottel and Blaser, 2011; Borella et al., 2021). Moreover, the progression of diseases has previously been shown to change the composition and diversity of the intestinal microbiota and associated metabolites. The authors used a mouse model of endometritis that involves an intrauterine administration of lipopolysaccharide (LPS). Using 16S rRNA gene sequencing and liquid chromatogram-mass spectrometry, they found that the relative abundance of some members of the microbiome was changed and resulted in the reduction of beneficial microorganisms in the intestinal tract.

At the same time, acute endometritis increased the relative abundance of pathogenic bacteria, altered the concentration of intestinal metabolites, and affected biological oxidation, energy metabolism, and biosynthesis of primary bile acids. Thus, the findings of this study have the potential to provide new strategies for the diagnosis of acute endometritis.

Amyotrophic lateral sclerosis (ALS) is a heterogeneous neuromuscular disorder with progressive degeneration of the upper and lower motor neurons (Hardiman, 2021). A combination of genetic and environmental factors, as well as age-related dysfunctions, are hypothesized to be involved in the ALS development (Masrori and Van Damme, 2020). Women are more affected by the disease with an estimation risk of 1:400 compared to 1:350 in men (Ryan et al., 2019; Masrori and Van Damme, 2020). Martin et al. contributed a review article that provides a comprehensive overview of the role that the microbiome may play in the ALS pathogenesis of. The authors explore existing evidence of gastrointestinal symptoms and microbial alterations in ALS pathogenesis from human and animal studies and discuss the possible therapeutic approaches to target specific diets, metabolites, and intestinal microbiome in ALS patients. They highlight innovative strategies for accurate diagnosis and better treatment for this challenging disease.

The last two articles discussed important aspects of fecal microbiota transplantation (FMT) in sepsis and bioinformatics tools for use in predictive models. Gai et al. reported that the FMT in sepsis model induced by cecal ligation and puncture is able to reestablish the gut microbiota diversity and decrease mortality by modulating the inflammatory response, restoring the epithelial barrier and function by upregulating the expression of tight junction proteins. Dahan et al. presented the EasyMap, an interactive online tool allowing for (1) running multiple multivariate linear regression models, with the same features and metadata; (2) visualizing the associations between microbial features and clinical metadata found in each model; and (3) comparing between the various models to identify critical metadata variables and select the optimal model. The EasyMap provides a side-by-side visualization of association results across the various models, each with additional metadata variables, enabling us to evaluate the impact of each metadata variable on the associated feature.

Collectively, the articles in this Research Topic demonstrated important aspects of the role played by the microbiomes from different sites (oral, gut and vagina) for women´s health, for a succeed pregnancy and fetal development, and for the prevention and treatment of diseases by using strategic approaches to modulate the microbiota, including nutritional interventions, probiotics and fecal microbiota transplantation. Furthermore, studies aimed at developing software for more accurate analyzes and prediction models are also necessary for the evolution of the microbiome field.

Author contributions

GO and ML wrote the initial draft of the editorial, MP and VN revised the manuscript, and all authors approved the final version of the editorial.

Acknowledgments

We would like to thank Dr. Alok K. Paul, University of Tasmania-Australia for assistance of an infographic using Biorender.com.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T. A., Coarfa, C., et al. (2012). A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One* 7, e36466. doi: 10.1371/journal.pone.0036466

Amir, M., Brown, J. A., Rager, S. L., Sanidad, K. Z., Ananthanarayanan, A., and Zeng, M. Y. (2020). Maternal microbiome and infections in pregnancy. *Microorganisms*. 8, 1996. doi: 10.3390/microorganisms8121996

Baezconde-Garbanati, L., Agurto, I., Gravitt, P. E., Luciani, S., Murphy, S., Ochoa, C., et al. (2019). Barriers and innovative interventions for early detection of cervical cancer. *Salud Publica Mex.* 61, 456–460. doi: 10.21149/10425

Bargiel, P., Szczuko, M., Stachowska, L., Prowans, P., Czapla, N., Markowska, M., et al. (2021). Microbiome metabolites and thyroid dysfunction. *J. Clin. Med.* 10, 3609. doi: 10.3390/jcm10163609

Bode, L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology* 22, 1147–1162. doi: 10.1093/glycob/cws074

Borella, F., Carosso, A. R., Cosma, S., Preti, M., Collemi, G., Cassoni, P., et al. (2021). Gut microbiota and gynecological cancers: A summary of pathogenetic mechanisms and future directions. *ACS Infect. Dis.* 7, 987–1009. doi: 10.1021/acsinfecdis.0c00839

Borgo, P. V., Rodrigues, V. A. A., Feitosa, A. C. R., Xavier, K. C. B., and Avila-Campos, M. J. (2014). Association between periodontal condition and subgingival microbiota in women during pregnancy: a longitudinal study. *J. Appl. Oral. Sci.* 22, 528–533. doi: 10.1590/1678-775720140164

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Cadenas-Sanchez, C., Henriksson, P., Henriksson, H., Delisle Nyström, C., Pomeroy, J., Ruiz, J. R., et al. (2017). Parental body mass index and its association with body composition, physical fitness and lifestyle factors in their 4-year-old children: results from the MINISTOP trial. *Eur. J. Clin. Nutr.* 71, 1200–1205. doi: 10.1038/ejcn.2017.62

Cobb, C. M., Kelly, P. J., Williams, K. B., Babbar, S., Angolkar, M., and Derman, R. J. (2017). The oral microbiome and adverse pregnancy outcomes. *Int. J. Womens Health* 9, 551–559. doi: 10.2147/IJWH.S142730

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and spatial variation of the human microbiota during pregnancy. *Proc. Natl. Acad. Sci. U S A.* 112, 11060–11065. doi: 10.1073/pnas.1502875112

Di Simone, N., Santamaria Ortiz, A., Specchia, M., Tersigni, C., Villa, P., Gasbarrini, A., et al. (2020). Recent insights on the maternal microbiota: Impact on pregnancy outcomes. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.528202

Edwards, S. M., Cunningham, S. A., Dunlop, A. L., and Corwin, E. J. (2017). The maternal gut microbiome during pregnancy. *MCN Am. J. Matern Child Nurs.* 42, 310–317. doi: 10.1097/NMC.0000000000000372

Fenneman, A. C., Rampanelli, E., Yin, Y. S., Ames, J., Blaser, M. J., Fliers, E., et al. (2020). Gut microbiota and metabolites in the pathogenesis of endocrine disease. *Biochem. Soc. Trans.* 48, 915–931. doi: 10.1042/BST20190686

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The vaginal microbiome and preterm birth. *Nat. Med.* 25, 1012–1021. doi: 10.1038/s41591-019-0450-2

Fujiwara, N., Tsuruda, K., Iwamoto, Y., Kato, F., Odaki, T., Yamane, N., et al. (2017). Significant increase of oral bacteria in the early pregnancy period in Japanese women. *J. Investig. Clin. Dent.* 8, e12189. doi: 10.1111/jicd.12189

Goltsman, D. S. A., Sun, C. L., Proctor, D. M., DiGiulio, D. B., Robaczewska, A., Thomas, B. C., et al. (2018). Metagenomic analysis with strain-level resolution reveals fine-scale variation in the human pregnancy microbiome. *Genome Res.* 28, 1467–1480. doi: 10.1101/gr.236000.118

Guignard, J., Deneux-Tharaux, C., Seco, A., Beucher, G., Kayem, G., Bonnet, M. P., et al. (2021). Gestational anaemia and severe acute maternal morbidity: a population-based study. *Anaesthesia.* 76, 61–71. doi: 10.1111/anae.15222

Hardiman, O. (2021). Major advances in amyotrophic lateral sclerosis in 2020. *Lancet Neurol.* 20, 14–15. doi: 10.1016/S1474-4422(20)30447-6

Jang, H., Patoine, A., Wu, T. T., Castillo, D. A., and Xiao, J. (2021). Oral microflora and pregnancy: a systematic review and meta-analysis. *Sci. Rep.* 11, 16870. doi: 10.1038/s41598-021-96495-1

Jehan, F., Sazawal, S., Baqui, A. H., Nisar, M. I., Dhingra, U., Khanam, R., et al. (2020). Multiomics characterization of preterm birth in low- and middle-income countries. *JAMA Netw. Open* 3, e2029655. doi: 10.1001/jamanetworkopen.2020.29655

Jiang, G., Zhou, Z., Li, X., Qian, Y., and Wang, K. (2021). The gut microbiome during pregnancy. *Matern Fetal Med.* 1–9 doi: 10.1097/FM9.0000000000000091

Knezevic, J., Starchl, C., Tmava Berisha, A., and Amrein, K. (2020). Thyroid-Gut-Axis: How does the microbiota influence thyroid function? *Nutrients.* 12, 1769. doi: 10.3390/nu12061769

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell.* 150, 470–480. doi: 10.1016/j.cell.2012.07.008

Kumar, M., Murugesan, S., Singh, P., Saadaoui, M., Elhag, D. A., Terranegra, A., et al. (2021). Vaginal microbiota and cytokine levels predict preterm delivery in Asian women. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.639665

Le Doare, K., Holder, B., Bassett, A., and Pannaraj, P. S. (2018). Mother's milk: A purposeful contribution to the development of the infant microbiota and immunity. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.00361

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). The vaginal microbiome during pregnancy and the postpartum period in a European population. *Sci. Rep.* 5, 8988. doi: 10.1038/srep08988

Marangoni, A., Laghi, L., Zagonari, S., Patuelli, G., Zhu, C., Foschi, C., et al. (2021). New insights into vaginal environment during pregnancy. *Front. Mol. Biosci.* 8. doi: 10.3389/fmolb.2021.656844

Martínez-Rodríguez, F., Limones-González, J. E., Mendoza-Almanza, B., Esparza-Ibarra, E. L., Gallegos-Flores, P. I., Ayala-Luján, J. L., et al. (2021). Understanding cervical cancer through proteomics. *Cells.* 10, 1854. doi: 10.3390/cells10081854

Martin, R., Makino, H., Cetinyurek Yavuz, A., Ben-Amor, K., Roelofs, M., Ishikawa, E., et al. (2016). Early-life events, including mode of delivery and type of feeding, siblings and gender, shape the developing gut microbiota. *PLoS One* 11, e0158498. doi: 10.1371/journal.pone.0158498

Masrori, P., and Van Damme, P. (2020). Amyotrophic lateral sclerosis: a clinical review. *Eur. J. Neurol.* 27, 1918–1929. doi: 10.1111/ene.14393

Nuriel-Ohayon, M., Neuman, H., Ziv, O., Belogolovski, A., Barsheshet, Y., Bloch, N., et al. (2019). Progesterone increases bifidobacterium relative abundance during late pregnancy. *Cell Rep.* 27, 730–736.e3. doi: 10.1016/j.celrep.2019.03.075

Pansieri, C., Pandolfini, C., Clavenna, A., Choonara, I., and Bonati, M. (2020). An inventory of European birth cohorts. *Int. J. Environ. Res. Public Health* 17, 3071. doi: 10.3390/ijerph17093071

Piler, P., Kandrnal, V., Kukla, L., Andrýsková, L., Švancara, J., Jarkovský, J., et al. (2017). Cohort profile: The European longitudinal study of pregnancy and childhood (ELSPAC) in the Czech republic. *Int. J. Epidemiol.* 46, 1379–1379f. doi: 10.1093/ije/dyw091

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic vaginitis-underestimated risk factor for cervical intraepithelial neoplasia. *Diagnostics (Basel).* 11, 97. doi: 10.3390/diagnostics11010097

Plottel, C. S., and Blaser, M. J. (2011). Microbiome and malignancy. *Cell Host Microbe* 10, 324–335. doi: 10.1016/j.chom.2011.10.003

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., et al. (2014). The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome.* 2, 18. doi: 10.1186/2049-2618-2-18

Ryan, M., Heverin, M., McLaughlin, R. L., and Hardiman, O. (2019). Lifetime risk and heritability of amyotrophic lateral sclerosis. *JAMA Neurol.* 76, 1367–1374. doi: 10.1001/jamaneurol.2019.2044

Saadaoui, M., Singh, P., and Al Khodor, S. (2021). Oral microbiome and pregnancy: A bidirectional relationship. *J. Reprod. Immunol.* 145, 103293. doi: 10.1016/j.jri.2021.103293

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic diversity in the dynamics of the vaginal microbiome during pregnancy. *Nat. Med.* 25, 1001–1011. doi: 10.1038/s41591-019-0465-8

Shi, H., Chen, L., Wang, Y., Sun, M., Guo, Y., Ma, S., et al. (2022). Severity of anemia during pregnancy and adverse maternal and fetal outcomes. *JAMA Netw. Open* 5, e2147046. doi: 10.1001/jamanetworkopen.2021.47046

Singh, A., and Mittal, M. (2020). Neonatal microbiome - a brief review. *J. Matern Fetal Neonatal Med.* 33, 3841–3848. doi: 10.1080/14767058.2019.1583738

Vieira-Baptista, P., Lima-Silva, J., Pinto, C., Saldanha, C., Beires, J., Martinez-de-Oliveira, J., et al. (2016). Bacterial vaginosis, aerobic vaginitis, vaginal inflammation and major pap smear abnormalities. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 657–664. doi: 10.1007/s10096-016-2584-1

Wiciński, M., Sawicka, E., Gębalski, J., Kubiak, K., and Malinowski, B. (2020). Human milk oligosaccharides: Health benefits, potential applications in infant formulas, and pharmacology. *Nutrients.* 12, 266. doi: 10.3390/nu12010266

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 30 August 2021
doi: 10.3389/fcimb.2021.734561

Insight Into the Potential Value of Gut Microbial Signatures for Prediction of Gestational Anemia

Hongcheng Wei[1,2†], *Siting Deng*[1,2†], *Yufeng Qin*[1,2], *Xu Yang*[1,2], *Ting Chen*[3], *Xu Wang*[4*] *and Yankai Xia*[1,2*]

[1] State Key Laboratory of Reproductive Medicine, School of Public Health, Nanjing Medical University, Nanjing, China, [2] Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing, China, [3] Nanjing Maternity and Child Health Care Institute, Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, Nanjing, China, [4] Department of Endocrinology, Children's Hospital of Nanjing Medical University, Nanjing, China

The gut microbiota alternations are associated with gestational anemia (GA); however, limited predictive value for the subsequent incidence of anemia in normal gestational women has been obtained. We sought to rigorously characterise gut dysbiosis in subjects with GA and explored the potential predictive value of novel microbial signatures for the risk of developing GA. A prospective cohort of subjects with GA (n = 156) and healthy control (n = 402), all of whom were free of GA in the second trimester, by 16S rRNA gene sequencing was conducted. Microbial signatures altered dramatically in GA compared with healthy control in the second trimester. *Megamonas*, *Veillonella*, and *Haemophilus* were confirmed to show differential abundances in GA after adjusting for covariates. On the contrary, *Lachnospiraceae* and *Blautia* were enriched in control. Microbial co-abundance group (CAG) network was constructed. Prospectively, CAG network relatively accurately predicted upcoming GA in normal pregnant women with an AUC of 0.7738 (95%CI: 0.7171, 0.8306) and the performance was further validated in Validation set (0.8223, 95%CI: 0.7573, 0.8874). Overall, our study demonstrated that alterations in the gut microbial community were associated with anemia in pregnancy and microbial signatures could accurately predict the subsequent incidence of anemia in normal pregnant women. Our findings provided new insights into understanding the role of gut microbiota in GA, identifying high-risk individuals, and modulating gut microbiota as a therapeutic target, thus improving quality of life and well-being of women and children.

Keywords: 16S rRNA gene sequencing, gut microbiota, co-abundance group, prediction, the risk of developing gestational anemia

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Huajun Zheng, Shanghai Institute for Biomedical and Pharmaceutical Technologies, China
Francesco Strati, European Institute of Oncology (IEO), Italy
Júlia Teixeira Cottas De Azevedo, Hemocentro Foundation of Ribeirão Preto, Brazil

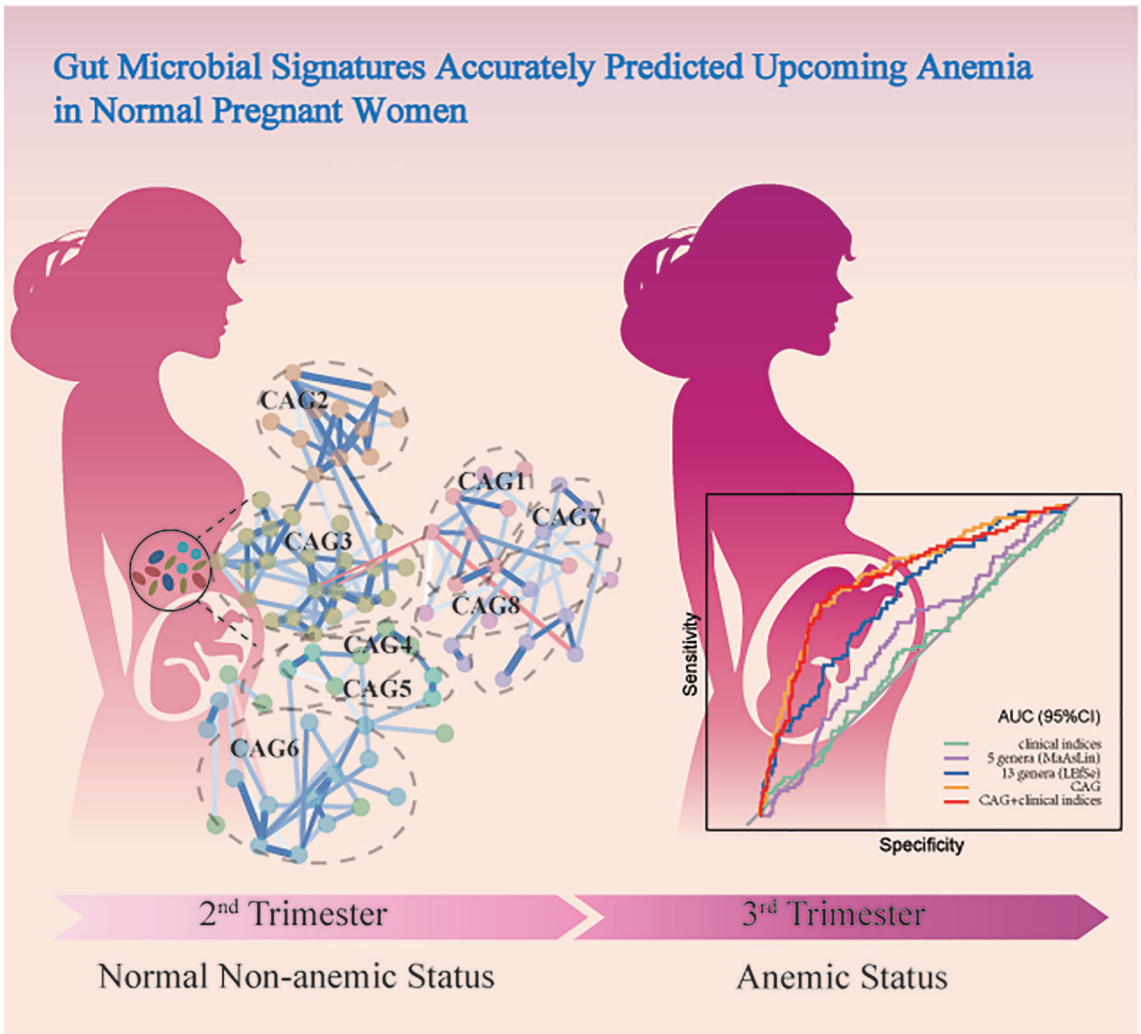
***Correspondence:**
Yankai Xia
yankaixia@njmu.edu.cn
Xu Wang
sepnine@njmu.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *01 July 2021*
Accepted: *09 August 2021*
Published: *30 August 2021*

Citation:
Wei H, Deng S, Qin Y, Yang X, Chen T, Wang X and Xia Y (2021) Insight Into the Potential Value of Gut Microbial Signatures for Prediction of Gestational Anemia. Front. Cell. Infect. Microbiol. 11:734561. doi: 10.3389/fcimb.2021.734561



GRAPHICAL ABSTRACT |

INTRODUCTION

Anemia is a serious public health problem affecting people all over the world, and is one of the most frequent complications involved in pregnancy, imposing a tremendous toll on well-being of approximately 40% of pregnant women in China (Li et al., 2018). Gestational anemia, according to the World Health Organization (WHO), is defined as a hemoglobin concentration (Hb) < 110 g L^{-1}. Gestational anemia mostly occurred in the second and third trimester (Zhao et al., 2018). Multiple factors account for gestational anemia, nutritional iron deficiency anemia (IDA) (approximately 75%) and folate megaloblastic deficiency anemia being the commonest (Sifakis and Pharmakides, 2000; Goonewardene et al., 2012).

Anemia in pregnancy has adverse effects on maternal and neonatal health. Weakness, fatigue, being vulnerable to infection, reduced work capacity, and productivity are typical symptoms during pregnancy (Prema et al., 1982; Hunt, 2002). Current evidence suggests severe gestational anemia could be associated with an increased risk of preterm birth, low birth weight, and even neonatal and maternal mortality (Khan et al., 2006; Rahman et al., 2016; Figueiredo et al., 2018; Guignard et al., 2021). Furthermore, it has been reported that babies born to anemic mothers are prone to exhibit future poor cognitive performance and delayed mental and motor development in adolescence and adulthood (Anker et al., 2009; Camaschella, 2015).

The current clinical method is generally applied to cross-sectional diagnosis rather than prediction in the long term. Although Hb concentration serves as a golden standard for the diagnosis of anemia in pregnancy, it has limitations in precise prediction of potential impending anemia in normal pregnant women. Additionally, it remains controversial to use Hb concentration to distinguish true or absolute anemia from relative anemia, ascribed to a normal physiologic increase of

plasma volume (Sifakis and Pharmakides, 2000). Thus, an accurate determination of gestational anemia is essential and of enormous clinical importance for prevention and management of anemia in pregnancy.

The human gut hosts an immense number of resident microorganisms, collectively termed as the microbiota (Structure, Function and Diversity of the Healthy Human Microbiome, 2012; Bäckhed et al., 2004). There has been accumulating evidence that the gut microbiota is implicated in enteric, metabolic, and psychiatric diseases (Imhann et al., 2018; Vich Vila et al., 2018; Thomas et al., 2019; Wirbel et al., 2019). Previous studies have documented that gut microbial dysfunction (e.g., deficiency in *lactobacillus*) is related to IDA and gut microbiota could promote hematopoiesis, underlying the close relationship between gut microbiota and anemia (Balamurugan et al., 2010; Khosravi et al., 2014). During pregnancy, host profoundly remodeling of the gut microbiota could cause symptoms of metabolic syndrome, which might be related to transportation and storage of host Fe, and ultimately lead to the occurrence of gestational anemia (Koren et al., 2012). Microbial signatures have been elucidated to function as novel biomarkers to discriminate patients suffering from illness and healthy individuals (Liu et al., 2019; Wei et al., 2020). To date, an anemia classifier in pregnancy has been constructed (Long et al., 2021); however, no efforts have been made to predict the subsequent incidence of anemia in normal gestational women.

To reach a better understanding of how gestational anemia is developing and regulated, herein, we conducted a prospective study to rigorously evaluate dynamic landscape of gut microbiota varying from healthy status to identified anemic status in pregnant women. Leveraging the discriminative microbial signatures in the early stage of pregnancy when anemia did not occur yet, we prospectively built an accurate prediction model for the subsequent incidence of gestational anemia. Our findings help identify pregnant women with high risk of anemia in general population and ultimately improve quality of life and well-being of women and children.

MATERIALS AND METHODS

Study Design and Participants

This study used data from the Mother and Child Microbiome Cohort (MCMC) Study, a prospective birth cohort study initiated and maintained in Nanjing Maternity and Child Health Care Hospital. The recruitment of eligible study pregnant women was from 2017 to 2018 (n = 1527), when they were all in the second trimester. This cohort aimed to explore the relationship between gut microbiota and maternal and children's health.

Among 1527 eligible women who were enrolled, 781 subjects have provided fecal samples both in the second and third trimester. Then, those with assisted conception (n = 19) or twin pregnancy (n = 8) were excluded. Additionally, women with pregnant complications containing diabetes mellitus and pathological anemia (e.g., aplastic anemia and hemolytic anemia) were excluded (n = 142). Women with diagnosed anemia in the second trimester were excluded (n = 54). In the final analysis, 558 participants were included (**Supplementary Figure 1**).

Though Hb < 110 g L^{-1} has been the accepted criterion for the diagnosis of gestational anemia, we reduced the diagnostic level to Hb < 100 g L^{-1} considering the increasing plasma volume during pregnancy, to reduce bias (Lund and Donovan, 1967). All subjects accepted treatment after they were diagnosed with anemia in our cohort.

Ethics

We obtained signed informed consent from all participants. The study was approved by the Ethics Committee of Nanjing Medical University [FWA00001501 No. (2017) 003].

TABLE 1 | Characteristics of the study cohort.

Characteristics	Healthy control (n = 402)	GA* (n = 156)	*P*
Age (years)	28.89 ± 3.19	29.39 ± 3.30	0.76
Pre pregnant BMI(kg/m^2)	22.44 ± 5.91	21.85 ± 4.85	0.23
Fetal gender			
Boy	204 (50.75)	79 (50.64)	0.98
Girl	198 (49.25)	77 (49.36)	
Fetal BMI(kg/m^2)	13.41 ± 1.26	13.79 ± 1.19	0.71
Gravidity			
1	238 (59.20)	90 (57.69)	0.75
≥2	164 (40.80)	66 (42.31)	
Parity			
0	303 (75.37)	122 (78.21)	0.48
≥1	99 (24.63)	34 (21.79)	
Family income (Chinese Yuan/year)			
<100,000	27 (6.72)	6 (3.85)	0.21
100,000–200,000	164 (40.80)	53 (33.97)	
>200,000	30 (7.46)	16 (10.26)	
Maternal education level			
College and below	86 (21.39)	36 (23.08)	0.81
University	156 (38.81)	57 (36.54)	
Graduate school and above	47 (11.69)	20 (12.82)	
Passive smoking			
Never	123 (30.60)	54 (34.62)	0.06
Seldom	159 (39.55)	64 (41.03)	
Always	32 (7.96)	4 (2.56)	
Antibiotic use before in the early stage			
No	167 (41.54)	55 (35.26)	0.10
Yes	205 (51.00)	94 (60.26)	
Folic acid and iron supplement use in the early stage			
No	28 (6.97)	4 (2.56)	0.09
Yes	374 (93.03)	152 (97.44)	
Alcohol use pre pregnancy			
No	246 (61.19)	94 (60.26)	0.97
Yes	18 (4.48)	7 (4.49)	
Caffeine use pre pregnancy			
No	158 (39.30)	64 (41.03)	0.42
Yes	125 (31.09)	42 (26.92)	
Alcohol use during pregnancy			
No	270 (67.16)	99 (63.46)	
Yes	0 (0.00)	0 (0.00)	
Caffeine use during pregnancy			
No	238 (59.20)	91 (58.33)	0.23
Yes	44 (10.95)	11 (7.05)	

Data presented by mean ± SD or n (%).
**GA (gestational anemia), based on the diagnosis of objects in the third trimester.*

Sample Collection and Sequencing Data

All samples were collected prior to anemia treatment. Fecal samples were obtained from each participant for measuring gut microbiota during the second (at 24 weeks of pregnancy) and third trimester (at 32 weeks of pregnancy), respectively, and were stored at -80°C until DNA extraction. Serum samples were obtained during the second trimester and were frozen in -20°C freezers. Serum ferritin levels were measured by electrochemiluminescence immunoassay on the immunoassay analyzer (Beckman Coulter Inc., Fullerton CA, USA) with the same batch of reagents.

16S rRNA sequencing was performed using primers (338F: 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3'). Each sample genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). From extracted DNA, we sequenced the hypervariable region 16S rRNA gene V3 to V4 regions by HiSeq2500 PE250 platform in Meiji Bioinformatics Technology Co. Ltd (Nanjing, China). Blinded positive quality control (QC) specimens were used across all sequencing batches for quality control.

The 16S rRNA sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME2 V.2020.6). The dada2 plugin was used to denoise sequences, and this quality control process will additionally filter any phiX reads (commonly present in marker gene Illumina sequence data) that were identified in the sequencing data, and will filter chimeric sequences. Amplicon sequence variants (ASVs) were obtained at 100% sequence homology; the taxonomy was assigned against the Silva database (Silva 138 release). To minimize the effect of spurious sequences, one case with too low sequence number was excluded. Representative sequences for each ASV were built into a phylogenetic tree with FastTree plugin. Alpha and beta diversity analyzes were conducted at a rarefied sampling depth of 31291.

Statistical Analysis

To compare maternal anemia information by characteristics, *t*-test for continuous variables and χ^2 test for categorical variables were used. Multivariate linear regression model was applied to multiple-factor analysis. Model 1 was crude model; Model 2 was adjusted for

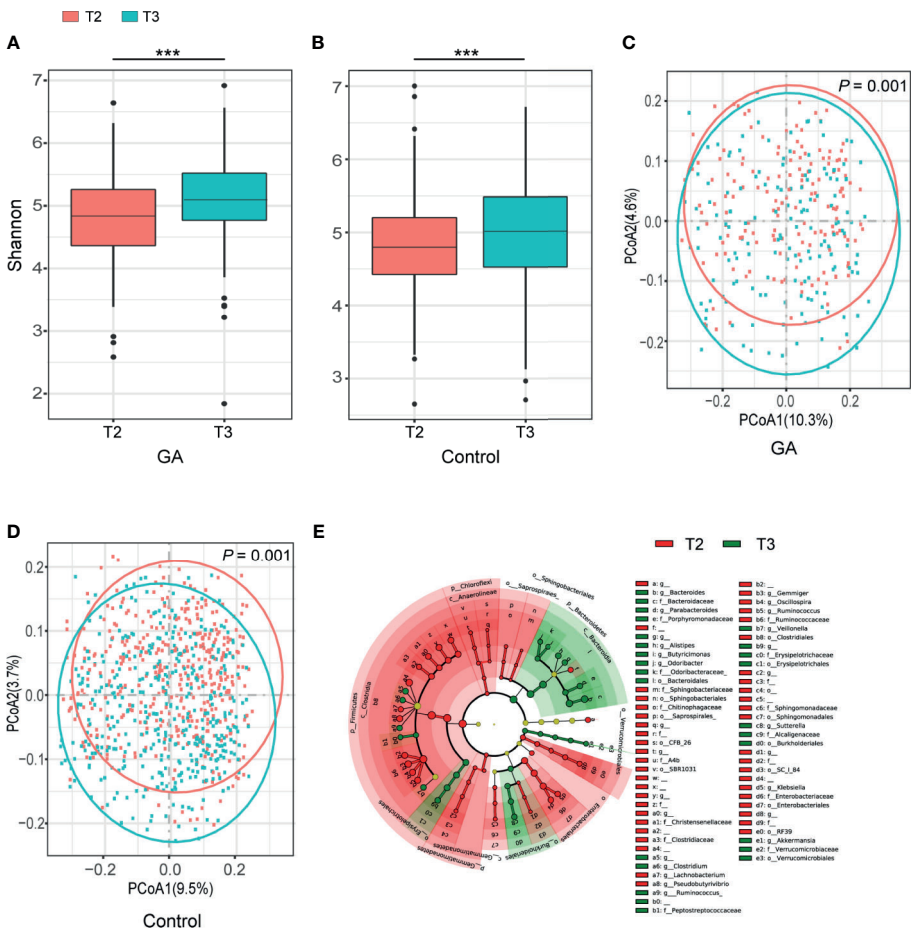


FIGURE 1 | Comparisons of alpha-diversity, beta-diversity, and variations of gut microbiota composition between T2 and T3. **(A)** Box plots based on Shannon diversity index in GA group. **(B)** Box plots based on Shannon diversity index in healthy control group. **(C)** PCoA based on unweighted UniFrac matrix of GA group. **(D)** PCoA based on unweighted UniFrac matrix of healthy control group. **(E)** The cladogram showed differently enriched taxa in the second trimester and the third trimester (FDR < 0.05). FDR, false discovery rate; PCoA, principal coordinate analysis; GA, gestational anemia; T2, the second trimester; T3, the third trimester; ***P < 0.001.

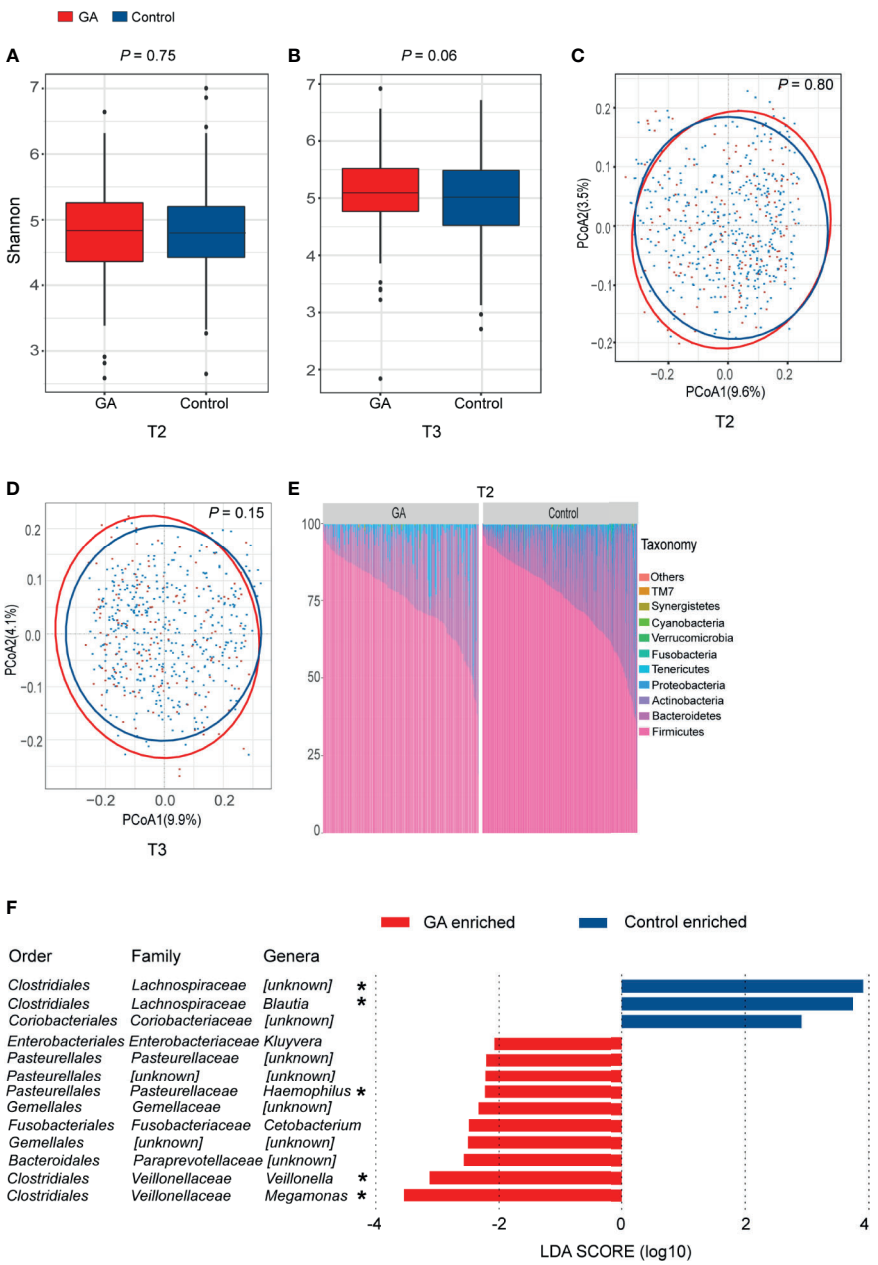


FIGURE 2 | Comparisons of alpha-diversity, beta-diversity, and variations of gut microbiota composition between GA and healthy control. **(A)** Box plots based on Shannon diversity index in the second trimester. **(B)** Box plots based on Shannon diversity index in the third trimester. **(C)** PCoA based on unweighted UniFrac matrix of T2 group. **(D)** PCoA based on unweighted UniFrac matrix of T3 group. **(E)** Relative proportions of bacterial phyla in GA and healthy control in the second trimester. **(F)** Histogram of the LDA scores computed for differentially abundant taxa between GA and healthy control in the second trimester (FDR < 0.05). FDR, false discovery rate; PCoA, principal coordinate analysis; GA, gestational anemia; T2, the second trimester; T3, the third trimester; LDA, linear discriminant analysis; *Genera remained significantly associated with GA after adjusting for covariates using multivariate association with linear models algorithm (MaAsLin). *$P < 0.05$.

maternal age, pre pregnant body mass index (BMI), parity, gravidity, family income, maternal education level, passive smoking, antibiotic use during pregnancy, folic acid and iron supplement use during pregnancy, alcohol and caffeine use pre pregnancy, and alcohol and caffeine use during pregnancy.

Wilcoxon rank-sum test was used to compare α-diversity, and permutational multivariate analysis of variance (PERMANOVA) using 9999 permutations was used to test for statistical significance between two groups. Given a false discovery rate (FDR) of 5%, linear discriminant analysis effect size (LEfSe) was used to identify bacteria

differentially abundant between anemic women and normal women (Segata et al., 2011). To further validate the results, after adjusting for maternal age, pre pregnant BMI, parity, gravidity, family income, maternal education level, passive smoking, antibiotic use during pregnancy, folic acid and iron supplement use during pregnancy, alcohol and caffeine use pre pregnancy, and alcohol and caffeine use during pregnancy, multivariate association with linear models algorithm (MaAsLin) analysis was performed (Morgan et al., 2012; Wei et al., 2020).

The top 99 most abundant genera were used to construct co-abundance group (CAG) network. Kendall correlation was calculated by the function "cor". CAG was defined with a Spearman correlation distance metric using the "Made4" R package. The appropriate number of clustering was selected based on significance testing among each group of the original Kendall correlation matrix using "adonis" function in "Vegan" R package. The sum of relative abundance of ASVs which belonged to the same CAG was calculated to represent the abundance of this CAG (Zhang et al., 2021). Subsequently, we used "qgraph" R package to construct the regularized partial correlation network based on least absolute shrinkage and selection operator (lasso) (Epskamp and Fried, 2018; Liang et al., 2020).

Spearman correlation was used to investigate relationships among continuous variables, and point biserial correlation was used to examine relationships between binary variable and continuous variables. Finally, we constructed receiving operational curve (ROC) and calculated area under curve (AUC) to assess the predictive performance of the model with the "pROC" R package.

RESULTS

Characteristics of the Study Population

Totally, 156 (27.96%) women diagnosed with gestational anemia (GA) and 402 healthy control in the third trimester, all of whom were non-anemic in the second trimester, constituted the study population for final analysis. Detailed demographic features of the cohort were summarized in **Table 1**.

Fecal Microbiota Altered Dramatically During Pregnancy

Shannon index indicated progression of pregnancy from the second trimester (T2) to the third trimester (T3) was accompanied by an increment in α-diversity (GA: $P < 0.001$,

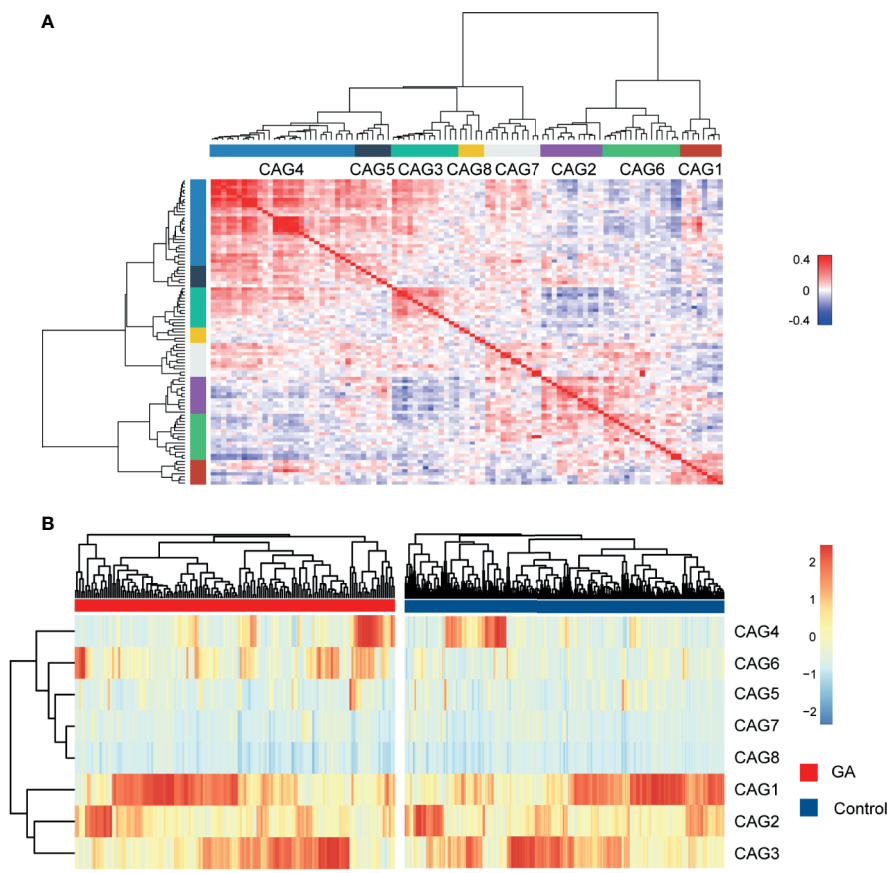


FIGURE 3 | Heatmap of microbial co-abundance group. **(A)** Kendall correlations coefficients between the top 99 most abundant genera in the second trimester were calculated, and eight CAGs were clustered based on Kendall correlation matrix. **(B)** CAG differently enriched in GA and healthy control in the second trimester. GA, gestational anemia; CAG, co-abundance group.

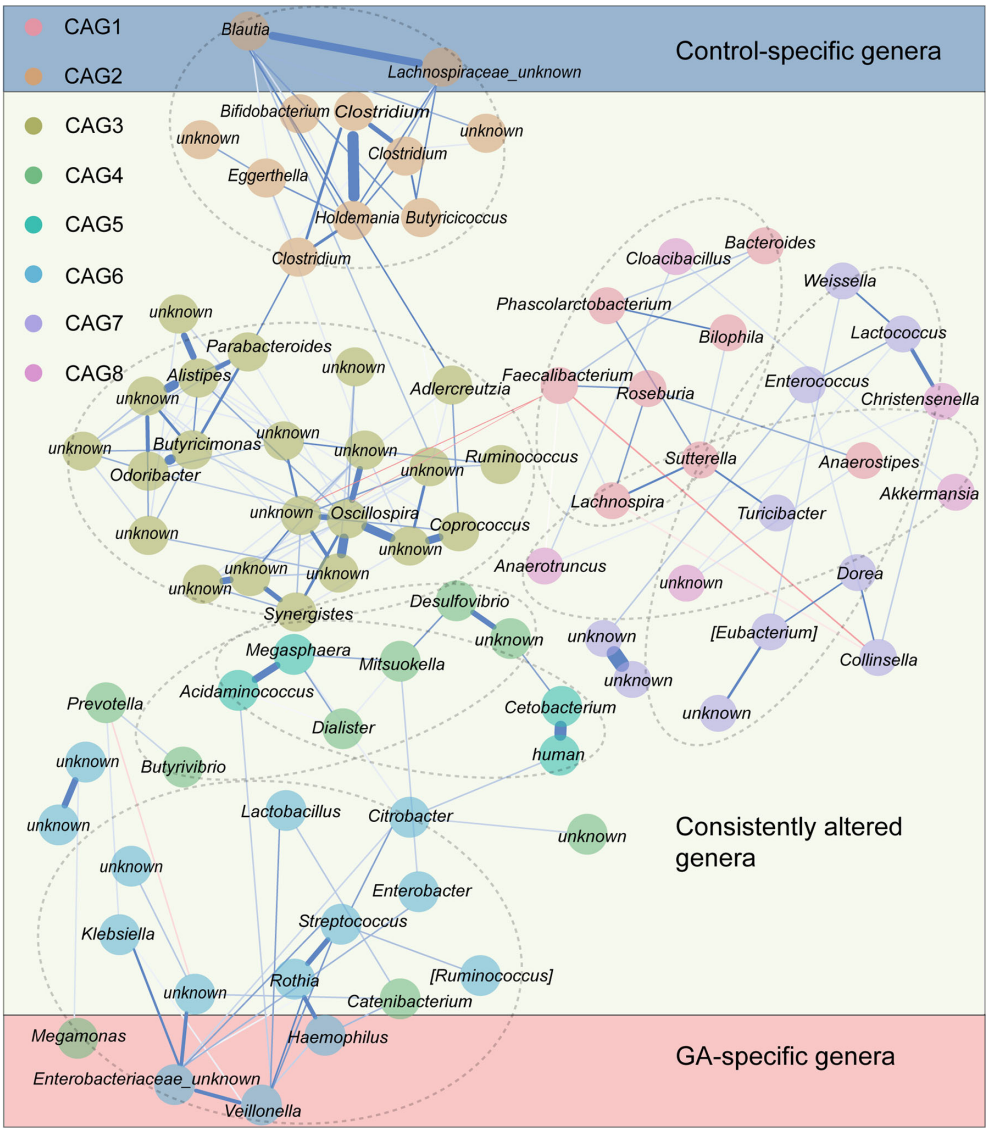


FIGURE 4 | Co-abundance group network reflecting microbial changes in GA and healthy control. Regularized partial correlation network of top altered taxa in GA in the second trimester. Each node represented a taxon, and each edge represented the strength of partial correlation between two taxa. Edge weights represented the partial correlation coefficients. Blue edge represented positive correlation, and red edge represented negative correlation. GA, gestational anemia.

Control: $P < 0.001$, **Figures 1A, B**) and the observed ASVs suggested the same trend (**Supplementary Table 1**). Principal coordinate analysis (PCoA) based on unweighted UniFrac distance was conducted to elaborate the overall structure of microbial composition. PERMANOVA manifested significant differences in structure and composition of the microbiota between T2 and T3 (GA: $P = 0.001$, Control: $P = 0.001$, **Figures 1C, D**). Such significant difference was also observed based on weighted UniFrac distance (**Supplementary Table 1**). LEfSe analysis revealed that remarkable differences were observed between T2 and T3 in pooled group (LDA > 2, FDR < 0.05, **Figure 1E** and **Supplementary Table 2**).

Changes in the Gut Microbiota Between GA and Healthy Control

Both Shannon index (T2: $P = 0.75$, T3: $P = 0.06$, **Figures 2A, B**) and the observed ASVs (**Supplementary Table 1**) in different trimesters showed that there was no difference in richness and diversity of the gut microbiota between GA and healthy control. No significant difference was observed between GA and healthy control based on unweighted or weighted UniFrac distance in both T2 and T3 (T2: $P = 0.80$, T3: $P = 0.15$, **Figures 2C, D** and **Supplementary Table 1**).

In both T2 and T3, overall microbiota composition of maternal fecal microbiota at phylum level showed that the maternal fecal

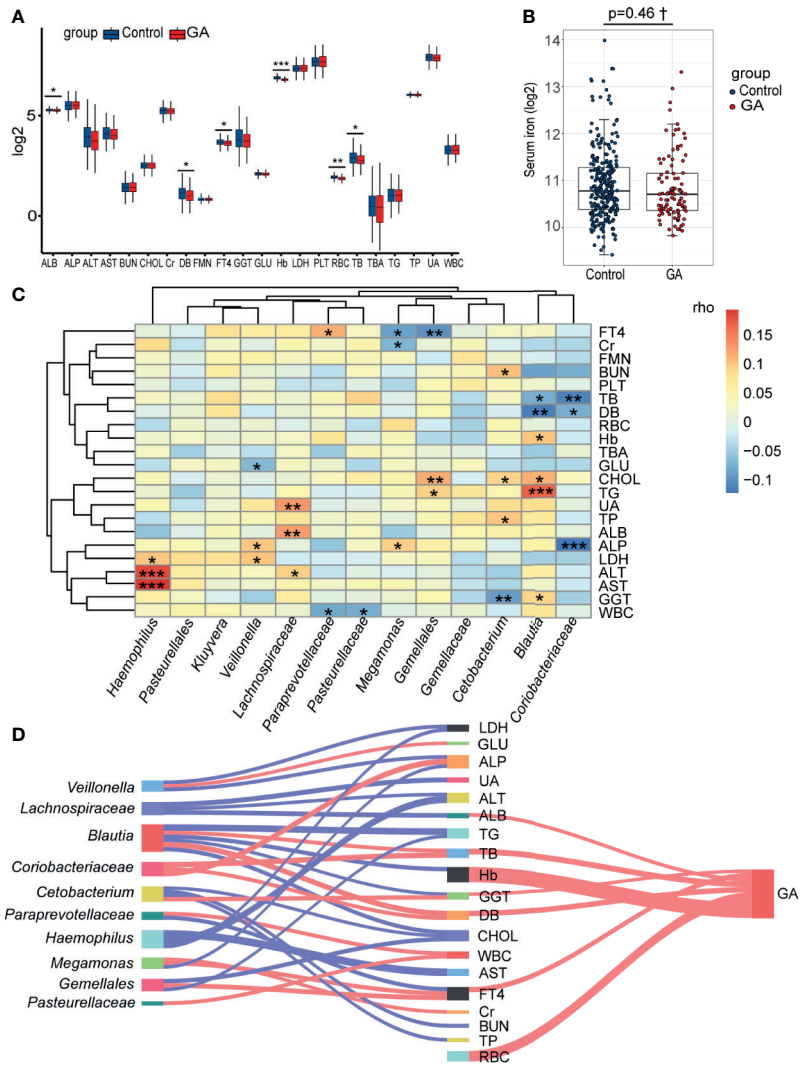


FIGURE 5 | Correlations among the gut microbiota, clinical indices, and GA. **(A)** The box plot showed that the clinical indices significantly changed between two groups. **(B)** The difference of levels of serum iron in the second trimester between the two groups. **(C)** The heatmap of the partial Spearman correlations between gut microbiota and clinical indices in the second trimester (FDR < 0.05). **(D)** Relationships among gut microbiota composition, clinical indices, and GA (only significant correlations were presented, FDR < 0.05). The width of lines represented the partial correlation coefficients. Red line represented negative correlation and blue line represented positive correlation. GA, gestational anemia; FDR, false discovery rate; *P < 0.05, **P < 0.01, ***P < 0.001; †Adjusted for potential covariates.

microbiota was dominated by *Firmicutes* and *Bacteroidetes*, implying there was no significant change between GA and healthy control at the phylum level (**Figure 2E** and **Supplementary Figure 2**). In the second trimester, 10 bacterial taxa, including *Megamonas*, *Veillonella*, *Paraprevotellaceae*, *Gemellales*, *Cetobacterium*, *Gemellaceae*, *Haemophilus*, *Pasteurellales*, *Pasteurellaceae*, and *Kluyvera* were observed enriched in GA versus control (LDA > 2, FDR < 0.05, **Supplementary Table 3**). *Megamonas*, *Veillonella*, and *Haemophilus* were confirmed to show differential abundances between GA and healthy control after validated by MaAsLin analysis. On the contrary, *Lachnospiraceae* and *Blautia* were enriched in control (**Figure 2F**). Detailed information was shown in **Supplementary Table 4**. In the third trimester, after adjusting for covariates, increased abundance in *Veillonella* and decreased abundance in *Lachnospiraceae* and *Blautia* were observed in GA (**Supplementary Figure 2**).

Microbial Co-abundance Group Network

Since bacteria work as functional groups (Zhang et al., 2015), in the second trimester, the top 99 most abundant genera were clustered into 8 CAGs according to their co-abundance correlations (**Figures 3A, B**), and the regularized partial correlation network based on lasso regression was constructed (**Figure 4**). CAG1, CAG2, CAG3, and CAG4, accounting for 86.36% of ASVs, were consistently abundant both in GA and healthy control. Intriguingly, the significantly enriched taxa in GA belonged to CAG6 and the significantly enriched taxa in healthy

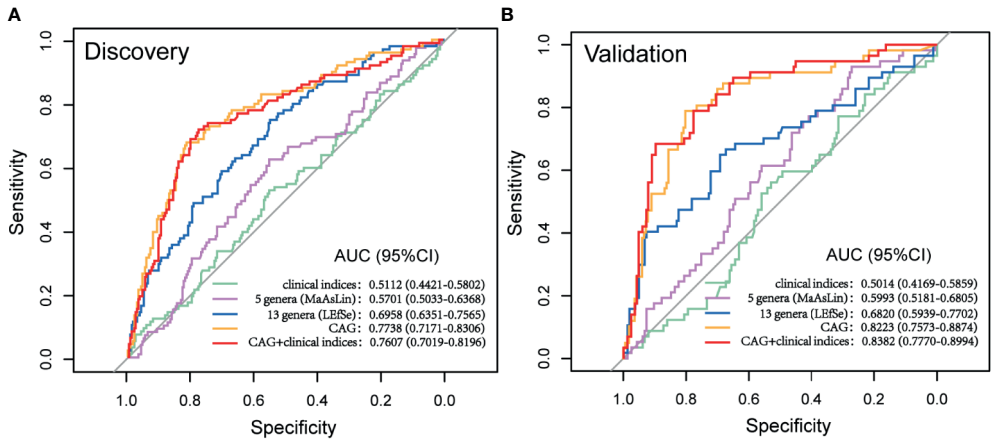


FIGURE 6 | Prediction model of GA based on microbial signatures. **(A)** Discovery set (GA: 91; control: 244). **(B)** Validation set (GA: 65, control: 158). GA, gestational anemia; AUC, area under curve.

control were specific to CAG2. These findings suggested a highly coordinated microbial regulatory network might underlie the occurrence of gestational anemia. Detailed information on CAG clusters was shown in **Supplementary Table 5**.

Interrelationship Between Gut Microbiota Composition, Clinical Indices, and GA

The abundance comparison adjusted for potential confounders showed decreased albumin (ALB), direct bilirubin (DB), free thyroxine (FT4), Hb, red blood cell (RBC), and total bilirubin (TB) were significantly associated with GA in the second trimester (**Figure 5A** and **Supplementary Table 6**). Intriguingly, no significant association was observed between GA and serum iron after adjusting for covariates (**Figure 5B** and **Supplementary Table 7**). Considering an FDR of 5%, the partial Spearman correlation delineated that 13 differentially abundant bacterial taxa were significantly correlated with clinical indices (**Figure 5C** and **Supplementary Tables 8**, **9**). As the Sankey plots demonstrated, in the second trimester, *Megamonas* was negatively correlated with FT4 and further negatively correlated with GA, and *Blautia* was positively correlated with Hb and further negatively correlated with GA, indicating that gut microbiota could be involved in occurrence of anemia by interacting with clinical indices (**Figure 5D**).

Potential Predictive Value of Gut Microbial Signatures for GA

The cohort was further randomly divided into a Discovery set (GA: 91; Control: 244) and a Validation set (GA: 65; Control: 158). As shown in **Figure 6**, clinical indices alone had a poor performance in predicting upcoming anemia (Discovery AUC: 0.5112, 95%CI: 0.4421, 0.5802; Validation AUC: 0.5014, 95%CI: 0.4169, 0.5859). Further on, the potential value of gut microbiota acting as predictor was assessed. Using five genera adjusted by MaAsLin generated an AUC of 0.5701 (95%CI: 0.5033, 0.6368) in Discovery set and 0.5993 (95%CI: 0.5181, 0.6805) in Validation set. Using 13 genera based on LEfSe yielded an AUC of 0.6958 (95%CI: 0.6351, 0.7565) in Discovery set and 0.6820 (95%CI: 0.5939, 0.7702) in Validation set. Of note, CAG accurately predicted an upcoming GA with an AUC of 0.7738 (95%CI: 0.7171, 0.8306) and the classifying ability was further validated in Validation set (0.8223, 95%CI: 0.7573, 0.8874). No significant improved predictive performance was observed when including the combination of CAG and clinical indices into the prediction model (Discovery AUC: 0.7607, 95%CI: 0.7019, 0.8196; Validation AUC: 0.8382, 95%CI: 0.7770, 0.8994).

DISCUSSION

In the current study, we delineated that GA microbial dysbiosis was characterized by several bacterial genera and structured CAG. A cross-sectional anemia classifier in the first trimester and second trimester has been constructed (Long et al., 2021); however, limited efforts have been made to prospectively predict future anemic women. The greatest advantage was that for the first time, a prediction model for upcoming anemia in normal pregnant women with relatively high discriminatory power was established based on novel gut microbial signatures.

It was challenging to determine the etiology of anemia and confirm IDA in pregnancy. Apart from Hb, more laboratory examinations (e.g., serum iron, transferrin receptor, transferrin saturation, and bone marrow iron) should be taken into consideration. The most common true anemia in pregnancy was IDA. In addition, there were no other types of pathological anemia (e.g., aplastic anemia and hemolytic anemia) remaining in our study. Thus, anemic women were basically postulated to suffer from IDA.

Clinical indices serve as generally accepted diagnostic criteria for GA cross-sectionally; however, they were confirmed to have very limited predictive value for potential impending anemia in normal pregnant women according to our study. Gut microbial

signatures exhibited impressive performance in the prediction model. Bacteria were significantly differently abundant in GA and healthy control in the second trimester. Of note, 93.03% of healthy control and 97.44% of anemic women took folic acid and iron supplement from conception to the second trimester, implying the sufficient iron storage. From the perspective of potential mechanism, the altered gut microbiota in the early stage was conjectured to be subsequently associated with the altered health condition (i.e., from iron sufficiency to iron deficiency or malnutrition) and further accounted for the upcoming anemia.

Megamonas, *Veillonella*, and *Haemophilus* were enriched in GA. *Megamonas* could act as a beneficial bacterium, and it has been reported that compared to healthy microbiota, canine anal furunculosis (CAF) microbiota showed a decreased abundance of *Megamonas* (Maldonado-Contreras et al., 2020). Nevertheless, another study illustrated infant vitamin D supplementation was associated with a lower abundance of *Megamonas* in gut microbiota, implying the potential competitive relationship between vitamin D and *Megamonas* (Drall et al., 2020). *Veillonella* species, documented as the Fe (III)-reducing genera, were capable of supplying Fe (II) to combine with oxygen in Hb (Jin et al., 2019). We hypothesized there might be a negative feedback regulation, host generating more *Veillonella* species when detecting less Hb combined with Fe (II). *Haemophilus* is a genus of Gram-negative, containing several markedly pathogenic bacteria, such as *Haemophilus influenzae* causing septicemia. Indigenous bacteria might inhibit host Fe transport and storage *via* producing metabolites that suppress hypoxia-inducible factor 2α (HIF-2α), assumed as a master transcription factor of intestinal Fe absorption and increasing the Fe-storage protein ferritin (Das et al., 2020). Decreased incidence of *Blautia* has been detected in the gut microbiota of obese children and *Blautia* genera might help to reduce inflammation causally linked to obesity-related complications (Benítez-Páez et al., 2020).

Microbial network has been an increasingly popular tool to explore microbial community structure (Röttjers and Faust, 2018). Ecologically, gut microbiota exists in functional groups named "guilds" rather than isolation and thrives in communities with large numbers and develops close interactions, which are critical evolutionary pressures for natural selection in microbial evolution (Faust and Raes, 2012; Ma et al., 2020). We sought to reduce the dimensionality of microbial datasets to identify GA more effectively based on CAG, and interestingly, the prediction model exhibited a much higher discriminatory power.

It is noticeable that normal physiologic changes in pregnancy lead to a relative or absolute reduction in Hb concentration. However, it is still an open question that this "anemia" is physiologic or pathologic. Given gut microbial signatures were free from influence of hydremia, a better diagnostic or predictive performance using gut microbial signatures could be achieved when it comes to either true anemia or physiologic anemia.

There were some advantages of our study. Firstly, samples were collected prior to treatment initiation in a large and well-characterized cohort. Secondly, antibiotics use was controlled in analysis. On the other hand, most of pregnant women enrolled in the study claimed antibiotics were used very early in pregnancy at a low dose and once they were discovered to be pregnant, antibiotics use was avoided as much as possible, which meant a possible lesser impact of antibiotics on gut microbiota. Thirdly, we constituted the first exploration to prospectively predict the risk of anemia in healthy subjects based on gut microbiota. Lastly, a much more accurate prediction model was built based on CAG network.

There were several limitations. Firstly, as we have discussed above, diagnostic evidence of IDA was not sufficient enough. Secondly, we did not construct a GA classifier using gut microbiota in the third trimester since that Hb was supposed to be a better alternative, quicker and costing lower. There was no information on vitamin D levels or supplementation in women, which was supposed to be a factor associated to gut dysbiosis and consequently GA. In addition, lack of metagenomics sequencing limited data interpretation from the angle of species level and bacterial function. Finally, there was not an independent cohort to verify the prediction model; our study merely provided evidence of association rather than causality and further studies are supposed to be conducted to validate the association.

CONCLUSIONS

Our results showed that alterations in the gut microbial community were associated with anemia in pregnancy. Moreover, microbial signatures relatively accurately predicted the subsequent incidence of anemia in normal pregnant women. Our findings could provide new insights into understanding the role of gut microbiota in GA, identifying high-risk individuals, and modulating gut microbiota as a therapeutic target, thus improving quality of life and well-being of women and children.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Nanjing Medical University [FWA00001501 No. (2017) 003]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: XW and YX. Formal analysis: HW and SD. Methodology: HW and YQ. Writing original draft: HW and SD. Verifying the underlying data: YQ and XY. Review and editing: YQ, XY, TC, XW, and YX. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by China-U.S. Program for Biomedical Collaborative Research (NSFC-NIH) (81961128022), the fifth phase of "333 High-level Talent Training Project" of the Jiangsu Province (BRA2020070), Postgraduate Research & Practice Innovation Program of Jiangsu Province (JX10313741), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

ACKNOWLEDGMENTS

The authors would like to acknowledge and are grateful for all the patient participants, infants, and their families that made this study possible.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.734561/full#supplementary-material

Supplementary Figure 1 | Flow chart of the inclusion of subjects.

Supplementary Figure 2 | (A) Relative proportions of bacterial phyla in GA and healthy control in the third trimester. **(B)** Histogram of the LDA scores computed for differentially abundant taxa between GA and healthy control in the third trimester. GA, gestational anemia; LDA, linear discriminant analysis; *Genera remained significantly associated with GA after adjusting for covariates using multivariate association with linear models algorithm (MaAsLin).

REFERENCES

Anker, S. D., Comin Colet, J., Filippatos, G., Willenheimer, R., Dickstein, K., Drexler, H., et al. (2009). Ferric Carboxymaltose in Patients With Heart Failure and Iron Deficiency. *N. Engl. J. Med.* 361 (25), 2436–2448. doi: 10.1056/NEJMoa0908355

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., et al. (2004). The Gut Microbiota as an Environmental Factor That Regulates Fat Storage. *Proc. Natl. Acad. Sci. U.S.A.* 101 (44), 15718–15723. doi: 10.1073/pnas.0407076101

Balamurugan, R., Mary, R. R., Chittaranjan, S., Jancy, H., Shobana Devi, R., and Ramakrishna, B. S. (2010). Low Levels of Faecal Lactobacilli in Women With Iron-Deficiency Anaemia in South India. *Br. J. Nutr.* 104 (7), 931–934. doi: 10.1017/s0007114510001637

Benítez-Páez, A., Gómez Del Pugar, E. M., López-Almela, I., Moya-Pérez, Á., Codoñer-Franch, P., and Sanz, Y. (2020). Depletion of Species in the Microbiota of Obese Children Relates to Intestinal Inflammation and Metabolic Phenotype Worsening. *mSystems* 5 (2), e00857–19. doi: 10.1128/mSystems.00857-19

Camaschella, C. (2015). Iron-Deficiency Anemia. *N. Engl. J. Med.* 372 (19), 1832–1843. doi: 10.1056/NEJMra1401038

Das, N. K., Schwartz, A. J., Barthel, G., Inohara, N., Liu, Q., Sankar, A., et al. (2020). Microbial Metabolite Signaling Is Required for Systemic Iron Homeostasis. *Cell Metab.* 31 (1), 115–130.e6. doi: 10.1016/j.cmet.2019.10.005

Drall, K. M., Field, C. J., Haqq, A. M., de Souza, R. J., Tun, H. M., Morales-Lizcano, N. P., et al. (2020). Vitamin D Supplementation in Pregnancy and Early Infancy in Relation to Gut Microbiota Composition and Colonization: Implications for Viral Respiratory Infections. *Gut Microbes* 12 (1), 1799734. doi: 10.1080/19490976.2020.1799734

Epskamp, S., and Fried, E. I. (2018). A Tutorial on Regularized Partial Correlation Networks. *Psychol. Methods* 23 (4), 617–634. doi: 10.1037/met0000167

Faust, K., and Raes, J. (2012). Microbial Interactions: From Networks to Models. *Nat. Rev. Microbiol.* 10 (8), 538–550. doi: 10.1038/nrmicro2832

Figueiredo, A., Gomes-Filho, I. S., Silva, R. B., Pereira, P. P. S., Mata, F., Lyrio, A. O., et al. (2018). Maternal Anemia and Low Birth Weight: A Systematic Review and Meta-Analysis. *Nutrients* 10 (5), 601. doi: 10.3390/nu10050601

Goonewardene, M., Shehata, M., and Hamad, A. (2012). Anaemia in Pregnancy. *Best Pract. Res. Clin. Obstet. Gynaecol.* 26 (1), 3–24. doi: 10.1016/j.bpobgyn.2011.10.010

Guignard, J., Deneux-Tharaux, C., Seco, A., Beucher, G., Kayem, G., and Bonnet, M. P. (2021). Gestational Anaemia and Severe Acute Maternal Morbidity: A Population-Based Study. *Anaesthesia* 76 (1), 61–71. doi: 10.1111/anae.15222

Human Microbiome Project Consortium. (2012). Structure, Function and Diversity of the Healthy Human Microbiome. *Nature* 486 (7402), 207–214. doi: 10.1038/nature11234

Hunt, J. M. (2002). Reversing Productivity Losses From Iron Deficiency: The Economic Case. *J. Nutr.* 132 (4 Suppl), 794s–801s. doi: 10.1093/jn/132.4.794S

Imhann, F., Vich Vila, A., Bonder, M. J., Fu, J., Gevers, D., Visschedijk, M. C., et al. (2018). Interplay of Host Genetics and Gut Microbiota Underlying the Onset and Clinical Presentation of Inflammatory Bowel Disease. *Gut* 67 (1), 108–119. doi: 10.1136/gutjnl-2016-312135

Jin, Z., Zhao, Z., and Zhang, Y. (2019). Potential of Direct Interspecies Electron Transfer in Synergetic Enhancement of Methanogenesis and Sulfate Removal in an Up-Flow Anaerobic Sludge Blanket Reactor With Magnetite. *Sci. Total Environ.* 677, 299–306. doi: 10.1016/j.scitotenv.2019.04.372

Khan, K. S., Wojdyla, D., Say, L., Gülmezoglu, A. M., and Van Look, P. F. (2006). WHO Analysis of Causes of Maternal Death: A Systematic Review. *Lancet* 367 (9516), 1066–1074. doi: 10.1016/s0140-6736(06)68397-9

Khosravi, A., Yáñez, A., Price, J. G., Chow, A., Merad, M., Goodridge, H. S., et al. (2014). Gut Microbiota Promote Hematopoiesis to Control Bacterial Infection. *Cell Host Microbe* 15 (3), 374–381. doi: 10.1016/j.chom.2014.02.006

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150 (3), 470–480. doi: 10.1016/j.cell.2012.07.008

Liang, L., Rasmussen, M. H., Piening, B., Shen, X., Chen, S., Röst, H., et al. (2020). Metabolic Dynamics and Prediction of Gestational Age and Time to Delivery in Pregnant Women. *Cell* 181 (7), 1680–1692.e1615. doi: 10.1016/j.cell.2020.05.002

Liu, H., Chen, X., Hu, X., Niu, H., Tian, R., Wang, H., et al. (2019). Alterations in the Gut Microbiome and Metabolism With Coronary Artery Disease Severity. *Microbiome* 7 (1), 68. doi: 10.1186/s40168-019-0683-9

Li, F., Zhang, C., Chen, Y. G., Shen, J. Y., Jiang, S., Li, J., et al. (2018). [Analysis on Maternal Anemia Rate and Related Factors in Taicang of Jiangsu Province in 2014-2016]. *Zhonghua Yu Fang Yi Xue Za Zhi* 52 (7), 703–708. doi: 10.3760/cma.j.issn.0253-9624.2018.07.005

Long, Y., Liang, F., Guo, R., Zhu, C., Zhao, X., Wang, X., et al. (2021). Gut Microbiota Signatures in Gestational Anemia. *Front. Cell Infect. Microbiol.* 11:549678. doi: 10.3389/fcimb.2021.549678

Lund, C. J., and Donovan, J. C. (1967). Blood Volume During Pregnancy. Significance of Plasma and Red Cell Volumes. *Am. J. Obstet Gynecol* 98 (3), 394–403. doi: 10.1016/0002-9378(67)90160-3

Maldonado-Contreras, A., Ferrer, L., Cawley, C., Crain, S., Bhattarai, S., Toscano, J., et al. (2020). Dysbiosis in a Canine Model of Human Fistulizing Crohn's Disease. *Gut Microbes* 12 (1), 1785246. doi: 10.1080/19490976.2020.1785246

Ma, B., Wang, Y., Ye, S., Liu, S., Stirling, E., Gilbert, J. A., et al. (2020). Earth Microbial Co-Occurrence Network Reveals Interconnection Pattern Across Microbiomes. *Microbiome* 8 (1), 82. doi: 10.1186/s40168-020-00857-2

Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., et al. (2012). Dysfunction of the Intestinal Microbiome in Inflammatory Bowel Disease and Treatment. *Genome Biol.* 13 (9), R79. doi: 10.1186/gb-2012-13-9-r79

Prema, K., Ramalakshmi, B. A., Madhavapeddi, R., and Babu, S. (1982). Immune Status of Anaemic Pregnant Women. *Br. J. Obstet. Gynaecol.* 89 (3), 222–225. doi: 10.1111/j.1471-0528.1982.tb03619.x

Rahman, M. M., Abe, S. K., Rahman, M. S., Kanda, M., Narita, S., Bilano, V., et al. (2016). Maternal Anemia and Risk of Adverse Birth and Health Outcomes in Low- and Middle-Income Countries: Systematic Review and Meta-Analysis. *Am. J. Clin. Nutr.* 103 (2), 495–504. doi: 10.3945/ajcn.115.107896

Röttjers, L., and Faust, K. (2018). From Hairballs to Hypotheses-Biological Insights From Microbial Networks. *FEMS Microbiol. Rev.* 42 (6), 761–780. doi: 10.1093/femsre/fuy030

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic Biomarker Discovery and Explanation. *Genome Biol.* 12 (6), R60. doi: 10.1186/gb-2011-12-6-r60

Sifakis, S., and Pharmakides, G. (2000). Anemia in Pregnancy. *Ann. N Y Acad. Sci.* 900, 125–136. doi: 10.1111/j.1749-6632.2000.tb06223.x

Thomas, A. M., Manghi, P., Asnicar, F., Pasolli, E., Armanini, F., Zolfo, M., et al. (2019). Metagenomic Analysis of Colorectal Cancer Datasets Identifies Cross-Cohort Microbial Diagnostic Signatures and a Link With Choline Degradation. *Nat. Med.* 25 (4), 667–678. doi: 10.1038/s41591-019-0405-7

Vich Vila, A., Imhann, F., Collij, V., Jankipersadsing, S. A., Gurry, T., Mujagic, Z., et al. (2018). Gut Microbiota Composition and Functional Changes in Inflammatory Bowel Disease and Irritable Bowel Syndrome. *Sci. Transl. Med.* 10 (472), eaap8914. doi: 10.1126/scitranslmed.aap8914

Wei, Y., Li, Y., Yan, L., Sun, C., Miao, Q., Wang, Q., et al. (2020). Alterations of Gut Microbiome in Autoimmune Hepatitis. *Gut* 69 (3), 569–577. doi: 10.1136/gutjnl-2018-317836

Wirbel, J., Pyl, P. T., Kartal, E., Zych, K., Kashani, A., Milanese, A., et al. (2019). Meta-Analysis of Fecal Metagenomes Reveals Global Microbial Signatures That are Specific for Colorectal Cancer. *Nat. Med.* 25 (4), 679–689. doi: 10.1038/s41591-019-0406-6

Zhang, Y., Chen, T., Zhang, Y., Hu, Q., Wang, X., Chang, H., et al. (2021). Contribution of Trace Element Exposure to Gestational Diabetes Mellitus Through Disturbing the Gut Microbiome. *Environ. Int.* 153, 106520. doi: 10.1016/j.envint.2021.106520

Zhang, C., Yin, A., Li, H., Wang, R., Wu, G., Shen, J., et al. (2015). Dietary Modulation of Gut Microbiota Contributes to Alleviation of Both Genetic and Simple Obesity in Children. *EBioMedicine* 2 (8), 968–984. doi: 10.1016/j.ebiom.2015.07.007

Zhao, S. Y., Jing, W. Z., Liu, J., and Liu, M. (2018). Prevalence of Anemia During Pregnancy in Chin-2016: A Meta-Analysis. *Zhonghua Yu Fang Yi Xue Za Zhi* 52 (9), 951–957. doi: 10.3760/cma.j.issn.0253-9624.2018.09.016

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 22 September 2021
doi: 10.3389/fcimb.2021.736204

Fecal Microbiota Transplantation Protects the Intestinal Mucosal Barrier by Reconstructing the Gut Microbiota in a Murine Model of Sepsis

*Xiaowei Gai[1,2,3], Huawei Wang[1], Yaqing Li[4], Haotian Zhao[5], Cong He[1], Zihui Wang[1] and Heling Zhao[1]**

[1] Department of Intensive Care Unit, Hebei General Hospital, Shijiazhuang, China, [2] Graduate School of Hebei Medical University, Hebei Medical University, Shijiazhuang, China, [3] Department of Intensive Care Unit, Qinhuangdao Jungong Hospital, Qinhuangdao, China, [4] Department of Infection, Hebei General Hospital, Shijiazhuang, China, [5] Department of Ultrasound, Hebei General Hospital, Shijiazhuang, China

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Helioswilton Sales-Campos, Universidade Federal de Goiás, Brazil
Parag Kundu, Institut Pasteur of Shanghai (CAS), China

***Correspondence:**
Heling Zhao
zhheling@sina.com

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *04 July 2021*
Accepted: *07 September 2021*
Published: *22 September 2021*

Citation:
Gai X, Wang H, Li Y, Zhao H, He C, Wang Z and Zhao H (2021) Fecal Microbiota Transplantation Protects the Intestinal Mucosal Barrier by Reconstructing the Gut Microbiota in a Murine Model of Sepsis. Front. Cell. Infect. Microbiol. 11:736204. doi: 10.3389/fcimb.2021.736204

The gastrointestinal (GI) tract has long been hypothesized to play an integral role in the pathophysiology of sepsis, and gut microbiota (GM) dysbiosis may be the key factor. Previous studies have shown that the gut flora was significantly altered in critically ill patients. This study aimed to observe what kind of GM dysbiosis is in the early stage of sepsis and whether the application of fecal microbiota transplantation (FMT) can reconstruct the GM of septic mice and restore its protective function on the intestinal mucosal barrier. The study investigated the effect of FMT on gut microbiota, mucosal barrier function, inflammatory response, and survival in a murine model of sepsis established by cecal ligation and puncture (CLP). It is found that FMT can not only reduce morbidity and mortality and restore the abundance and diversity of the gut flora in septic mice, but can also improve the intestinal barrier function by reducing epithelial cell apoptosis, improving the composition of the mucus layer, upregulating the expression of tight junction proteins, and reducing intestinal permeability and the inflammatory response. After FMT, Lachnospiraceae contributed the most to intestinal protection through enhancement of the L-lysine fermentation pathway. FMT offers a microbe-mediated survival advantage in a murine model of sepsis. Therefore, an improved understanding of the connection between microbiota, and systemic illness may yield new therapeutic strategies for patients with sepsis.

Keywords: sepsis, fecal microbiota transplantation, intestinal mucosal barrier, gut microbiota, critical care

INTRODUCTION

Sepsis continues to be the leading cause of mortality in the intensive care unit (Fay et al., 2017; Haak and Wiersinga, 2017). The World Health Organization has recognized sepsis as a global health priority (Fay et al., 2019). Despite significant advancement in our understanding of the pathophysiology of sepsis, treatment of sepsis is still limited to antibiotics, aggressive fluid resuscitation, vasopressor administration, and supportive care, and no targeted therapeutics for sepsis are approved for usage in patients (Ames et al., 2018).

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (Ames et al., 2018; Fay et al., 2019; Gong et al., 2019). The syndrome can be induced by a wide variety of microbes by definition and the gastrointestinal tract is the largest pool of bacteria (Fay et al., 2019). It is known that the human gastrointestinal tract contains trillions of bacteria that comprise a complex ecosystem known as the intestinal microbiota that has relevant implications in human health and disease (Bassetti et al., 2020). The symbiotic relationship between microbiota and the host is mutually beneficial (Ekmekciu et al., 2017). The host provides an important habitat and nutrients for the microbiome, and the gut microbiota supports the development of the metabolic system and the maturation of the intestinal immune system by providing beneficial nutrients, for example, by the synthesis of vitamins and short-chain fatty acids (SCFAs) (Shi et al., 2017). Resident microbiota can out-compete pathogens for space, metabolites, and nutrients, and inhibit pathogens by calibrating the host immune response (Bassetti et al., 2020). However, the microbiome is markedly altered in critical illness. Studies have shown that the microbial diversity is diminished within 6 hours of admission to the intensive care unit, and this lack of diversity has been associated with poor outcomes in critically ill patients (Fay et al., 2019).

Considering GM dysbiosis is one of the most important factors that can lead to pathological bacterial translocation and systemic infection, it may be feasible to develop novel therapeutic strategies against gut-derived sepsis by modulating the microbiota (Wang et al., 2019). More than 90% of the commensal organisms may be lost during the early stage of critical illness, making it nearly impossible that a single or several probiotic species would be able to completely replenish the diversity of the GM without intervention. Transfer of healthy donor feces containing thousands of microbial species, termed FMT, facilitates the replenishment of diminished commensal bacteria and may guide the patient's microbiota toward a healthy state (Wang et al., 2019). Fecal microbiota transplantation has been successfully applied to a series of diseases (Otani and Coopersmith, 2019; Zeng et al., 2019). Even though the evidence is limited to some case reports on the treatment of septic patients, the improved clinical outcomes following FMT are promising (Schmidt et al., 2018; Limketkai et al., 2019; Wang et al., 2019). However, during sepsis, the exact mechanism of action for the use of FMT on the intestine is still unknown (Haussner et al., 2019). Considering the important role of microbiota in sepsis, we wonder whether the use of fecal microbiota transplantation in the early stage of sepsis can inhibit or even reverse the clinical outcome.

MATERIALS AND METHODS

Animals Experiments

All experimental procedures were performed by the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health) and were approved by the Animal Ethics Committee of Hebei Medical University (identification number: 202152). Male C57BL/6 mice, approximately 6–8-weeks old and weighing 20 –25 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (license number: SCXK(京)2016-0006). The animals were housed in a temperature-controlled environment (20°C-23°C and 45%-55% humidity) with a 12 h light-dark cycle. The mice were allowed to acclimate to the housing conditions for one week before the experiments started. The experiment was divided into two parts: an acute experiment and a 7-day mortality observation experiment. All experimental animals were randomly divided into four groups: the sham operation group, the sepsis model group, the fecal microbiota transplantation group, and the normal group (healthy donor mice). The ten normal mice were only used to collect feces and make fecal bacteria liquid. The mice were euthanized at 12, 24, and 48 hours following cecal ligation and puncture for acute studies, respectively (Sham: n=6 per time point; CLP: n= 10/11 per time point; FMT: n=10/11 per time point). Ten mice in each group of the sham group, the CLP group, and the FMT group were used for a 7-day mortality observation experiment. All animals had free access to food and water. All surgery was performed under anesthesia, and every effort was made to minimize suffering.

Sepsis Model

Sepsis was induced by cecal ligation and puncture as previously described (Rittirsch et al., 2009; Khailova et al., 2013). Mice were anesthetized using an intraperitoneal injection at 50 mg/kg of 2% sodium pentobarbital. To perform the surgery, the cecum was exposed by a 1.5 cm midline incision in the abdomen. The cecum was ligated at 1cm from the cecal tip using a single suture, punctured with a squeeze to extrude a small amount (droplet) of feces from the perforation sites, and returned to the peritoneal cavity. The location of the cecal ligation and the size of the puncture or hole was determined in each mouse. The amount of extruded cecal content was kept the same to ensure the consistency of the model. The laparotomy was closed with silk sutures. Sham controls were subjected to the same procedures, except that there was no ligation or puncture of the cecum. Animals were resuscitated by subcutaneously injecting pre-warmed normal saline (37°C; 5 mL per 100 g body weight). Recovery of these mice was assessed and recorded 2 hours after surgery, and their survival was recorded daily, including diet, fur, bloating, defecation, mobility, abnormal behavior, and response to stimuli. Tissue, blood, and feces samples of all mice were collected when they were euthanized.

Fecal Microbiota Transplantation

Fresh feces were collected from ten healthy C57BL/6 mice, homogenized in 10 mL of sterile phosphate-buffered saline (PBS), and centrifuged for 30 sec at 800 ×g, 4°C, to pellet the

particulate matter. The optical density (OD) value of the supernatant slurry was checked to calculate the concentration of total bacteria (OD = 0.5 represents 10^8 cells). For each mouse, $1\times10^{9.8}$ bacterial cells (sum of the total bacterial population within 2 g cecal contents) were centrifuged for 5 min at 13,000 ×g, 4°C, and then bacterial pellets were resuspended in 0.2 mL PBS (Li et al., 2015; Li et al., 2017). Mice in the FMT group received a single dose of fecal microbiota just prior to cecal ligation and puncture and were treated for three consecutive days (Khailova et al., 2013). The mice in the CLP group and the Sham group were gavaged with 0.2 mL PBS once a day as a control.

Serum IL-6, IL-10, and TNF-α Analysis

Enzyme-linked immunosorbent assay was used to determine the concentrations of TNF-α (Lot: RXQJYSXLD4, Elabscience, China), IL-6 (Lot: 449268JEI6, Elabscience, China), and IL-10 (Lot: C8QEY2KAVE, Elabscience, China) in serum according to the manufacturer's instructions. Serum was collected after centrifuging blood for 10 min at 4°C and 800 ×g and stored at -80°C until the assay was performed.

Sample Processing for Animal Experiments

After the mice were euthanized, their GI tracts were quickly removed. The colons were gently separated, by cutting at the cecum-colon junction and rectum and divided into two parts. One-half of each colon was immediately frozen in liquid nitrogen and then stored at -80°C until further use. The other part ones were preserved in Carnoy's fixative (dry methanol: chloroform: glacial acetic acid in the ratio 60:30:10) (Johansson et al., 2011; Desai et al., 2016). The Carnoy's fixative was made fresh with anhydrous methanol, chloroform, and glacial acetic acid. The colons were fixed in Carnoy's solution for 3 h followed by transfer to fresh Carnoy's solution for 2-3 h. The colons were then washed in dry methanol for 2 h, placed in cassettes, and stored in fresh dry methanol at 4°C. Samples were then embedded in paraffin, and cut into sections (5μm thick). Cecal contents from each animal were divided into replicates, and they were all instantly flash-frozen in liquid nitrogen and then stored at -80°C until the use for microbiome evaluation.

Immunohistochemistry and Immunofluorescence

The colonic sections were mounted onto polylysine-coated slides, deparaffinized, rehydrated, and placed in a 3% citrate buffer to repair antigens. After being pretreated with 3% H2O2 for 30 min, the sections were blocked with goat serum for 20 min. Sections were incubated overnight at 4°C with a rabbit polyclonal to MUC2 blocking antibody (MUC2, ab90007, Abcam Ltd.; Occludin, ab168986, Abcam Ltd; caspase 3, ab44976, Abcam Ltd.). The sections were washed with PBS and incubated with a secondary antibody for 30 min, rewashed, and incubated with peroxidase-conjugated streptavidin for 30 min. DAB developed, hematoxylin counterstained, dehydrated, and mounted. The secondary antibody of MUC 2 was prepared in 0.5% Triton X-100 PBS buffer, and the Alexa Fluor™ 488 donkey anti-rabbit antibody was diluted 1:1000. The specimen was incubated in this solution at 37°C for 1 h. We aspirated the secondary antibody and rinsed the specimen three times in PBS for 5 min each and covered the specimen with a DAPI coverslip.

Transmission Electron Microscopy (TEM)

For TEM, two 0.5×0.5 cm mini-segments of intestinal tissue from each group were excised and placed in a fixative for TEM at 4°C for 2-4 h. The segments were washed in 0.1 M PBS three times for 15 min each time, postfixed in 1% osmium tetroxide in PBS, dehydrated in a graduated series of ethanol solution, and embedded by baking in an oven at 60°C for 48 h. Samples were cut into 60-80 nm sections and stained with uranyl acetate and lead citrate. The sections were analyzed by electronic microscopy (HT7700 TEM; Hitachi Inc., Tokyo, Japan).

Western Blot

The snap-frozen tissues were subjected to homogenization in 250μL of lysis buffer as previously described. Samples (30 μg of protein for each condition) were transferred onto PVDF membranes and then incubated with antibodies (Li et al., 2017). The following were used as primary antibodies: caspase 3 (ab323519, Abcam), myeloid differentiation factor 88 (MyD88) (SC74532, Santa Cruz), toll-like receptor 4 (TLR4) (AF7017, Affinity), ZO-1 (AF 5145, Affinity), occludin (DF7504, Affinity), and NF-κB(ab16502, Abcam). Immunoreactive bands were revealed using a 1:10,000 dilution of secondary antibody conjugated to horseradish peroxidase (goat anti-rabbit IgG, BE0101, Bioeasy; goat anti-mice IgG, BE0102, Bioeasy). The blots were re-probed with antibodies against β-actin (EASYBIO) and GAPDH (Bioworld) to ensure equal loading and transfer of proteins. All critical blots and immunoprecipitation experiments were repeated at least three times.

Real-Time PCR

Total RNA was isolated from colonic tissue using the RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) as described in the manufacturer's protocol. The RNA concentrations were quantified at 260 nm, and their purity and integrity were determined using a NanoDrop. Reverse transcription and real-time PCR assays were performed to quantify steady-state messenger RNA levels of TLR4, MyD88, and NF-κB at 48 h following CLP. Complementary DNA was synthesized from 0.1 μg of total RNA. The following cycling protocol was used: denaturation at 95°C (15 min) and 40 cycles of 95°C (10 s), 60°C (32 s). The reporter dye emission (SYBR green) was detected by an automated sequence detector combined with ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Microbiome Evaluation

Fecal samples were collected at the time mice were euthanized and frozen until DNA extraction samples were sent to the Shanghai Personal Biotechnology Co., Ltd., where bacterial DNA was extracted. PCR amplification of the bacterial 16S rRNA genes V3-V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The Illumina MiSeq sequencing platform was amplified (Illumina, San Diego, CA, USA) using the NovaSeq-PE250 sequencing strategy. Sequence denoising or OTU clustering was performed according to the

QIIME2 dada 2 analysis process or the Vsearch software analysis process. The resulting sequences were clustered with 100% similarity against the GreenGenes to the database (v. 13.8; https://greengenes.secondgenome.com/) (Fay et al., 2019) to assign bacterial operational taxonomic units. Alpha and beta diversity comparisons were performed using QIIME2 and taxonomic summaries were generated with QIIME2. Comparison of sample compositions and identification of statistically significant differences were performed with LEfSe using the correction for independent comparisons. Microbial functions were predicted by using PICRUSt (Phylogenetic investigation of communities by reconstruction of unobserved states) based on high-quality sequences.

Statistical Analysis

The Kaplan-Meier estimator was used to draw the survival curve of the mice, and the log-rank method was used to compare the survival rates between different groups. The measurement data had a normal distribution, the variance was uniform, and the one-way ANOVA and LSD tests were used for comparison between multiple groups. The Student's t-test was used to compare the two independent groups, and the measurement result was expressed as the mean ± standard deviation (mean ± sd). The Kruskal-Wallis H test was used for data with non-normal distribution and/or uneven variance. The results were expressed in medians (interquartile range). SPSS 21.0, GraphPad Prism 8.0, Photoshop CS5, Image-Pro Plus, and ImageJ were used for data analysis, and $P < 0.05$ was considered statistically significant.

RESULTS

Mortality Among Three Groups

The survival rates of the Sham group, CLP group, and FMT group were compared 7 days following sepsis modeling. The CLP group had a mortality rate of 30% at 24 h and a 50% survival rate at 7 days. There were no deaths at 24 h in the FMT group, and a 90% survival at 7 days, and no deaths in the Sham group. Compared with the Sham group and the FMT group, the mortality of the CLP group was significantly higher, $P < 0.05$ (**Figure 1A**).

Apoptosis

The expression of caspase 3 in the CLP group was significantly higher than that of the other two groups at 12 and 24 h, but the average integral optical density (IOD) of caspase 3 in the FMT group was close to that of the CLP group at 48 h (**Figure 1B**). Therefore, we performed a quantitative analysis of caspase 3 protein. The results of the western blot showed that the expression of caspase 3 in the CLP group at 24 and 48 h was higher than that in the FMT group, $P < 0.001$ (**Figure 1C**).

Mouse Serum Inflammatory Factors TNF-α, IL-6, and IL-10

The expression of IL-6 in the CLP group was the highest at 24 h after modeling, and then decreased, but it was still higher than that in the Sham group at 48 h. Expression of IL-6 in the FMT group was slightly higher than that in the CLP group at 12 h, but there was no significant difference, to the contrary, IL-6 levels were markedly lower than the CLP group at 24 and 48 h. The TNF-α level in the CLP group continued to increase, and it was the highest among the three groups at 48 h, however, the IL-10 level was lowest at 24 h after modeling (**Figure 2**).

The Thickness of the Mucus Layer (nm) and MUC2 Expression

The AB-PAS method was used to detect the colonic mucus layer thickness at 12, 24, and 48 h after sepsis modeling in the three groups. The mucus layer thickness of mice in the CLP group was significantly lower than that of the Sham group during the same timepoint ($P < 0.0001$). The thickness of the mucus layer in the

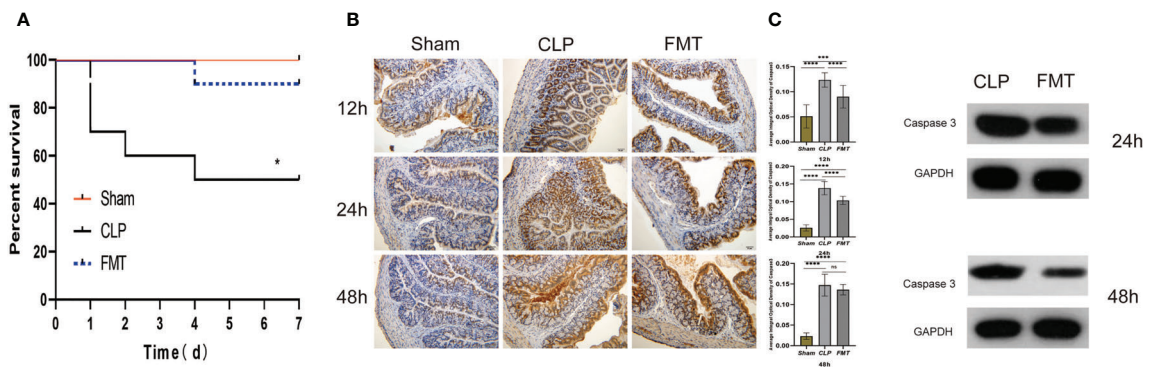


FIGURE 1 | Survival analysis, and caspase 3 expressions among the Sham, CLP, and FMT groups. **(A)** Seven-day mortality observations in the Sham (red), CLP (black), and FMT groups (blue). There were significant differences between the CLP group and the other two groups, $P < 0.05$, but there was no significant difference between the FMT group and the Sham group; **(B)** Immunohistochemistry of the colon [under a digital microscope (20 × 10)]. The average integral optical density of caspase 3 between the Sham, CLP, and FMT groups at 12, 24, and 48 h are shown on the right side. **(C)** Relative expression of caspase 3 compared with GAPDH between the CLP and FMT groups at 24 and 48 h. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, ns, no statistical difference, respectively.

FMT group was significantly greater than that in the CLP group ($P < 0.01$). Compared with the Sham group, the thickness of the mucus layer in the FMT group had no difference at 24 h ($P = 0.4473$) but had a significant difference at 12 and 48 h ($P < 0.0001$, and $P < 0.05$, respectively) (**Figure 3A**). The fluorescence expressions of MUC2 at 12, 24, and 48 h in the three groups were observed by digital microscope and recorded, and the gray value of the green channel was calculated and statistically analyzed. Except that the expression levels of MUC2 in the Sham group and the FMT group were not statistically different at 12 h, there was a significant difference between each pair at 24 and 48 h ($P < 0.0001$). The expression of MUC2 in the CLP group at 12, 24, and 48 h was lower than that of the other two groups (**Figure 3B**).

Transmission Electron Microscope

Intestinal epithelial cells and intracytoplasmic organelles in the CLP group were significantly swollen, and the microvilli were arranged neatly, with uniform thickness and partial shedding. The tight junctions and the structure of the intermediate junctions were blurred, and the gap between junctions was slightly widened in the CLP group. Portions of the desmosomes and tension wires had disappeared. The intercellular space was widened and the mitochondria had swelled. Intestinal epithelial cells and intracytoplasmic organelles in the FMT group were slightly swollen. The microvilli were arranged neatly and uniformly in thickness, and the local area was slightly detached. The tight junctions between epithelial cells and the structure of the intermediate junctions were fuzzy, and the gap was slightly widened in the FMT group. The number of desmosomes was slightly reduced, the surrounding tension filaments were abundant, and the mitochondria were slightly swollen (**Figure 4**).

Tight Junction Proteins

We compared the average integral OD of occludin between the CLP and the FMT groups at 12, 24, and 48 h. The results showed that occludin expression in the CLP group at 12, 24, and 48 h was significantly lower than expression in the FMT group (**Figure 5A**). We further verified occludin and ZO-1 protein expression by western blot test. The results showed the relative expression of these two proteins in the CLP group was significantly lower than that in the other two groups at 24 or 48 h, ($P < 0.001$). The relative expression of the two proteins was highest in the Sham group, intermediate in the FMT group and lowest in the CLP group (**Figure 5B**).

TLR4, MyD88, and NF-κB Protein Levels and mRNA Levels

We analyzed and compared the expression of TLR4, MyD88, and NF-κB relative to GAPDH at 24 and 48 h in the three groups. Except that there was no difference in the expression of TLR4 protein between the Sham and FMT groups, expression levels of the other two proteins in the three groups were significantly different compared with each other. Expression of the three proteins in the CLP group at 24 and 48 h was significantly higher than those in both the Sham and the FMT groups at the same time points ($P < 0.05$) (**Figure 6A**). Coincidently, mRNA expression trends in the three groups were similar to the trends in protein expression. The relative expressions of TLR4, MyD88, and NF-κB, in the CLP group, were significantly higher than those in the Sham and FMT groups. Expression of MyD88, and NF-κB had a significant difference in the CLP group compared with the Sham and FMT groups($P < 0.05$). There was no difference in the expression of the three indicators between the Sham group and the FMT group (**Figure 6B**).

16SrRNA Sequence Analysis

Krona Species Composition

Firmicutes and Bacteroidetes were the main bacteria, accounting for 55% and 33% of the abundance found in the normal mice, respectively. The Sham group was dominated by Firmicutes, at 66%. Proteobacteria was the dominant bacteria in the CLP group. The relative abundance of Proteobacteria observed in the mortality observation of the CLP groups following sepsis modeling at 12 h, 24 h, and 7-day were 48%, 66%, and 42%, respectively. The relative abundance of unclassified bacteria in the CLP group at 48 h and the 7-day mortality observation in the FMT group was 70% and 41%. The FMT group at 24 and 48 h were dominated by Firmicutes and Bacteroidetes, accounting for

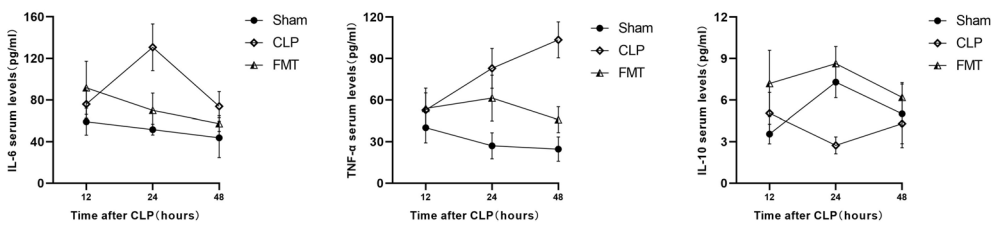


FIGURE 2 | The serum IL-6, TNF-α, and IL-10 among the Sham, CLP, and FMT groups at 12, 24, and 48 h. The concentration of serum IL-6 (pg/mL) and IL-10 (pg/mL) in the FMT group at 12 h after sepsis modeling were significantly higher than in the Sham group ($P < 0.05$), and there was no significant difference in TNF-α levels (pg/mL) among the three groups. The serum IL-6 level in the CLP group was significantly higher than in the Sham group and the FMT group ($P < 0.001$) at 24 h. The TNF-α levels in both the CLP and FMT groups were higher than in the Sham group ($P < 0.001$ and $P < 0.01$, respectively). The IL-10 level in the CLP group was lower than in the Sham group and the FMT group ($P < 0.001$). The serum IL-6 level in the CLP group was higher than in the Sham group at 48 h ($P < 0.01$). The TNF-α level in the CLP group was higher than in the Sham group and FMT group ($P < 0.001$). The TNF-α level in the FMT group was higher than in the Sham group ($P < 0.05$). There was no significant difference among the three groups in the IL-10 level at 48 h.

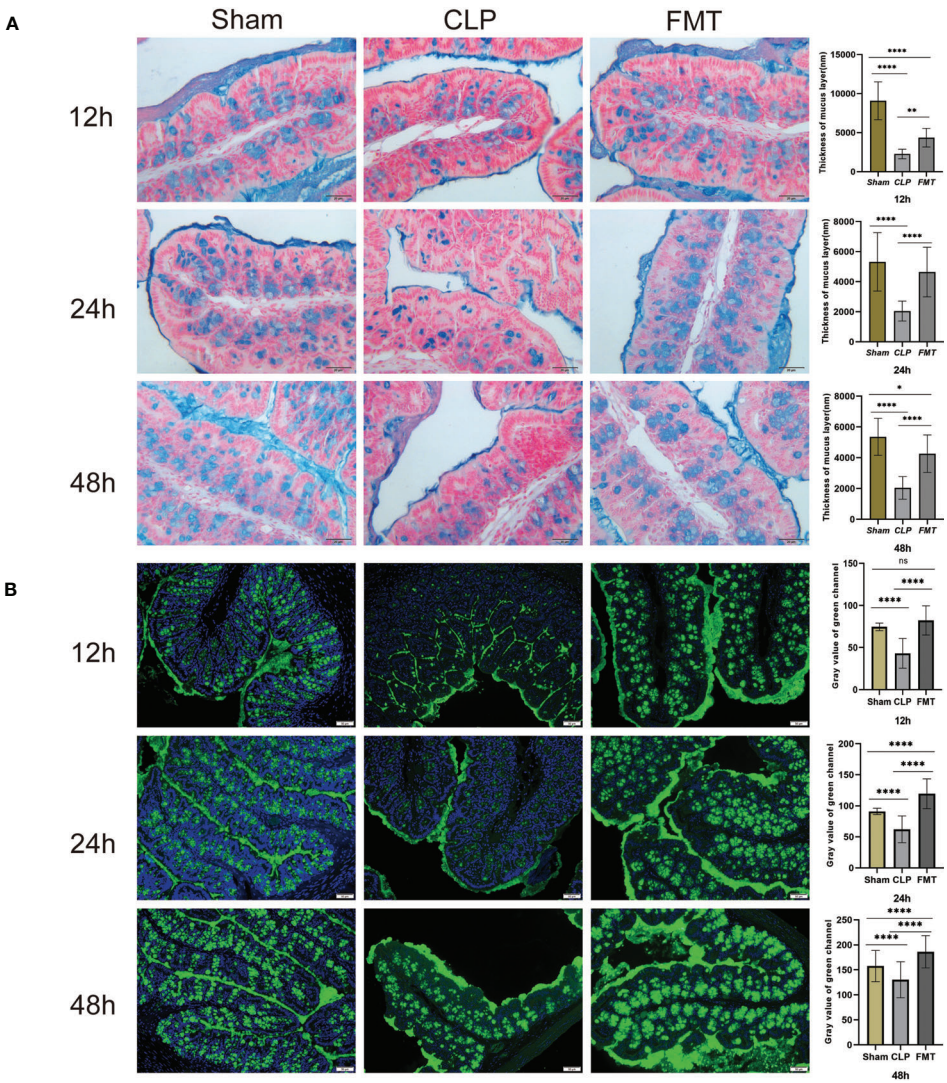


FIGURE 3 | The mucus layer thickness and MUC2 expression in the Sham, CLP, and FMT groups at 12, 24, and 48 h. **(A)** The AB-PAS method was used to measure the thickness of the mucus layer in the Sham, CLP, and FMT groups at 12, 24, and 48 h under a digital microscope (20 × 10). The blue color indicates goblet cell secretion and a mucus layer. The histogram on the right shows the statistical results of the comparison of the three groups. There was no significant difference between the Sham group and the FMT group in mucus layer thickness at 24 h. **(B)** MUC2 expression (green fluorescence) was observed in the three groups at 12, 24, and 48 h. The histogram shows the statistical results of all three groups. There was no significant difference between the Sham group and the FMT group at 12 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, ns, no significant difference.

48%, 27%, and 37%, 26%, and the composition closely resembled the composition found in mice. The main bacteria in the FMT group at 12 h were Proteobacteria and Verrucomicrobiae, with a relative abundance of 33% and 39%, respectively. Verrucomicrobiae accounted for 22% in the CLP group at 24 h (**Figure 7A**).

Alpha Diversity Analysis

The 12 h Sham group had a similar fecal richness and diversity compared with normal mice. The fecal richness and diversity were significantly lower in the CLP group at 12, 24, and 48 h and 7-day mortality than those of normal mice. We observed that the fecal richness and diversity were lowest at 48 h in the CLP group, but there was a slight recovery in the 7-day still alive mice in the CLP group, a significant difference compared with the normal mice. The richness and diversity of the flora in the FMT group were higher than that observed in the CLP group at all time points, and there was more fecal diversity than the normal mice at 24 h in the FMT group. There was no significant difference in fecal richness and diversity in the 7-day mortality FMT mice compared with the CLP group at the same timepoint (**Figure 7B**).

Beta Diversity Analysis

The dimensionality reduction of multi-dimensional microbial data was performed through NMDS analysis, and the main

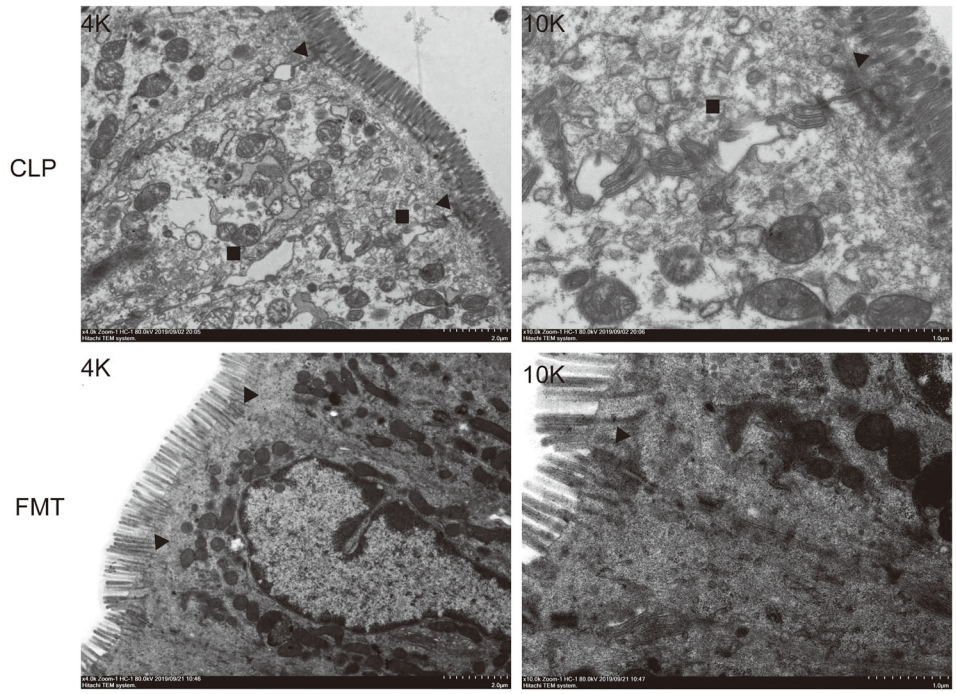


FIGURE 4 | Intestinal epithelial cell junctions. Intestinal epithelial cell junctions in the CLP group and FMT group at 48 h. The images on the left were enlarged 4K, and those on the right were enlarged 10K. ▲ Tight junction and the intermediate junction between intestinal epithelial cells. ■ The space between intestinal epithelial cells.

trends of data changes were displayed through the distribution of samples on a continuous sorting axis. The data were also classified by cluster analysis. In the NMDS analysis, the clusters within the group were well and the difference between the groups was large (**Figure 7C**).

LefSe (LDA Effect Size) Analysis

LEfSe analysis can directly perform simultaneous difference analysis on all classification levels, and at the same time, it emphasizes finding robust differences between groups, that is, marker species. The main species found in normal mice were Firmicutes and Bacteroides. The species with high levels in the Sham group were Firmicutes and Lactobacillus, while the differential species in the CLP group were Bacillales and Staphylococcaceae at 12 h, Enterobacteriales and Proteobacteria at 24 h, Planococcaceae at 48 h, and Deltaproteobacteria, Desulfovibrionales, and Erysipelatoclostridium at 7-day mortality. The FMT group was modeled with high levels of bacteria, including Verrucomicrobiae, Akkermansia, and Ruminococcus at 12 h, Lachnospiraceae group, Bifidobacteriales, Actinobacteria at 24 h, and Burkholderiaceae, Bacteroides, and Butyricimonas at 48 h (**Figure 7D**).

Functional Analysis

The core of the KEGG database is a biological metabolic pathway analysis database, in which metabolic pathways are classified into six categories, including metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, and human diseases. We found that infectious diseases had the highest relative abundance among human diseases in the KEGG secondary functional pathway (**Figure 7E**).

Metabolite Analysis

We further analyzed the species composition of the differential pathways, and found that the Lachnospiraceae contributed the most to L-lysine fermentation to acetate and butanoate (**Figure 7F**).

DISCUSSION

The commensal microbiome has been shown to play a key role in intestinal immunity because microbes regulate the maturation of the mucosal immune system, support local mucosal immunity, regulate cell growth, and maintain the epithelial barrier function (Haussner et al., 2019). Sepsis alters the composition of the flora and disrupts the balance between the host and the gut (Schmidt et al., 2018; Liu et al., 2019). Striking abnormalities have been reported in the intestinal microbiota of critically ill patients with sepsis, with a wide inter-individual variation and a low bacterial diversity (Avila et al., 2020; Bassetti et al., 2020). In this context, FMT is an effective strategy for adjusting the dysbiosis and restoring the normal gut microflora in patients with sepsis (Avila et al., 2020).

Fecal microbiota transplantation refers to the transplantation of functional bacteria from the feces of healthy donors into the patient's GI tract to restore the intestinal micro ecological

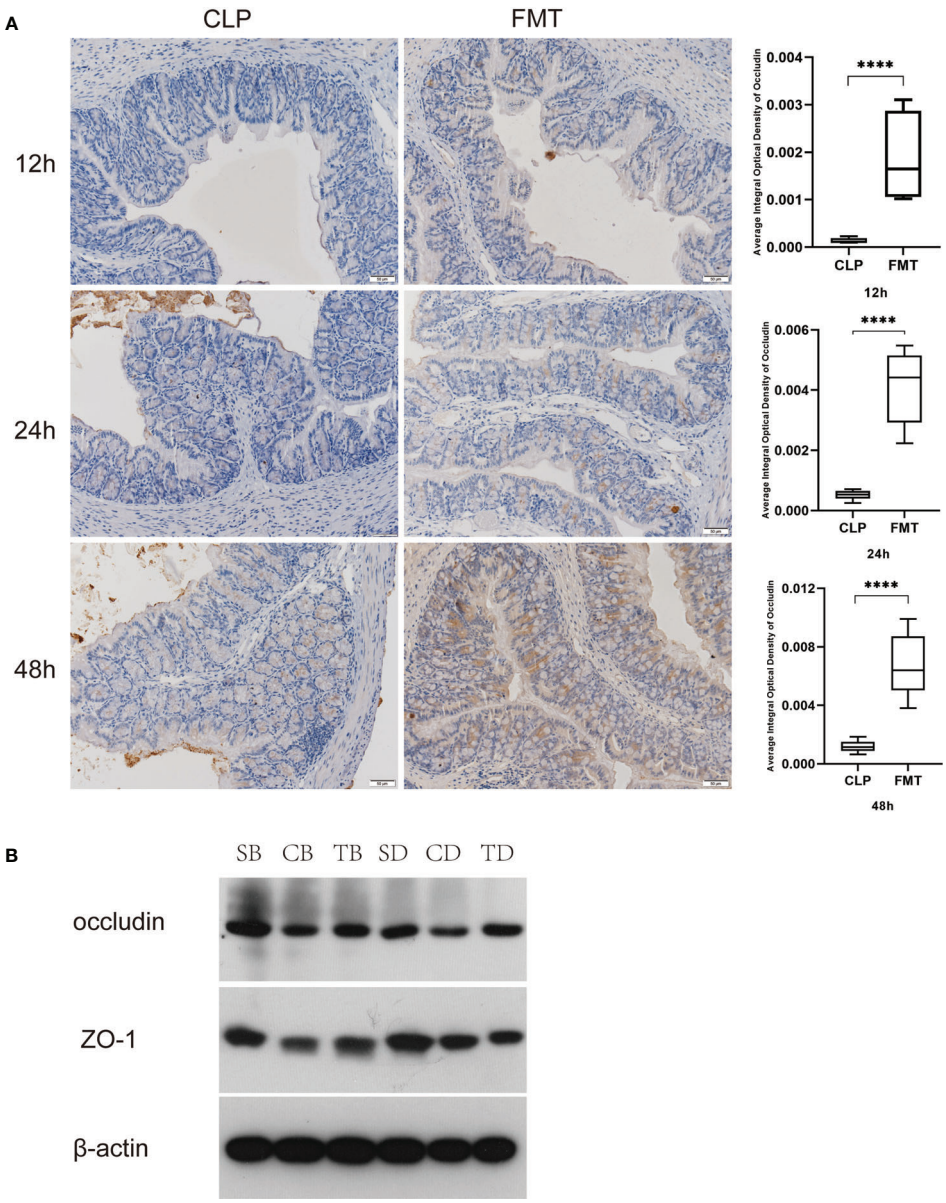


FIGURE 5 | Comparison of tight junction protein expression. **(A)** Comparison of occluding expression in the CLP and FMT groups at 12, 24 add 48 h, respectively, and observed under a digital microscope (20 × 10). Significant results are shown in the box plot on the right, ****$P < 0.0001$; **(B)** Relative expression of occludin and ZO-1 compared with β-actin expression in the Sham, CLP, and FMT groups at 24 or 48 h. SB, CB, TB, SD, CD, and TD represent the 24 h Sham, CLP, and FMT groups and 48 h Sham, CLP, and FMT groups, respectively.

balance and subsequently to treat diseases related to microbial imbalances (Wang et al., 2019; Zeng et al., 2019). This study was an attempt to explore whether FMT can maintain the integrity of the intestinal flora and protect the intestinal barrier function in mice with sepsis. We found that there was a flora imbalance 12-h after the modeling of sepsis. Proteobacteria had an absolute advantage, but Firmicutes and Bacteroidetes decreased, which was consistent with previous studies (Otani and Coopersmith, 2019). The relative abundance of Firmicutes and Bacteroidetes recovered slightly over time, but the amount of Proteobacteria in the intestinal flora of the 7-day mortality CLP group still accounted for 42%, which was significantly higher than that of normal mice. The analysis of the bacterial flora in the FMT group revealed that Firmicutes and Bacteroidetes were the most prevalent and the relative abundance of Proteobacteria was low, meaning that the bacterial composition is similar to that of normal mice. Besides, the Alphaproteobacterial load increased and the Betaproteobacterial load decreased in the FMT group. All of these observations indicate that the intestinal flora of septic mice had been replenished after FMT. It is believed that FMT can

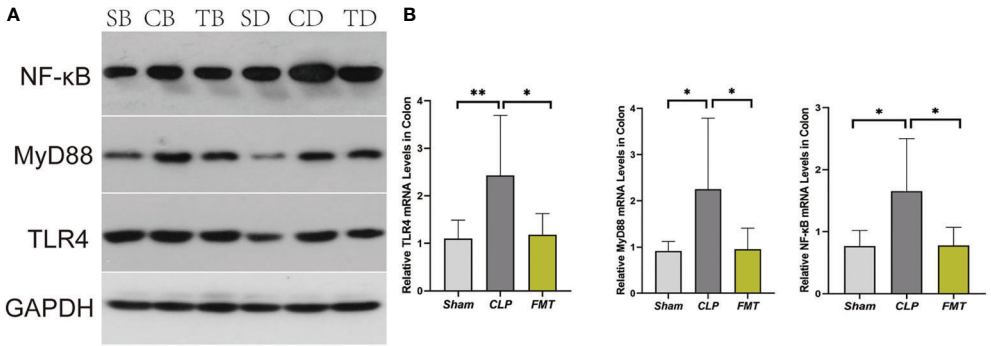


FIGURE 6 | TLR4, MyD88, and NF-κB expression and mRNA levels. **(A)** Expression of TLR4, MyD88, and NF-κB compared to GAPDH protein among Sham, CLP, and FMT groups at 24 and 48 h. SB, CB, TB, SD, CD, and TD represent 24 h Sham, CLP, and FMT groups and 48 h Sham, CLP, and FMT groups, respectively. **(B)** Relative TLR4, MyD88, and NF-κB levels in the colon. $^{*}P < 0.05$, $^{**}P < 0.01$.

maintain the intestinal bacterial balance by increasing the diversity of the flora and restoring and protecting intestinal flora from external interference (Jeon et al., 2018). However, in the observation of the 7-day mortality CLP group, the richness and the diversity of bacterial flora were both lower than those of the FMT group at 24 -h and 48 -h. As aforementioned, the dose and the number of infusions of FMT may have contributed to this result (Jeon et al., 2018). This experiment confirmed that the 7-day mortality rate of septic mice was significantly higher than that of the FMT group. Early application of FMT can effectively reduce the mortality of septic mice, most likely due to the reconstruction of intestinal flora.

The GM supports the development of the metabolic system and the maturation of the intestinal immune system by providing beneficial nutrients, such as synthesizing vitamins and short-chain fatty acids (SCFAs) (Shi et al., 2017). Butyrate, especially, can promote the release of the mucoprotein so as to maintain the mucus barrier (Johansson and Hansson, 2016; Wang et al., 2019). Studies have shown that SCFAs have anti-inflammatory and immunoregulatory activities and may reduce butyrate-producing bacteria such as Ruminococcaceae, Faecalibacterium, and Roseburia (Cammarota et al., 2015). Therefore, we compared the relative abundance of Ruminococcaceae in each group, and the results suggest that fecal bacteria significantly reduced in the sepsis model group, while in the FMT group, the bacterial counts were similar to or even slightly higher than those found in the normal mice. (In our study, the relative abundance of the normal mice was 0.07%, 0.0025% in the CLP group, and 0.067% in the FMT group in 48-h). It appears that Ruminococcaceae plays a role in the inflammatory response, and fecal microbiota transplantation can effectively improve the intestinal bacteria composition, thereby improving the inflammatory state. We found through the functional prediction that the relative abundance of intestinal flora was significantly different in infectious diseases. Further analysis of the species composition of the different pathways revealed that Lachnospiraceae contributed the most to L-lysine fermentation to acetate and butanoate, consistent with previous studies (Shen et al., 2018). This indicated that Lachnospiraceae may be the key bacteria for the effectiveness of FMT.

Tight junctions play an important role in maintaining the integrity of the mucosal epithelium (Zihni et al., 2016; Xu et al., 2018), and it is generally defined as a life-threatening organ failure in the setting of critical illness (Guttman and Finlay, 2009; Vermette et al., 2018). Critical illness induces hyper-permeability of the gut barrier which begins as early as 1 hour after the onset of sepsis and lasts at least 48 hours (Yoseph et al., 2016; Otani and Coopersmith, 2019). We compared transmission electron microscopy results in 48-h between the CLP and the FMT groups. The TJs of the septic mice were blurred more than those of the FMT mice. The cell gap was significantly wider, and both cells and organelles were swollen. We suppose that the mice had not only intestinal barrier dysfunction but also cellular dysfunction. The main functions of occludin are regulating and sealing the TJs (Shawki and McCole, 2017; Wang et al., 2017). That ZO-1 outperformed other TJ markers may reflect the concomitant organ epithelial injury which occurs in MODS (Vermette et al., 2018). Therefore, we observed the expression of the above two proteins in 24-h and 48-h in the three groups, and the results showed that occludin and ZO-1 in the CLP group were significantly lower than those in the Sham group and the FMT group. Levels of the two indicators in the FMT group were higher than those in the CLP group, or they were similar to those in the Sham group.

Research has shown that the thickness of the mucus layer is dependent on commensal bacteria (Otani and Coopersmith, 2019). We performed blinded thickness measurements of the colonic mucus layer in each mouse using Alcian blue-stained sections. We further validated the thickness of the mucus layer by immunofluorescence staining of the MUC2 mucins using a-MUC2 antibody (Desai et al., 2016) and observed that the thickness of the mucus layer in septic mice significantly reduced, and the thickness in the FMT group was significantly increased. This change was consistent with the changes in the flora of the two groups.

TLR4 is the best-characterized pathogen-recognition receptor. Its downstream effects are varied, and the TLR/MyD88/p38 MAPK/NF-κB pathway is popularly believed to

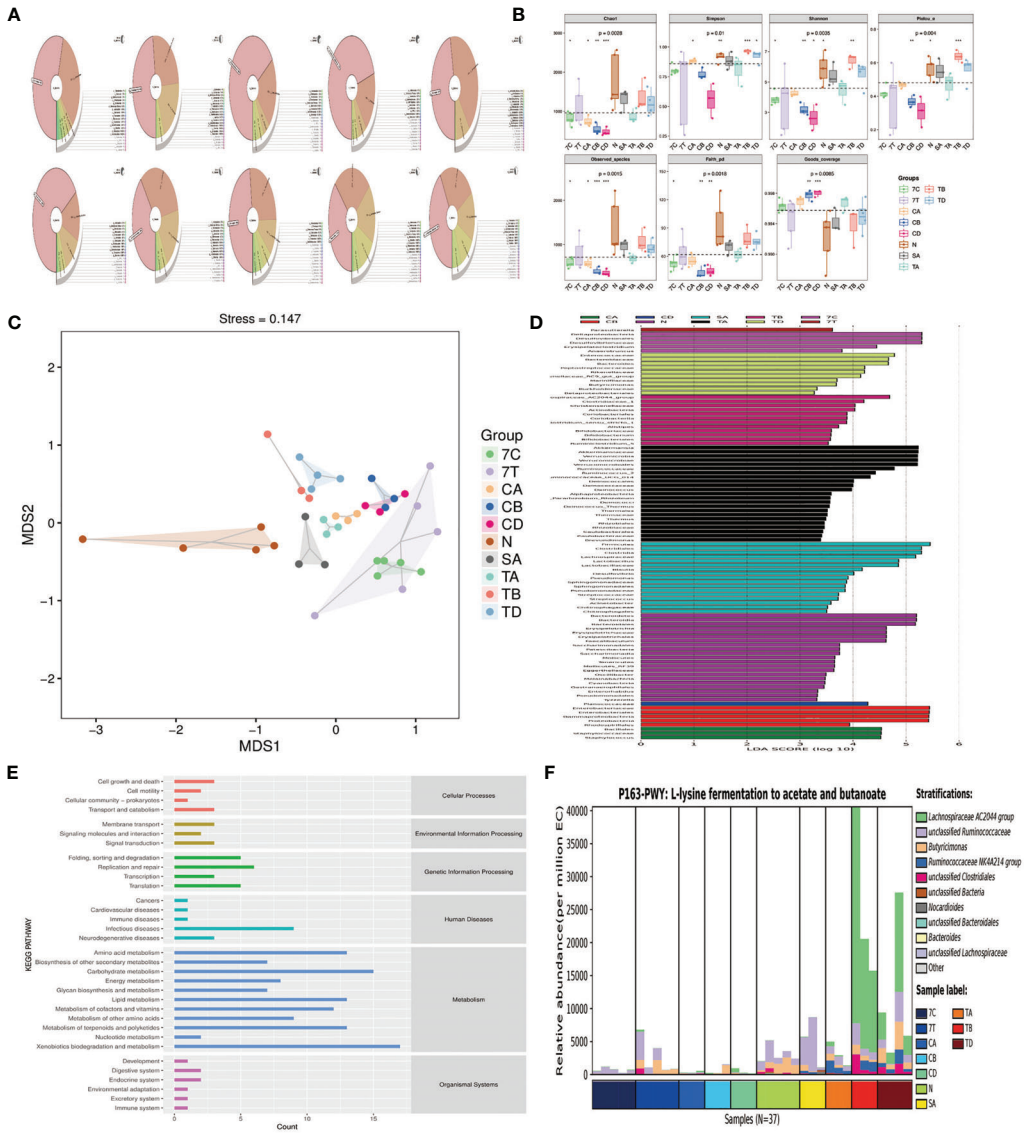


FIGURE 7 | Microbiota analysis. **(A)** Krona species at phylum among the Normal, Sham, CLP, and FMT groups. **(B)** Alpha diversity. *P < 0.05, **P < 0.01 ***P < 0.001. **(C)** NMDS two-dimensional sorting diagram. The closer the distance between the two points in the figure, the smaller the difference between the microbial communities in the two samples. **(D)** LEfSe (LAD Effect Size). A longer bar denotes a more significant difference in the taxon. The color of the bar graph indicates the most abundant sample group corresponding to the taxon. **(E)** Predicted abundance map of KEGG secondary functional pathways. **(F)** Species composition map of differential MetaCys metabolic pathways. The abscissa shows different groups. The order of the samples in the groups was sorted according to the similarity of the data; the ordinate was the relative abundance of the metabolic pathways, and the species different levels of contribution to the metabolic pathways were displayed in different colors at different levels. (In order to show the results better, we used abbreviations to represent each group: N = normal mice, 7C = CLP group mortality observation, 7T = FMT group mortality observation, SA = Sham group 12 h; CA, CB, and CD = CLP group at 12, 24, and 48 h time points, respectively, and TA, TB, and TD = FMT group at 12, 24, and 48 h time points, respectively).

play a critical role in the inflammatory response (Piton and Capellier, 2016; Tian et al., 2016). In this study, the TLR4/MyD88/NF-κB pathway in septic mice increased significantly at both the protein level and the gene level, and the inflammatory factors TNF-α and IL-6 increased at different levels in 24-h and 48-h after modeling, while IL-10 decreased in 24-h and 48-h after modeling. FMT treatment decreased the inflammatory response. This is consistent with the pathological score of bowel injury in the three groups. It was observed that the amount of apoptosis protein caspase 3 was different between the CLP group and the FMT group, and the amount of apoptosis in the CLP group was significantly higher than that of the FMT group.

This study has certain limitations: First, we selected the C57BL/6 mouse as our animal model. It is of common knowledge that the human gut microbiota varies greatly, and it is affected by many factors, including host-intrinsic, host-extrinsic, and

environmental. Precise microbiome modulation, therefore, is still in its infancy (Schmidt et al., 2018). Second, FMT has been shown to be highly efficient in the treatment of recurrent *C. difficile* infection (Jeon et al., 2018; Schmidt et al., 2018), but its application in sepsis has only been rarely reported. FMT is often preceded by preparatory antibiotic treatment in clinical practice, which makes it difficult to disentangle its effects (Schmidt et al., 2018). While antimicrobials are one of the fundamental and often life-saving modalities in septic patients, they can also pave the way for subsequent harm because of the resulting damage to the gut microbiome (Bhalodi et al., 2019). It has been reported that the association between antibiotic exposure and subsequent sepsis is related to microbiome depletion, rather than the severity of illness (Haak et al., 2018). Therefore, our initial experiment did not involve the use of antibiotics, but its combined application with FMT was included in subsequent studies. Third, it has been reported that the biological activity of the fresh fecal microbiota liquid is not affected by two hours of storage on ice (Hamilton et al., 2012). In our experiment, the fresh fecal bacteria liquid was kept on ice and was transplanted within 1 hour. It is speculated that its biological activity is not affected, but this needs to be confirmed in further research. Fourth, We did not attempt to discern the complete reconstruction time of intestinal flora after initiating FMT, nor did we compare the frequency and the number of transplantation, but it appears that early application of FMT has a protective effect on the intestinal function of septic mice. Finally, in view of the limited predictive function of PICRUSt, we will further use the full RNA transcriptome from both stool and mucosa-adherent microbiota for verification.

CONCLUSION

GM imbalance exists early in sepsis. Fecal microbiota transplantation can not only improve morbidity and effectively reduce mortality in septic mice, but can also effectively reduce epithelial cell apoptosis, improve the composition of the mucus layer, upregulate the expression of TJ proteins, and reduce intestinal permeability and the inflammatory response, thus protecting the intestinal barrier function. In our study, after FMT, the abundance and diversity of the gut flora were restored, and the microbial characteristics of the donors changed. Lachnospiraceae contributes the most to intestinal protection through enhancement of the L-lysine fermentation pathway, resulting in the production of acetate and butanoate, and maybe the key bacteria in short-chain fatty acid metabolism that promotes the success of fecal microbiota transplantation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accessionnumber(s) can be found below: NCBI repository, accession number SRP336491: PRJNA762235.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Ethics Committee of Hebei Medical University.

AUTHOR CONTRIBUTIONS

XG, HW, and HLZ conceived and designed the experiments, participated in its design and coordination, and helped to draft and revise the manuscript. XG, YL, HTZ, CH, and ZW performed the mice studies and analyzed the data. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This article was submitted as a preprint to BioRxiv as Gai et al. 2020.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.736204/full#supplementary-material

REFERENCES

Ames, S. G., Davis, B. S., Angus, D. C., Carcillo, J. A., and Kahn, J. M. (2018). Hospital Variation in Risk-Adjusted Pediatric Sepsis Mortality. *Pediatr. Crit. Care Med.* 19 (5), 390–396. doi: 10.1097/PCC.0000000000001502

Avila, P. R. M., Michels, M., Vuolo, F., Bilesimo, R., Burger, H., Milioli, M. V. M., et al. (2020). Protective Effects of Fecal Microbiota Transplantation in Sepsis are Independent of the Modulation of the Intestinal Flora. *Nutrition* 73, 110727. doi: 10.1016/j.nut.2020.110727

Bassetti, M., Bandera, A., and Gori, A. (2020). Therapeutic Potential of the Gut Microbiota in the Management of Sepsis. *Crit. Care* 24 (1), 105. doi: 10.1186/s13054-020-2780-3

Bhalodi, A. A., van Engelen, T. S. R., Virk, H. S., and Wiersinga, W. J. (2019). Impact of Antimicrobial Therapy on the Gut Microbiome. *J. Antimicrob. Chemother.* 74 (Suppl 1), i6–i15. doi: 10.1093/jac/dky530

Cammarota, G., Ianiro, G., Cianci, R., Bibbo, S., Gasbarrini, A., and Curro, D. (2015). The Involvement of Gut Microbiota in Inflammatory Bowel Disease Pathogenesis: Potential for Therapy. *Pharmacol. Ther.* 149, 191–212. doi: 10.1016/j.pharmthera.2014.12.006

Desai, M. S., Seekatz, A. M., Koropatkin, N. M., Kamada, N., Hickey, C. A., Wolter, M., et al. (2016). A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell* 167 (5), 1339–1353.e1321. doi: 10.1016/j.cell.2016.10.043

Ekmekciu, I., von Klitzing, E., Fiebiger, U., Escher, U., Neumann, C., Bacher, P., et al. (2017). Immune Responses to Broad-Spectrum Antibiotic Treatment and Fecal Microbiota Transplantation in Mice. *Front. Immunol.* 8, 397. doi: 10.3389/fimmu.2017.00397

Fay, K. T., Ford, M. L., and Coopersmith, C. M. (2017). The Intestinal Microenvironment in Sepsis. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863 (10 Pt B), 2574–2583. doi: 10.1016/j.bbadis.2017.03.005

Fay, K. T., Klingensmith, N. J., Chen, C.-W., Zhang, W., Sun, Y., Morrow, K. N., et al. (2019). The Gut Microbiome Alters Immunophenotype and Survival From Sepsis. *FASEB J.* 33 (10), 11258–11269. doi: 10.1096/fj.201802188R

Gai, X., Wang, H., Li, Y., Zhao, H., He, C., Wang, Z., et al. (2020). Fecal Microbiota Transplantation Reconstructs the Gut Microbiota of Septic Mice and Protects the Intestinal Mucosal Barrier. *bioRxiv* 2020.2006.2022.164541.

Gong, S., Yan, Z., Liu, Z., Niu, M., Fang, H., Li, N., et al. (2019). *Et Al*: Intestinal Microbiota Mediates the Susceptibility to Polymicrobial Sepsis-Induced Liver Injury by Granisetron Generation in Mice. *Hepatology* 69 (4), 1751–1767. doi: 10.1002/hep.30361

Guttman, J. A., and Finlay, B. B. (2009). Tight Junctions as Targets of Infectious Agents. *Biochim. Biophys. Acta* 1788 (4), 832–841. doi: 10.1016/j.bbamem.2008.10.028

Haak, B. W., Prescott, H. C., and Wiersinga, W. J. (2018). Therapeutic Potential of the Gut Microbiota in the Prevention and Treatment of Sepsis. *Front. Immunol.* 9:2042. doi: 10.3389/fimmu.2018.02042

Haak, B. W., and Wiersinga, W. J. (2017). The Role of the Gut Microbiota in Sepsis. *Lancet Gastroenterol. Hepatol.* 2 (2), 135–143. doi: 10.1016/s2468-1253(16)30119-4

Hamilton, M. J., Weingarden, A. R., Sadowsky, M. J., and Khoruts, A. (2012). Standardized Frozen Preparation for Transplantation of Fecal Microbiota for Recurrent Clostridium Difficile Infection. *Am. J. Gastroenterol.* 107 (5), 761–767. doi: 10.1038/ajg.2011.482

Haussner, F., Chakraborty, S., Halbgebauer, R., and Huber-Lang, M. (2019). Challenge to the Intestinal Mucosa During Sepsis. *Front. Immunol.* 10, 891. doi: 10.3389/fimmu.2019.00891

Jeon, S. R., Chai, J., Kim, C., and Lee, C. H. (2018). Current Evidence for the Management of Inflammatory Bowel Diseases Using Fecal Microbiota Transplantation. *Curr. Infect. Dis. Rep.* 20 (8), 21. doi: 10.1007/s11908-018-0627-8

Johansson, M. E., and Hansson, G. C. (2016). Immunological Aspects of Intestinal Mucus and Mucins. *Nat. Rev. Immunol.* 16 (10), 639–649. doi: 10.1038/nri.2016.88

Johansson, M. E., Larsson, J. M., and Hansson, G. C. (2011). The Two Mucus Layers of Colon are Organized by the MUC2 Mucin, Whereas the Outer Layer is a Legislator of Host-Microbial Interactions. *Proc. Natl. Acad. Sci. U.S.A.* 108 Suppl 1, 4659–4665. doi: 10.1073/pnas.1006451107

Khailova, L., Frank, D. N., Dominguez, J. A., and Wischmeyer, P. E. (2013). Probiotic Administration Reduces Mortality and Improves Intestinal Epithelial Homeostasis in Experimental Sepsis. *Anesthesiology* 119 (1), 166–177. doi: 10.1097/ALN.0b013e318291c2fc

Li, M., Liang, P., Li, Z., Wang, Y., Zhang, G., Gao, H., et al. (2015). Fecal Microbiota Transplantation and Bacterial Consortium Transplantation Have Comparable Effects on the Re-Establishment of Mucosal Barrier Function in Mice With Intestinal Dysbiosis. *Front. Microbiol.* 6, 692. doi: 10.3389/fmicb.2015.00692

Li, X., Li, X., Shang, Q., Gao, Z., Hao, F., Guo, H., et al. (2017). Fecal Microbiota Transplantation (FMT) Could Reverse the Severity of Experimental Necrotizing Enterocolitis (NEC) *via* Oxidative Stress Modulation. *Free Radic. Biol. Med.* 108, 32–43. doi: 10.1016/j.freeradbiomed.2017.03.011

Limketkai, B. N., Hendler, S., Ting, P. S., and Parian, A. M. (2019). Fecal Microbiota Transplantation for the Critically Ill Patient. *Nutr. Clin. Pract.* 34 (1), 73–79. doi: 10.1002/ncp.10228

Liu, Z., Li, N., Fang, H., Chen, X., Guo, Y., Gong, S., et al. (2019). Enteric Dysbiosis is Associated With Sepsis in Patients. *FASEB J.* 33 (11), 12299–12310. doi: 10.1096/fj.201900398RR

Otani, S., and Coopersmith, C. M. (2019). Gut Integrity in Critical Illness. *J. Intensive Care* 7, 17. doi: 10.1186/s40560-019-0372-6

Piton, G., and Capellier, G. (2016). Biomarkers of Gut Barrier Failure in the ICU. *Curr. Opin. Crit. Care* 22 (2), 152–160. doi: 10.1097/MCC.0000000000000283

Rittirsch, D., Huber-Lang, M. S., Flierl, M. A., and Ward, P. A. (2009). Immunodesign of Experimental Sepsis by Cecal Ligation and Puncture. *Nat. Protoc.* 4 (1), 31–36. doi: 10.1038/nprot.2008.214

Schmidt, T. S. B., Raes, J., and Bork, P. (2018). The Human Gut Microbiome: From Association to Modulation. *Cell* 172 (6), 1198–1215. doi: 10.1016/j.cell.2018.02.044

Shawki, A., and McCole, D. F. (2017). Mechanisms of Intestinal Epithelial Barrier Dysfunction by Adherent-Invasive Escherichia Coli. *Cell Mol. Gastroenterol. Hepatol.* 3 (1), 41–50. doi: 10.1016/j.jcmgh.2016.10.004

Shen, Z. H., Zhu, C. X., Quan, Y. S., Yang, Z. Y., Wu, S., Luo, W. W., et al. (2018). Relationship Between Intestinal Microbiota and Ulcerative Colitis: Mechanisms and Clinical Application of Probiotics and Fecal Microbiota Transplantation. *World J. Gastroenterol.* 24 (1), 5–14. doi: 10.3748/wjg.v24.i1.5

Shi, N., Li, N., Duan, X., and Niu, H. (2017). Interaction Between the Gut Microbiome and Mucosal Immune System. *Mil Med. Res.* 4, 14. doi: 10.1186/s40779-017-0122-9

Tian, Z., Liu, J., Liao, M., Li, W., Zou, J., Han, X., et al. (2016). Beneficial Effects of Fecal Microbiota Transplantation on Ulcerative Colitis in Mice. *Dig Dis. Sci.* 61 (8), 2262–2271. doi: 10.1007/s10620-016-4060-2

Vermette, D., Hu, P., Canarie, M. F., Funaro, M., Glover, J., and Pierce, R. W. (2018). Tight Junction Structure, Function, and Assessment in the Critically Ill: A Systematic Review. *Intensive Care Med. Exp.* 6 (1), 37. doi: 10.1186/s40635-018-0203-4

Wang, L., Cui, Y. L., Zhang, Z., Lin, Z. F., and Chen, D. C. (2017). Rhubarb Monomers Protect Intestinal Mucosal Barrier in Sepsis *via* Junction Proteins. *Chin. Med. J. (Engl)* 130 (10), 1218–1225. doi: 10.4103/0366-6999.205855

Wang, G., Huang, S., Wang, Y., Cai, S., Yu, H., Liu, H., et al. (2019). Bridging Intestinal Immunity and Gut Microbiota by Metabolites. *Cell Mol. Life Sci.* 76 (20), 3917–3937. doi: 10.1007/s00018-019-03190-6

Wang, C., Li, Q., and Ren, J. (2019). Microbiota-Immune Interaction in the Pathogenesis of Gut-Derived Infection. *Front. Immunol.* 10:1873. doi: 10.3389/fimmu.2019.01873

Xu, J., Liu, Z., Zhan, W., Jiang, R., Yang, C., Zhan, H., et al. (2018). Recombinant TsP53 Modulates Intestinal Epithelial Barrier Integrity *via* Upregulation of ZO1 in LPSinduced Septic Mice. *Mol. Med. Rep.* 17 (1), 1212–1218. doi: 10.3892/mmr.2017.7946

Yoseph, B. P., Klingensmith, N. J., Liang, Z., Breed, E. R., Burd, E. M., Mittal, R., et al. (2016). Mechanisms of Intestinal Barrier Dysfunction in Sepsis. *Shock* 46 (1), 52–59. doi: 10.1097/SHK.0000000000000565

Zeng, W., Shen, J., Bo, T., Peng, L., Xu, H., Nasser, M. I., et al. (2019). Cutting Edge: Probiotics and Fecal Microbiota Transplantation in Immunomodulation. *J. Immunol. Res.* 2019, 1603758. doi: 10.1155/2019/1603758

Zihni, C., Mills, C., Matter, K., and Balda, M. S. (2016). Tight Junctions: From Simple Barriers to Multifunctional Molecular Gates. *Nat. Rev. Mol. Cell Biol.* 17 (9), 564–580. doi: 10.1038/nrm.2016.80

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.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 17 December 2021
doi: 10.3389/fcimb.2021.791373

Combined Intestinal Metabolomics and Microbiota Analysis for Acute Endometritis Induced by Lipopolysaccharide in Mice

Yuqing Dong[1,2], Yuan Yuan[1,3], Yichuan Ma[1], Yuanyue Luo[1], Wenjing Zhou[1], Xin Deng[1], Jingyu Pu[1], Binhong Hu[1] and Songqing Liu[1]**

[1] College of Chemistry and Life Sciences, Chengdu Normal University, Chengdu, China, [2] College of Forestry, Sichuan Agricultural University, Chengdu, China, [3] College of Life Science, Sichuan Agricultural University, Yaan, China

Endometritis is generally caused by bacterial infections, including both acute and chronic infections. In the past few decades, accumulated evidence showed that the occurrence of diseases might be related to gut microbiota. The progression of diseases is previously known to change the composition and diversity of intestinal microbiota. Additionally, it also causes corresponding changes in metabolites, primarily by affecting the physiological processes of microbiota. However, the effects of acute endometritis on intestinal microbiota and its metabolism remain unknown. Thus, the present study aimed to assess the effects of acute endometritis on intestinal microbes and their metabolites. Briefly, endometritis was induced in 30 specific pathogen-free (SPF) BALB/c female mice *via* intrauterine administration of lipopolysaccharide (LPS) after anesthesia. Following this, 16S rRNA gene sequencing and liquid chromatogram-mass spectrometry (LC-MS) were performed. At the genus level, the relative abundance of *Klebsiella*, *Lachnoclostridium_5*, and *Citrobacter* was found to be greater in the LPS group than in the control group. Importantly, the control group exhibited a higher ratio of *Christensenellaceae_R–7_group* and *Parasutterella*. Furthermore, intestinal metabolomics analysis in mice showed that acute endometritis altered the concentration of intestinal metabolites and affected biological oxidation, energy metabolism, and biosynthesis of primary bile acids. The correlation analysis between microbial diversity and metabolome provided a basis for a comprehensive understanding of the composition and function of the microbial community. Altogether, the findings of this study would be helpful in the prevention and treatment of acute endometritis in the future.

Keywords: acute endometritis, intestinal microbiota, metabolomics, lipopolysaccharide, mice

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Zhentian Lei, University of Missouri, United States
Helioswilton Sales-Campos, Universidade Federal de Goiás, Brazil

****Correspondence:***
Binhong Hu
binhong.hu86@mail.ru
Songqing Liu
songqingliu@cdnu.edu.cn

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *08 October 2021*
Accepted: *24 November 2021*
Published: *17 December 2021*

Citation:
Dong Y, Yuan Y, Ma Y, Luo Y, Zhou W, Deng X, Pu J, Hu B and Liu S (2021) Combined Intestinal Metabolomics and Microbiota Analysis for Acute Endometritis Induced by Lipopolysaccharide in Mice. Front. Cell. Infect. Microbiol. 11:791373. doi: 10.3389/fcimb.2021.791373

INTRODUCTION

Endometritis is a disease caused by bacterial pathogens, such as Chlamydia trachomatis, Enterococcus, Escherichia coli, Gardnerella vaginalis, Klebsiella pneumoniae, Mycoplasma hominis, Neisseria gonorrhoeae, Staphylococcus, and Streptococcus (Moreno et al., 2018). These bacterial pathogens are known to cause persistent inflammation of the uterus, which could further

result in infertility in severe cases (Wallach and Czernobilsky, 1978; Kitaya et al., 2018). Among these, E. coli and Staphylococcus aureus are particularly recognized as important factors in the infection of endometritis (Andrews et al., 2006; Kitaya et al., 2017). Importantly, lipopolysaccharide (LPS) produced by E. coli is considered to be the triggering factor for various inflammatory reactions, which play a vital role in the establishment of inflammatory models (Chen et al., 2010; Wu et al., 2016; Wang and Zhang, 2018).

Throughout long-term evolution, the mammalian intestines have evolved into a complex environment. The intestines usually consist of a dense microbiome that is strongly associated with host health and disease status (Marchesi et al., 2016). Intestinal microbiota not only affects normal metabolism (Sharon et al., 2014) but also regulates host immunity (Rooks and Garrett, 2016), nervous system (Tan et al., 2020), and even cancer development (Yachida et al., 2019). In particular, it acts as a barrier against pathogen invasion, protects the intestinal structure, and maintains normal physiological function. In fact, any imbalance in the gut microbiome can cause corresponding physiological effects. Previous studies have shown that imbalances in the gut microbiota could lead to an increase in estrogen secretion (Plottel and Blaser, 2011), which was associated with endometriosis, endometrial cancer, and other uterine diseases (Borella et al., 2021). However, its role in endometritis remains poorly understood.

The microbiome represents a dynamic community, whose composition is mainly influenced by age, disease status, eating habits, and other factors. These factors influence the composition and diversity of microorganisms. Additionally, these also affect the physiological processes of microorganisms, such that the distribution of their metabolites also changes. Variations in the microbial metabolic spectrum could further cause physiological changes in both host and pathogenic microorganisms, thereby affecting the progression of the disease (Cameron and Sperandio, 2015). Consequently, metabolism studies and metabolic profiling are used as markers for the diagnosis of diseases (Ursell et al., 2014). In particular, plasma trimethylamine nitrogen oxide (TMAO) has been previously identified as a marker for cardiovascular disease (CVD) (Koeth et al., 2013), whereas 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) was identified in the plasma of patients with gestational diabetes, impaired glucose tolerance, and type 2 diabetes (Prentice et al., 2014). However, no previous studies reported the identification of metabolites associated with endometritis.

The present study aimed to assess the effects of acute endometritis on intestinal microbes and their metabolites. In particular, it was hypothesized that administration of lipopolysaccharide (LPS) would induce endometritis and affect the intestinal microbiota and metabolites in the mice model. Herein, the intestinal tissues of acute endometritis mice were subjected to 16S rRNA high-throughput sequencing technology and liquid chromatogram-mass spectrometry (LC-MS) non-targeted metabolism technology, to assess the distribution of intestinal metabolites and unravel the composition of microbiota present in the mice with acute endometritis. Altogether, the results of the study would provide possible molecular markers for the diagnosis of acute endometritis.

MATERIALS AND METHODS

Animals

All experimental procedures, including animal handling, welfare monitoring, and euthanasia, were performed following the ARRIVE guidelines and regulations and were approved by the Animal Care Office of Chengdu Normal University, Chengdu, China. Specific pathogen-free (SPF) BALB/c female mice aged 6–8 weeks were purchased from the Chengdu Dossy Experimental Animals Co., Ltd.

Experimental Processing

A total of 30 mice were placed at a temperature of 25 ± 3°C, under 75 ± 5% humidity, fixed with 12-h light/12-h dark treatment daily, and provided adequate food and water. The mice were randomly categorized into five groups: the LPS group (3, 6, 12, and 24 h) and the control group. The murine model of endometritis was established as previously described (Wu et al., 2016; Wu et al., 2018). Briefly, each side of the mouse uterus was perfused with 20 μl of LPS (3 mg/ml) under anesthesia. The LPS was brought from Sigma-Aldrich (USA). The control group mice were intraperitoneally injected with the same amount of phosphate-buffered saline (PBS; Beijing Labgic Technology Co., Beijing, China). The uterine and intestinal tissues were collected at 3, 6, 12, and 24 h. The samples were frozen under liquid nitrogen immediately after collection at −80°C.

Inflammation Analysis

The uterine tissues were fixed with paraformaldehyde and then pruned, dehydrated, and paraffin-embedded. Five sections were stained with hematoxylin and eosin (H&E) before microscopic observation (Nikon, Eclipse Ci-L, Japan). The expressions of interleukin (IL)-6, IL-1β, and tumor-necrosis factor-alpha (TNF-α) were detected by quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from the uterine tissues by using the MiniBEST Universal RNA Extraction Kit (Takara, Japan) and reverse transcribed into cDNA. The cDNA product was diluted with the Fast qPCR Master Mix (High Rox, BBI, ABI) on the StepOne Plus Fluorescent Quantitative PCR instrument (ABI, Foster, CA, USA). Primers (listed in **Supplementary Table 1**) were designed using the Primer Premier 5.0 software, and the relative quantification of the target gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using the GraphPad Prism 8 (GraphPad InStat Software, USA). Comparison between the groups was performed using *t*-test, and the data were expressed as mean ± SD. $p < 0.05$ was considered to indicate statistical significance.

DNA Extraction and Library Construction

Total genomic DNA was extracted according to the instructions for the QIAamp 9 PowerFecalQIAcube HT Kit (Qiagen, 51531).

The concentration of DNA was verified by the Nano Drop system (Thermo Fisher, 2000) and agarose gel. Using the genomic DNA as a template, according to the selection of sequencing V3–V4 variable regions [primers 343F: 5′-TACGGRAGGCAGCAG-3′, 798R: 5′-AGGGTATCTAATCCT-3′ (Nossa et al., 2010)], TksGflex DNA polymerase (Takara, R060B) and specific primers with barcode were used for PCR. The quality of amplifiers was confirmed by gel electrophoresis, purified by the AMPoule XP Bead (Agencourt), followed by another round of PCR amplification. The Qubit dsDNA Analysis Kit (Life Technologies, Q32854) was used to quantify the final amplifier after purification of the ampoule XP bead. An equal number of purified amplifiers were assembled for subsequent sequencing.

Analysis of Intestinal Microbiota

The Trimmomatic software was used to preprocess the paired-end reads (Bolger et al., 2014), pruned, and assembled by the FLASH software after trimming (Reyon et al., 2012). The assembly parameters were as follows: minimum overlap, 10 bp; maximum overlap, 200 bp; and maximum error ratio, 20%. Abandoned homologous sequences are those <200 bp; 75% of the base readings above Q20 were retained. The chimera readings were detected and removed by using the UCHIME (Caporaso et al., 2010). Vsearch software was used to generate operational taxonomic units (OTU) by removing the primer sequences and clustering with a cutoff value of 97% similarity (Rognes et al., 2016). The representative reading of each OTU was selected using the QIIME package. The RDP classifier was used to annotate the species of all representative reads according to the Silva database (version 123) (confidence threshold 70%) (Wang et al., 2007).

Metabolomics Processing

We accurately weighed 15 mg of the tissue samples into 1.5-ml of the EP tube and added the inner standard (FMOC-L-2-Chlorophe, 0.3 mg/ml; Lyso PC17: 0, 0.01 mg/ml, all configured with methanol) of 20 μl and added 400 μl of methanol–water (v/v = 4:1). After grinding, centrifugation, supernatant absorption, filtration, and transfer to the LC sample vial, the solution was stored at −80°C until LC-MS analysis.

For data processing, the metabolic profiling in positive and negative electrospray ionization (ESI) modes was analyzed by using the liquid-mass spectrometry system consisting of the Dionex U3000 UHPLC High-Resolution Mass Spectrometer and the QE plus (Thermo Fisher Scientific, Waltham, MA, USA). The determination was performed on the ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 μm) with a mobile phase consisting of A-water (containing 0.1% formic acid, v/v) and B-acetonitrile (containing 0.1% formic acid, v/v). The flowrate was set to 0.35 ml/min, and the column temperature was 45°C. The injection volume was 2 μl. Data acquisition was performed in the full-scan mode (m/z ranges from 70 to 1,000) combined with the IDA mode.

Metabolomics Data Analysis

The Progenesis Qi V2.3 software (Nonlinear Dynamics, Newcastle, UK) was used to process the metabolic raw data after collection by Unifi 1.8.1. The compounds were identified based on the accurate mass number, secondary fragments, and isotope distribution using The Human Metabolome Database (HMDB), Lipidmaps (V2.3), METLIN databases, and self-built databases. The qualitative compounds were screened according to the qualitative results score of the compounds. The screening standard was 36 points (full score = 60 points), and the qualitative results <36 points were considered to be inaccurate and deleted.

Principle component analysis (PCA) and (orthogonal) partial least-square-discriminant analysis (O) PLS-DA were performed to observe the overall metabolic differences among the groups. The Hotelling's T2 region demonstrated an ellipse in the model score, which was defined at a 95% confidence interval for model variation. In the OPLS-DA analysis, variable importance in the projection (VIP) was employed to measure the influence and explanatory ability of the samples in each group; VIP >1 was regarded as the screening criteria. We selected differential metabolites according to the threshold of statistically significant variables obtained from the OPLS-DA model on (VIP) values and p-values obtained from two-tailed Student's *t*-test of normalized peak areas. Metabolites with VIP values >1.0 and $p < 0.05$ were considered to indicate differential metabolites.

RESULTS

Effect of LPS on Inflammation of Mouse Uterus

The effect of LPS on uterine inflammation in mice was assessed by evaluating histopathological conditions. As shown in **Figures 1A–E**, the histopathological assessment showed that the morphology of uterine tissue was normal in the control group. An increase in LPS treatment time resulted in a gradual enhancement of the pathological manifestations, and the pathological situation was reported to be most serious at 12 h. After 12 h, the pathological condition of uterine tissue weakened. In addition to this, changes in cytokine-related expression levels were also observed (**Figure 1F**). In fact, LPS treatment for 12 h significantly increased the levels of IL-6, IL-1β, and TNF-α.

Effects of LPS on Diversity, Richness, and Composition of Intestinal Microorganisms in Mice

Following quality controlled processing of the original sequencing results obtained from 16S rRNA sequencing, the data volume obtained for clean tags was distributed between 89734–93578. After removing chimeras, the valid tags obtained for analysis were allocated between 80742 and 85484. Valid tags were divided into OTU according to 97% similarity, and a total of 4,373 OTUs were obtained. According to the results of the Shannon index and Chao1 index (**Figure 2**), no significant differences were recorded between the diversity of gut microbiome in mice treated with LPS and normal mice.

In particular, a total of 27 bacterial phyla were detected for the classification of the resulting OTUs. Among these, *Bacteroidetes* and *Firmicutes* were found to be the dominant ones, which accounted

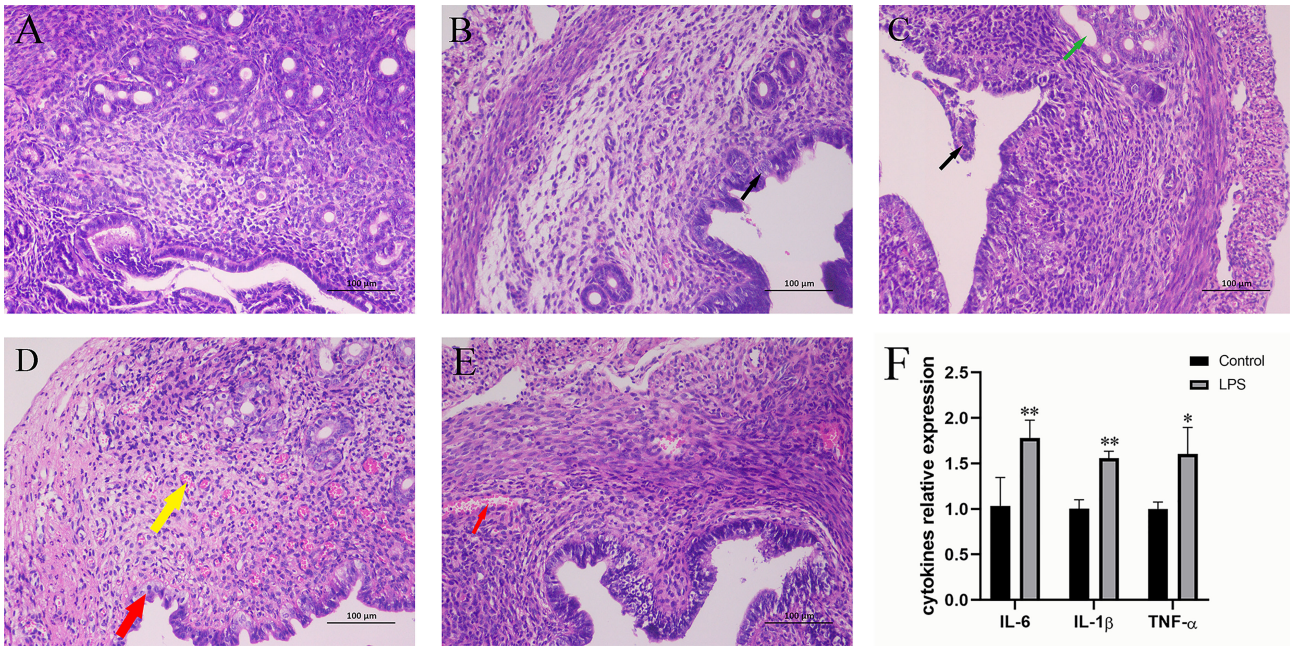


FIGURE 1 | Effect of LPS on inflammation of the mouse uterus. **(A)** Control group. **(B)** LPS group (3 h). A small amount of endometrial epithelial cells seems swollen, and the cytoplasm is loose and light-stained (black arrow). **(C)** LPS group (6 h). Bits of endometrial epithelial cells are shed (black arrow), and a small number of uterine glands are slightly dilated (green arrow). **(D)** LPS group (12 h). The endometrial epithelium and glandular epithelium are swollen, the cytoplasm has loosened and lightly stained (red arrow), and a large number of capillaries in the lamina propriety are congested and dilated (yellow arrow). **(E)** LPS group (24 h). A spot of blood stasis in the lamina propria (red arrow). (Hematoxylin and eosin staining; magnification, 200×). **(F)** The expression of inflammatory cytokines IL-6, IL-1β, and TNF-α. Mean ± SD was employed for data processing. Three replicates were processed in each group. *$p < 0.05$, **$p < 0.01$ vs. control group.

for 59.56% and 29.86%, respectively. At the genus level, a total of 529 bacterial genera were detected. Among these, the dominant species were *Bacteroides* (51.25%), *Faecalibacterium* (9.05%), *Lachnospiraceae_NK4A136_group* (4.07%), *Lachnoclostridium* (2.99%), *Helicobacter* (2.66%), and *Paraprevotella* (2.02%) (**Figure 3**). In addition to this, the study also analyzed different species present in each group using the Wilcoxon test (**Figure 4**). At the phylum level, *Elusimicrobia* was identified as a distinct phylum. Furthermore, at the generic level, *Klebsiella*, *Lachnoclostridium_5*, *Citrobacter*, *Enterobacter*, *Treponema_2*, *Christensenellaceae_R−7_group*, and *Parasutterella* were found to be different in the two groups.

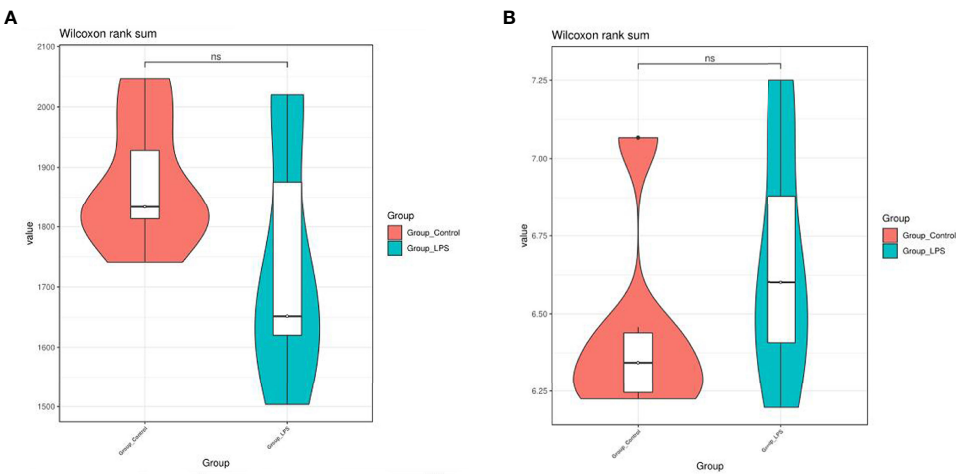


FIGURE 2 | Effects of LPS on the diversity and richness of intestinal microbiota in mice. **(A)** Chao1 index. **(B)** Shannon index. ns means no significant difference.

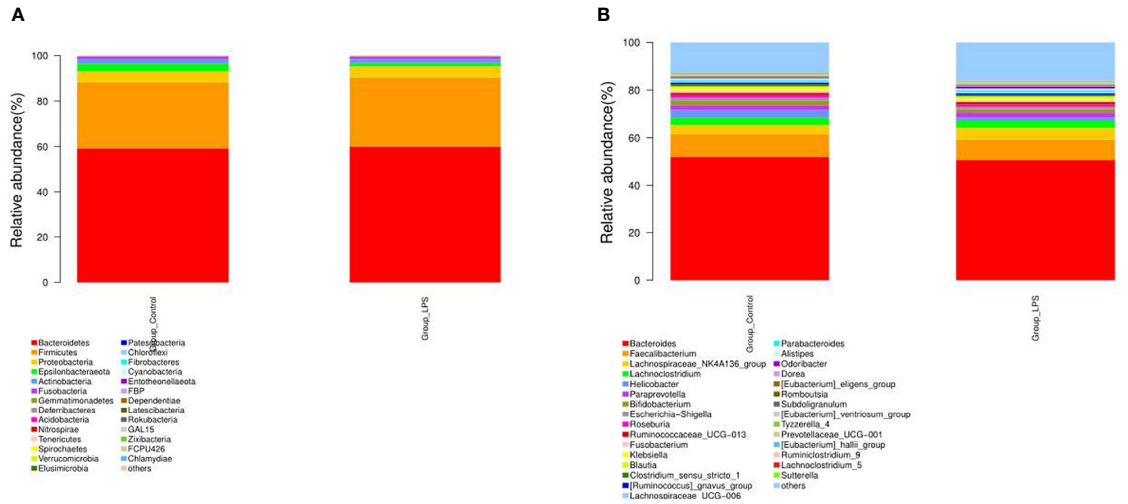


FIGURE 3 | Effect of LPS on the composition of intestinal microbiota in mice. **(A)** Phylum level. **(B)** Genus level.

Effects of LPS on Intestinal Metabolomics in Mice

LC-MS non-targeted metabolomics analysis was conducted for the intestinal samples of the control group and LPS group. The principal component analysis showed a separation between the samples obtained from these two groups (**Figure 5A**). OPLS-DA was utilized to verify differential metabolites between the two groups, and multivariate analysis was supervised. Furthermore, the score plot showed/revealed significant differences in OPLS-DA score for the two groups of samples (**Figure 5B**). As shown in **Figure 5C**, the OPLS-DA fitting model did not show overfitting of the model. Meanwhile, for the displacement test, the values for R2Y (0.867) and Q2Y (−0.222 < 0) also indicated the validity of the model.

The study also compared the metabolites (**Supplementary Table 2**) between the two groups, by *t*-test and multivariate analysis combined with variable influence on projection (VIP). A total of 187 different metabolites were identified (**Supplementary Table 3**). Among the 76 positive ion metabolites, two were identified as benzenoids, 23 were lipids and function-like molecules, and one belonged to nucleosides, nucleotides, and analogs. Additionally, these positive ion metabolites included nine kinds of organic acids and derivatives, four types of organic oxygen compounds, four types of organoheterocyclic compounds, one organosulfur compound, and two phenylpropanoids and polyketides. In the case of negative-ion metabolites, a total of 111 such metabolites were identified, which were divided into alkaloids and derivatives, benzenoids,

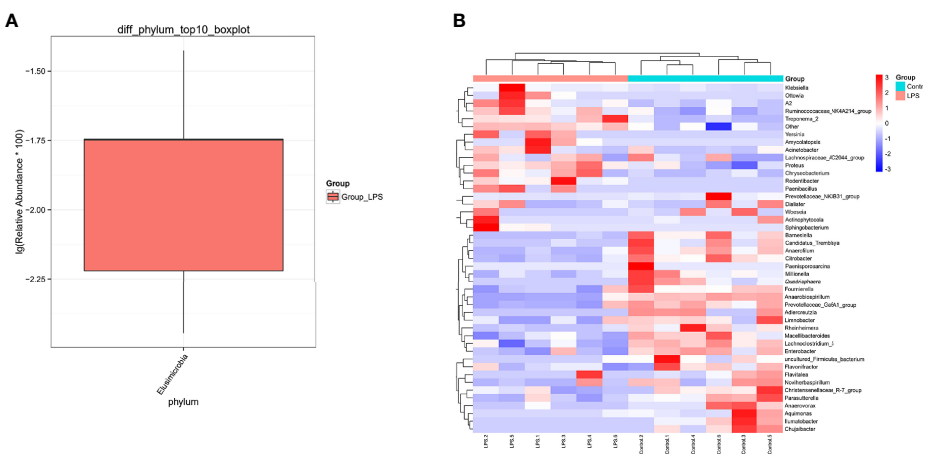


FIGURE 4 | Differential species of the intestinal microbiota of mice in the LPS group and control group. **(A)** The phylum level is evident from the box drawing. **(B)** The genus level is represented by a heat map. Orange indicates relatively high species abundance, while blue indicates relatively low species abundance.

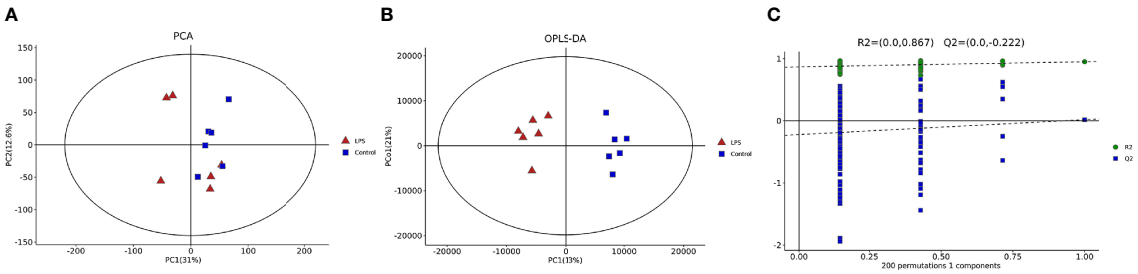


FIGURE 5 | Multivariate statistical analyses of intestinal metabolites in mice. **(A)** Principal component analysis (PCA). **(B)** Orthogonal partial least-squares-discriminant analysis (OPLS-DA) diagram. **(C)** Validation diagram obtained from the permutation test.

lipids, and lipid-like molecules, nucleosides, nucleotides, and their analogs, organic acids and derivatives, organic compounds, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides, and others.

The volcano diagram showed dramatic up- and downregulation of metabolites in the endometritis group and normal group (**Figure 6A**). To display the relationship between samples and differences in the expression of metabolites among different samples, hierarchical clustering analysis (HCA) was performed on the expression levels of top 50 significantly different metabolites, and the clustering results were shown in terms of a heat map (**Figure 6B**). In the positive ion mode, LPS treatment increased the expression of metabolites, such as cholesterol, 11-deoxycortisol, and N-phenylacetylphenylalanine, and reduced the expression of Darunavir, PE (19:1(9Z)/0:0), and LysoPE (24:1(15Z)/0:0). However, in the negative ion mode, LPS treatment resulted in an increase in metabolites, like trans-piceid and Psilocybin, and decreased the levels of NAD, MET-enkephalin, and Isokobusone. In addition to this, KEGG ID for differential metabolites was used for pathway enrichment analysis (**Figure 7**). In particular, the metabolic pathways for cholesterol metabolism, primary bile acids biosynthesis, and vitamin digestion and absorption were markedly affected by LPS.

Importantly, changes in the metabolic spectrum of the microbiome reflect changes in the dynamics of the microbiome. Therefore, the present study analyzed the correlation between microbial diversity and metabolomics to understand the composition and function of microorganisms in a better way. As shown in **Figure 8**, *Treponema_2* positively correlated with the levels of cholesterol in the microbial community, with higher genera expression abundance in the LPS group, and negatively correlated with NAD. In the normal control group, *Parasutterella* was found to be positively correlated with NAD and Isokobusone and negatively correlated with 8-Epiiridotrial glucoside in the microbial community, with superior generic-level expression abundance.

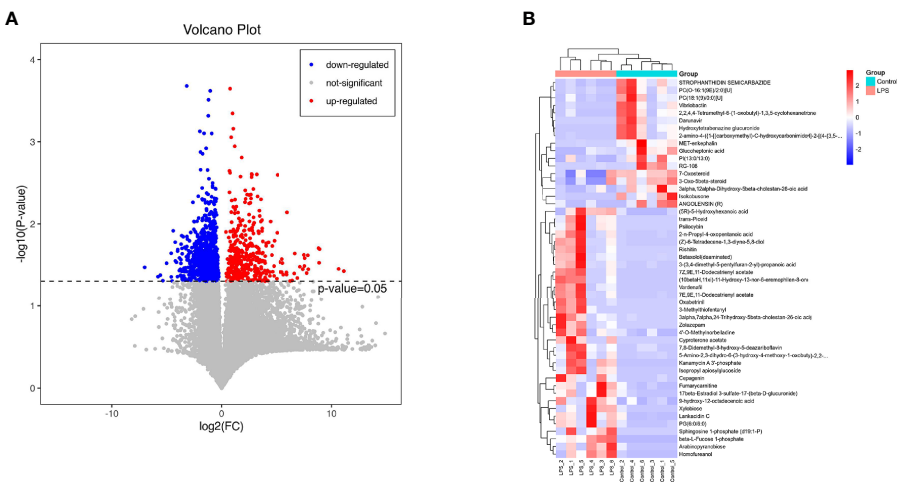


FIGURE 6 | Effects of LPS on the intestinal metabolites in mice. **(A)** Significantly different metabolites, in which the red dots represent significantly upregulated differential metabolites in the LPS group, the blue dots represent significantly downregulated differential metabolites, and the gray dots represent insignificant differential metabolites. **(B)** HCA. The abscissa expresses the sample name, and the ordinate represents the differential metabolites. The color ranges from blue to red, indicating the expression abundance of metabolites from minimal to high, that is, the greater the intensity of the red color, the higher is the expression abundance of differential metabolites.

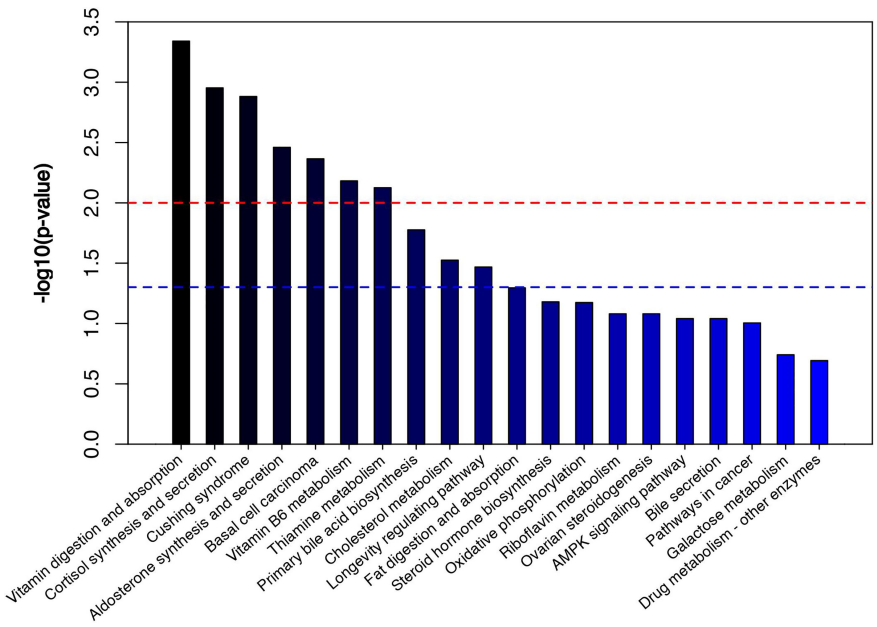


FIGURE 7 | Metabolic pathway enrichment diagram. The red line indicates p = 0.01, while the blue line indicates p = 0.05. When the top of the bar is higher than that of the blue line, the signal pathway represented is significant.

DISCUSSION

As a bacterial infectious disease, endometritis is known to primarily affect the life of women and modern agricultural production, which is detrimental to human health and economic development (Zhao et al., 2017). Lipopolysaccharide (LPS) obtained from the cell wall of Gram-negative bacteria has been previously shown to play an important role in the pathogenesis of endometritis (Li et al., 2019; Zhou et al., 2019). Thus, it is used to induce endometritis in mice. Currently, the gold standard used for

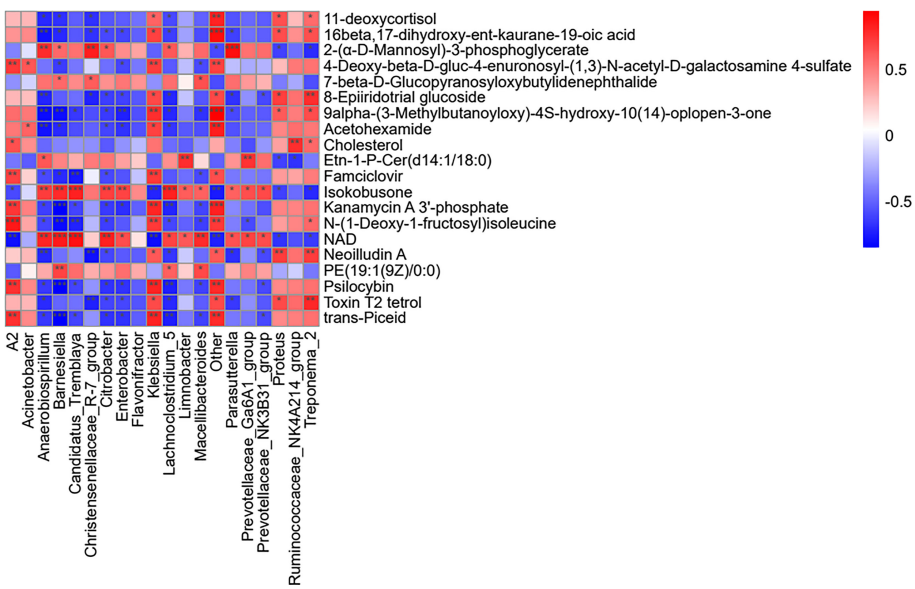


FIGURE 8 | Analysis of the effects of LPS on the microbial genus and metabolite concentration. For each species with distinct behaviors and the corresponding metabolites in each column, the orange color indicates a positive correlation, while blue indicates a negative correlation. The darker the color, the greater is the correlation. The closer the color is to white, the closer is the correlation to zero. ***p < 0.001, **p < 0.01, and *p < 0.05 (i.e., significance of correlation).

endometritis is histopathological examination (Kiviat et al., 1990; Kitaya and Yasuo, 2011). In the present study, H&E staining of the tissue samples and assessment of tissue morphology were used for the evaluation of the tissue samples. Briefly, after 12 h of LPS treatment, endometrium and uterine glandular epithelial cells were found to be swollen, the cytoplasm was loose and lightly stained, and a large number of capillaries in lamina propriety stasis and dilation were recorded as the most serious pathological conditions. Consequently, the tissues treated with LPS for 12 h were used for subsequent analysis. Since LPS can induce the production of cytokines, such as IL-6, IL-1β, and TNF-α (Ahmad et al., 2019), the present study also assessed the related expression levels of cytokines in uterine tissues at 12 h, after LPS induction. The results showed that LPS treatment for 12 h significantly increased the levels of IL-6, IL-1β, and TNF-α, indicating the successful establishment of the LPS-induced endometritis model in mice.

The occurrence of diseases has been previously shown to cause changes in the gut microbiota and its metabolism (Rooks and Garrett, 2016). No previous studies investigated the relation between acute endometritis and gut microbiota. The present study reported changes in intestinal microbiome and metabolism of mice with acute endometritis, using 16SrRNA high-throughput sequencing and LC-MS technology. The study also reported differences in the composition and metabolites of the microbiome structure and metabolites present in the intestines of mice with acute endometritis, induced by LPS. *Elusimicrobia* is known to exert a potential negative impact on health, and it is often used as a biomarker for intestinal damage (Carbonero et al., 2019). At the genus level, a decrease in beneficial bacteria, such as *Christensenellaceae_R.7_group* and *Parasutterella*, was recorded in the LPS group in the present case. An increase in the relative abundance of *Treponema_2*, *Klebsiella*, *Lachnoclostridium_5*, and *Citrobacter* was also recorded. In general, *Christensenellaceae_R.7_Group* is a microorganism that is widely present in humans and animals. It is primarily associated with obesity and inflammatory bowel disease (Waters and Ley, 2019). The relative abundance of *Christensenellaceae_R.7_group* has been reported to be comparatively low in obese patients (Valdes et al., 2018). However, obese people generally exhibit a higher risk of endometritis as compared to the general population (Kalkanbaeva et al., 2017). *Parasutterella* is an important microorganism that maintains the health of the human gastrointestinal tract. It is associated with many diseases (Blasco-Baque et al., 2017; Ju et al., 2019). In comparison to this, *Klebsiella* is a pathogen that primarily causes severe suppurative community-acquired pneumonia, which can infect almost any part of the body and is extremely lethal (Sahly et al., 2002). *Lachnoclostridium_5* is usually associated with digestive diseases (Wang et al., 2018). *Citrobacter* is an extracellular intestinal pathogen that is specifically designed to mimic human pathogenic *E. coli* and inflammatory bowel disease infections (Mullineaux-Sanders et al., 2019). In the present study, these bacteria genera exhibited differential expression in the LPS group, which indicated that after acute endometritis, the abundance of intestinal pathogenic bacteria increased, while the beneficial bacteria decreased, and the protection provided by intestinal barrier also reduced. Consequently, the possibility of the disease increased.

In addition to this, the amount of intestinal metabolite cholesterol was recorded to be higher in the LPS group. Cholesterol is known to be an essential molecule in the animal body, which participates in various biochemical processes, such as cell membrane synthesis and cell growth and differentiation (Russell, 1992). However, the inadequate ability of animals to decompose cholesterol can lead to an increase in the level of cholesterol that can further result in diseases, such as atherosclerosis (Schade et al., 2020). Besides this, acute endometritis also resulted in reduced levels of NAD and Isokobusone. Nicotinamide adenine dinucleotide (NAD) is known for its role in redox reactions, particularly as a hydrogen carrier for cellular oxidoreductases. It also acts as a signaling molecule that regulates hundreds of key processes, including energy metabolism and cell survival, *via* regulation of NAD^+ sensing enzymes. NAD^+ levels usually decline with age, resulting in metabolic changes and increased susceptibility to disease (Rajman et al., 2018). A previous study reported that Isokobusone could activate pregnane X receptor (PXR) and constitutive androstane receptor (CAR) and thus induced the expression of drug metabolism enzymes and inhibited the expression of LPS-induced inflammatory mediators (Kittayaruksakul et al., 2013). Therefore, acute endometritis altered the levels of metabolites in the body, which further affected normal biological redox responses, decreased the inhibition of inflammatory response, and increased susceptibility to the disease.

The combination of microbiology and metabolomics revealed the changes in the composition of the intestinal microbiome in acute endometritis, which suggested that the activity of the gut microbiome might be related to intestinal metabolism. It was found that the abnormal expression of *Treponema_2* in the LPS group positively correlated with cholesterol, wherein Treponema cells could obtain cholesterol from the erythrocytic membranes of eukaryotes (Stanton and Cornell, 1987) and produced exogenous cholesterols during growth. Cholesterol depletion or incorporation of cholesterol molecules might harm the intestinal epithelial membrane. In addition to this, acute endometritis also decreased the relative abundance of *Parasutterella*. *Parasutterella* has been previously shown to be associated with bile acid homeostasis (Ju et al., 2019). Biosynthesis of primary bile acids is generally involved in the pathogenesis of cervicitis. Cervicitis is known to provoke/induce a variety of diseases, including endometritis (Zhang et al., 2018). In addition to this, enrichment analysis of metabolic pathways revealed that LPS could alter the biosynthesis of primary bile acids, indicating an important role in bile acid metabolism. However, the specific mechanism of action remains to be explored in the future. In addition to this, it needs to be explored whether the altered gut microbiota and its metabolites also affected the internal environment of the uterus.

Altogether, the present study reported the induction of acute endometritis in mice upon LPS treatment, and changes were reported in intestinal microbiota and metabolites by 16S rRNA high-throughput sequencing and LC-MS untargeted

metabolism. At the generic level, acute endometritis resulted in the reduction in beneficial microorganisms in the intestinal tract. At the same time, it increased the relative abundance of pathogenic bacteria, altered the metabolic levels of cholesterol and NAD, and affected the corresponding biological REDOX reactions and other biochemical processes. Thus, the findings of this study would provide new strategies for the diagnosis of acute endometritis.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Sequence Read Archive of the National Center for Biotechnology Information repository, accession number PRJNA782690.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Office of Chengdu Normal University, Chengdu, China.

AUTHOR CONTRIBUTIONS

Conceptualization: YD. Methodology: YY, YM, YL, and WZ. Formal analysis: YD, YY. Investigation: XD and JP. Validation: YY and YD. Data curation: BH and YD. Writing—original draft preparation: YD and YY. Writing—review and editing: YM, BH, and SL. Funding acquisition: BH and SL. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Chengdu Normal University scientific research project (No. 111-153701) and the Agricultural Ecology and Green Food Development project (CSCXTD2020B11). The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank the funding support of Chengdu Normal University and the investigators and staff of the Shanghai OE Biotech Co., Ltd for technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.791373/full#supplementary-material

REFERENCES

Ahmad, A., Wu, J., Niu, P., Zhao, Y., Cheng, Y., Chen, W., et al. (2019). Impact of miR-223-3p and miR-2909 on Inflammatory Factors IL-6, IL-1ß, and TNF-α, and the TLR4/TLR2/NF-κb/STAT3 Signaling Pathway Induced by Lipopolysaccharide in Human Adipose Stem Cells. *PloS One* 14 (2), e0212063. doi: 10.1371/journal.pone.0212063

Andrews, W. W., Hauth, J. C., Cliver, S. P., Conner, M. G., Goldenberg, R. L., and Goepfert, A. R. (2006). Association of Asymptomatic Bacterial Vaginosis With Endometrial Microbial Colonization and Plasma Cell Endometritis in Nonpregnant Women. *Am. J. Obstet. Gynecol.* 195 (6), 1611–1616. doi: 10.1016/j.ajog.2006.04.010

Blasco-Baque, V., CoupÃ©, B.r., Fabre, A., Handgraaf, S., Gourdy, P., Arnal, J.-F., et al. (2017). Associations Between Hepatic miRNA Expression, Liver Triacylglycerols and Gut Microbiota During Metabolic Adaptation to High-Fat Diet in Mice. *Diabetologia* 60 (4), 690–700. doi: 10.1007/s00125-017-4209-3

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* 30 (15), 2114–2120. doi: 10.1093/bioinformatics/btu170

Borella, F., Carosso, A. R., Cosma, S., Preti, M., Collemi, G., Cassoni, P., et al. (2021). Gut Microbiota and Gynecological Cancers: A Summary of Pathogenetic Mechanisms and Future Directions. *ACS Infect. Dis.* 7 (5), 987–1009. doi: 10.1021/acsinfecdis.0c00839

Cameron, E. A., and Sperandio, V. (2015). Frenemies: Signaling and Nutritional Integration in Pathogen-Microbiota-Host Interactions. *Cell Host Microbe* 18 (3), 275–284. doi: 10.1016/j.chom.2015.08.007

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* 7 (5), 335–336. doi: 10.1038/nmeth.f.303

Carbonero, F., Mayta, A., Bolea, M., Yu, J.-Z., Lindeblad, M., Lyubimov, A., et al. (2019). Specific Members of the Gut Microbiota Are Reliable Biomarkers of Irradiation Intensity and Lethality in Large Animal Models of Human Health. *Radiat. Res.* 191 (1), 107–121. doi: 10.1667/RR14975.1

Chen, H., Bai, C., and Wang, X. (2010). The Value of the Lipopolysaccharide-Induced Acute Lung Injury Model in Respiratory Medicine. *Expert Rev. Respir. Med.* 4 (6), 773–783. doi: 10.1586/ers.10.71

Ju, T., Kong, J. Y., Stothard, P., and Willing, B. P. (2019). Defining the Role of Parasutterella, A Previously Uncharacterized Member of the Core Gut Microbiota. *ISME J.* 13 (6), 1520–1534. doi: 10.1038/s41396-019-0364-5

Kalkanbaeva, C. K., Shoonaeva, N. D., Asakeeva, R., Niyazova, F., and Dzhakypova, A. (2017). Assessment of Premorbid Background in Pregnant Women With Obesity of Various Degrees. *Kazan Med. J.* 98 (6), 913–917. doi: 10.17750/KMJ2017-913

Kitaya, K., Matsubayashi, H., Takaya, Y., Nishiyama, R., Yamaguchi, K., Takeuchi, T., et al. (2017). Live Birth Rate Following Oral Antibiotic Treatment for Chronic Endometritis in Infertile Women With Repeated Implantation Failure. *Am. J. Reprod. Immunol.* 78 (5). doi: 10.1111/aji.12719

Kitaya, K., Takeuchi, T., Mizuta, S., Matsubayashi, H., and Ishikawa, T. (2018). Endometritis: New Time, New Concepts. *Fertil. Steril.* 110 (3), 344–350. doi: 10.1016/j.fertnstert.2018.04.012

Kitaya, K., and Yasuo, T. (2011). Immunohistochemistrical and Clinicopathological Characterization of Chronic Endometritis. *Am. J. Reprod. Immunol.* 66 (5), 410–415. doi: 10.1111/j.1600-0897.2011.01051.x

Kittayaruksakul, S., Zhao, W., Xu, M., Ren, S., Lu, J., Wang, J., et al. (2013). Identification of Three Novel Natural Product Compounds That Activate PXR and CAR and Inhibit Inflammation. *Pharm. Res.* 30 (9), 2199–2208. doi: 10.1007/s11095-013-1101-9

Kiviat, N. B., WÃ¸lner-Hanssen, P.l., Eschenbach, D. A., Wasserheit, J. N., Paavonen, J. A., Bell, T. A., et al. (1990). Endometrial Histopathology in Patients With Culture-Proved Upper Genital Tract Infection and Laparoscopically Diagnosed Acute Salpingitis. *Am. J. Surg. Pathol.* 14 (2), 167–175. doi: 10.1097/00000478-199002000-00008

Koeth, R. A., Wang, Z., Levison, B. S., Buffa, J. A., Org, E., Sheehy, B. T., et al. (2013). Intestinal Microbiota Metabolism of L-Carnitine, A Nutrient in Red

Meat, Promotes Atherosclerosis. *Nat. Med.* 19 (5), 576–585. doi: 10.1038/nm.3145

Li, R., Maimai, T., Yao, H., Liu, X., He, Z., Xiao, C., et al. (2019). Protective Effects of Polydatin on LPS-Induced Endometritis in Mice. *Microb. Pathog.* 137, 103720. doi: 10.1016/j.micpath.2019.103720

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., et al. (2016). The Gut Microbiota and Host Health: A New Clinical Frontier. *Gut* 65 (2), 330–339. doi: 10.1136/gutjnl-2015-309990

Moreno, I., Cicinelli, E., Garcia-Grau, I., Gonzalez-Monfort, M., Bau, D., Vilella, F., et al. (2018). The Diagnosis of Chronic Endometritis in Infertile Asymptomatic Women: A Comparative Study of Histology, Microbial Cultures, Hysteroscopy, and Molecular Microbiology. *Am. J. Obstetrics Gynecol.* 218 (6), 602.e601–602.e616. doi: 10.1016/j.ajog.2018.02.012

Mullineaux-Sanders, C., Sanchez-Garrido, J., Hopkins, E. G. D., Shenoy, A. R., Barry, R., and Frankel, G. (2019). Citrobacter Rodentiumâ€"Hostâ€"Microbiota Interactions: Immunity, Bioenergetics and Metabolism. *Nat. Rev. Microbiol.* 17 (11), 701–715. doi: 10.1038/s41579-019-0252-z

Nossa, C. W., Oberdorf, W. E., Yang, L., Aas, J., Paster, B. J., DeSantis, T. Z., et al. (2010). Design of 16S rRNA Gene Primers for 454 Pyrosequencing of the Human Foregut Microbiome. *World J. Gastroenterol.: WJG* 16 (33), 4135–4144. doi: 10.3748/wjg.v16.i33.4135

Plottel, C. S., and Blaser, M. J. (2011). Microbiome and Malignancy. *Cell Host Microbe* 10 (4), 324–335. doi: 10.1016/j.chom.2011.10.003

Prentice, K. J., Luu, L., Allister, E. M., Liu, Y., Jun, L. S., Sloop, K. W., et al. (2014). The Furan Fatty Acid Metabolite CMPF Is Elevated in Diabetes and Induces Î² Cell Dysfunction. *Cell Metab.* 19 (4), 653–666. doi: 10.1016/j.cmet.2014.03.008

Rajman, L., Chwalek, K., and Sinclair, D. A. (2018). Therapeutic Potential of NAD-Boosting Molecules: The In Vivo Evidence. *Cell Metab.* 27 (3), 529–547. doi: 10.1016/j.cmet.2018.02.011

Reyon, D., Tsai, S. Q., Khayter, C., Foden, J. A., Sander, J. D., and Joung, J. K. (2012). FLASH Assembly of TALENs for High-Throughput Genome Editing. *Nat. Biotechnol.* 30 (5), 460–465. doi: 10.1038/nbt.2170

Rognes, T.r., Flouri, T.Å., Nichols, B., Quince, C., and MahÃ©, F. (2016). VSEARCH: A Versatile Open Source Tool for Metagenomics. *PeerJ* 4, e2584. doi: 10.7717/peerj.2584

Rooks, M. G., and Garrett, W. S. (2016). Gut Microbiota, Metabolites and Host Immunity. *Nat. Rev. Immunol.* 16 (6), 341–352. doi: 10.1038/nri.2016.42

Russell, D. W. (1992). Cholesterol Biosynthesis and Metabolism. *Cardiovasc. Drugs Ther.* 6 (2), 103–110. doi: 10.1007/BF00054556

Sahly, H., Podschun, R., and Ullmann, U. (2002). Klebsiella Infections in the Immunocompromised Host. *Biol. Pathol. Innate Immun. Mech.* 479, 237–249. doi: 10.1007/0-306-46831-X_21

Schade, D. S., Shey, L., and Eaton, R. P. (2020). Cholesterol Review: A Metabolically Important Molecule. *Endocrine Pract.* 26 (12), 1514–1523. doi: 10.4158/EP-2020-0347

Sharon, G., Garg, N., Debelius, J., Knight, R., Dorrestein, P. C., and Mazmanian, S. K. (2014). Specialized Metabolites From the Microbiome in Health and Disease. *Cell Metab.* 20 (5), 719–730. doi: 10.1016/j.cmet.2014.10.016

Stanton, T. B., and Cornell, C. P. (1987). Erythrocytes as a Source of Essential Lipids for Treponema Hyodysenteriae. *Infect. Immun.* 55 (2), 304–308. doi: 10.1128/IAI.55.2.304-308.1987

Tan, H.-E., Sisti, A. C., Jin, H., Vignovich, M., Villavicencio, M., Tsang, K. S., et al. (2020). The Gutâ€"Brain Axis Mediates Sugar Preference. *Nature* 580 (7804), 511–516. doi: 10.1038/s41586-020-2199-7

Ursell, L. K., Haiser, H. J., Van Treuren, W., Garg, N., Reddivari, L., Vanamala, J., et al. (2014). The Intestinal Metabolome: An Intersection Between Microbiota and Host. *Gastroenterology* 146 (6), 1470–1476. doi: 10.1053/j.gastro.2014.03.001

Valdes, A. M., Walter, J., Segal, E., and Spector, T. D. (2018). Role of the Gut Microbiota in Nutrition and Health. *BMJ (Clinical Res. ed.)* 361, k2179–k2179. doi: 10.1136/bmj.k2179

Wallach, E., and Czernobilsky, B. (1978). Endometritis and Infertility. *Fertil. Steril.* 30 (2), 119–130. doi: 10.1016/s0015-0282(16)43448-5

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences Into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.* 73 (16), 5261–5267. doi: 10.1128/AEM.00062-07

Wang, C.-S.-E., Li, W.-B., Wang, H.-Y., Ma, Y.-M., Zhao, X.-H., Yang, H., et al. (2018). VSL3 Can Prevent Ulcerative Colitis-Associated Carcinogenesis in Mice. *World J. Gastroenterol.* 24 (37), 4254–4262. doi: 10.3748/wjg.v24.i37.4254

Wang, X., and Zhang, Y. (2018). Resveratrol Alleviates LPS-Induced Injury in Human Keratinocyte Cell Line HaCaT by Up-Regulation of miR-17. *Biochem. Biophys. Res. Commun.* 501 (1), 106–112. doi: 10.1016/j.bbrc.2018.04.184

Waters, J. L., and Ley, R. E. (2019). The Human Gut Bacteria Christensenellaceae are Widespread, Heritable, and Associated With Health. *BMC Biol.* 17 (1), 83–83. doi: 10.1186/s12915-019-0699-4

Wu, H., Dai, A., Chen, X., Yang, X., Li, X., Huang, C., et al. (2018). Leonurine Ameliorates the Inflammatory Responses in Lipopolysaccharide-Induced Endometritis. *Int. Immunopharmacol.* 61, 156–161. doi: 10.1016/j.intimp.2018.06.002

Wu, H., Zhao, G., Jiang, K., Li, C., Qiu, C., and Deng, G. (2016). Engeletin Alleviates Lipopolysaccharide-Induced Endometritis in Mice by Inhibiting TLR4-Mediated NF-kappaB Activation. *J. Agric. Food Chem.* 64 (31), 6171–6178. doi: 10.1021/acs.jafc.6b02304

Yachida, S., Mizutani, S., Shiroma, H., Shiba, S., Nakajima, T., Sakamoto, T., et al. (2019). Metagenomic and Metabolomic Analyses Reveal Distinct Stage-Specific Phenotypes of the Gut Microbiota in Colorectal Cancer. *Nat. Med.* 25 (6), 968–976. doi: 10.1038/s41591-019-0458-7

Zhang, X., He, M., Lei, S., Wu, B., Tan, T., Ouyang, H., et al. (2018). An Integrative Investigation of the Therapeutic Mechanism of Ainsliaea Fragrans Champ. In Cervicitis Using Liquid Chromatography Tandem Mass Spectrometry Based on a Rat Plasma Metabolomics Strategy. *J. Pharm. Biomed. Anal.* 156, 221–231. doi: 10.1016/j.jpba.2018.04.048

Zhao, G., Jiang, K., Wu, H., Qiu, C., Deng, G., and Peng, X. (2017). Polydatin Reduces Staphylococcus Aureus Lipoteichoic Acid-Induced Injury by Attenuating Reactive Oxygen Species Generation and TLR2-NFkappaB Signalling. *J. Cell Mol. Med.* 21 (11), 2796–2808. doi: 10.1111/jcmm.13194

Zhou, M., Yi, Y., and Hong, L. (2019). Oridonin Ameliorates Lipopolysaccharide-Induced Endometritis in Mice via Inhibition of the TLR-4/NF-Kappabpathway. *Inflammation* 42 (1), 81–90. doi: 10.1007/s10753-018-0874-8

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 18 January 2022
doi: 10.3389/fcimb.2021.795924

Metabolic and Microbial Changes Associated With Diet and Obesity During Pregnancy: What Can We Learn From Animal Studies?

Caitlin Dreisbach[1], *Hailey Morgan*[2], *Caroline Cochran*[3], *Adwoa Gyamfi*[4], *Wendy Ann Henderson*[4,5] *and Stephanie Prescott*[2]*

[1] Data Science Institute, Columbia University, New York, NY, United States, [2] College of Nursing, University of South Florida, Tampa, FL, United States, [3] School of Nursing, Columbia University, New York, NY, United States, [4] School of Medicine, University of Connecticut, Farmington, CT, United States, [5] School of Nursing, University of Connecticut, Storrs, CT, United States

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Sabrina Casarotti, Federal University of Rondonópolis, Brazil
Helioswilton Sales-Campos, Universidade Federal de Goiás, Brazil
Daria Peleg-Raibstein, ETH Zürich, Switzerland

***Correspondence:**
Stephanie Prescott
prescotts@usf.edu

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *15 October 2021*
Accepted: *13 December 2021*
Published: *18 January 2022*

Citation:
Dreisbach C, Morgan H, Cochran C, Gyamfi A, Henderson WA and Prescott SM (2022) Metabolic and Microbial Changes Associated With Diet and Obesity During Pregnancy: What Can We Learn From Animal Studies? Front. Cell. Infect. Microbiol. 11:795924. doi: 10.3389/fcimb.2021.795924

The intestinal microbiota changes throughout pregnancy and influences maternal metabolic adaptations to support fetal growth. Obesity induces alterations to the microbiota that include decreased microbial diversity and shifts in microbial composition, though specific species changes are inconsistent between published studies. In animal models, probiotics and exercise moderate maternal weight gain and partially correct the maternal microbiota. Supplemental *Escherichia coli*, however, exacerbate maternal obesity during the perinatal period, lending weight to the theory that inflammation-induced gut epithelial barrier leak influences metabolic dysregulation. Although birth weight is not always altered when offspring are exposed to an obesogenic diet during gestation, insulin resistance and lipid metabolism are impacted through adulthood in association with this exposure and can lead to increased body weight in adulthood. Postnatal offspring growth is accelerated in response to maternal overnutrition during lactation. Offspring microbiota, metabolism, and behavior are altered in response to early exposure to high fat and high sucrose diets. Consequences to this exposure include impaired glucose and insulin homeostasis, fatty liver, and neurobehavioral deficits that can be ameliorated by improving the microbial environment. In this mini review, we provide an overview of the use of translational animal models to understand the mechanisms associated with changes to the gastrointestinal microbiota due to maternal obesity and the microbial impact on the metabolic changes of pregnancy.

Keywords: obesity, microbiota, pregnancy, metabolism, diet

INTRODUCTION

Worldwide obesity rates have almost tripled in the last 35 years (World Health Organization (WHO), 2021). Nearly two billion adults over the age of 18 are overweight (B.M.I. 24-29 kg/m^2), and 650 million are obese (B.M.I.> 30 kg/m^2) (World Health Organization (WHO), 2021). Additionally, over 340 million children and adolescents aged 5-18 are overweight or obese (World Health Organization

(WHO), 2021). These statistics contribute to the 2.6 million people who die each year due, in part, to having an overweight or obese BMI (World Health Organization (WHO), 2021). More specifically, maternal obesity, or obesity directly before or during pregnancy, is associated with an increased risk of complications during gestation, obesity, and neurodevelopmental abnormalities in offspring (Kim et al., 2017). The composition of the maternal intestinal microbiota changes throughout pregnancy and lactation as maternal metabolism adjusts to support the needs of the mother and the developing infant (Edwards et al., 2017). Obesity leads to altered microbial colonization, which may impact not only fecundity and pregnancy outcomes but the metabolic and developmental programming of offspring (Zhou and Xiao, 2018). While many studies focus on either human subjects or animal models, little work has been done to integrate the translational nature of these research questions.

Understanding the changes in the maternal microbiota because of obesity and overnutrition may elucidate the relationship between the microbiota and obesity-related adverse metabolic outcomes. Animal models have long been used to understand the mechanisms that may be involved in human pathophysiology as they 1) allow rigorous experimental control; 2) permit environmental, genetic, and experimental manipulation that is intolerable, unethical, or unsafe to humans, particularly when in vulnerable conditions such as pregnancy; and 3) facilitate molecular study at the tissue level of biologic phenomenon. Therefore, the purpose of this mini review is to synthesize recent research on the importance of the maternal microbiota to metabolic functioning during pregnancy, examine the influence of maternal obesity on offspring microbiota, and evaluate the potential of translational animal models to inform human clinical studies.

OBESITY AND MATERNAL MICROBIOTA DURING GESTATION

Not surprisingly, because of its role in energy extraction, metabolism, hormone regulation, and inflammatory crosstalk, the maternal intestinal microbial community is altered soon after pregnancy begins and continues to change throughout gestation (Nuriel-Ohayon et al., 2016). The gut microbiome typically consists of several phyla including Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Rinninella et al., 2019). Of these, Firmicutes and Bacteroidetes make up approximately 90% of the microbial environment within the gut (Rinninella et al., 2019). The relative abundance of specific microbes is often stable in individuals, barring changes as a result of disease processes and antibiotic use, and is influenced by an individual's dietary habits, cultural lifestyles, physical environment, exercise habits, and age (Rinninella et al., 2019). During pregnancy, maternal fecal within-sample bacterial diversity (alpha diversity) decreases significantly throughout gestation. Maternal obesity reduces alpha diversity further, though species affected are different across studies. Notably, as women progress through pregnancy, they have dramatically expanded intestinal beta diversity (high levels of between-individual variation), which is globally distinct from early gestation or nonpregnant controls (Rinninella et al., 2019). This increase in intestinal beta diversity during the third trimester of pregnancy is largely independent of maternal health status, suggesting that it is driven by pregnancy itself (Rinninella et al., 2019). Proteobacteria and Actinobacteria are enriched in the third trimester and induce inflammation at the intestinal mucosal interface leading to increases in inflammatory cytokines, including TNFa (Rinninella et al., 2019). In a study examining the human maternal microbiome across pregnancy and into the postpartum period, researchers transferred third-trimester microbiota to germ-free mice, which resulted in increased inflammation, decreased glucose tolerance, and increased adiposity compared to germ-free mice colonized with first-trimester microbiota (Rinninella et al., 2019). High intestinal beta diversity persists into the postpartum period, which may impact infant colonization and energy harvesting capabilities.

During the gestational period, the high fat/high fat high sugar (HF/HFHS) diet has a temporal effect on microbial composition in animal studies. A study comparing mice on a HF diet to mice genetically predisposed to obesity (ob/ob) on a regular diet found a trend toward increased Firmicutes in the ob/ob mice, but a significant proportional increase in Firmicutes from baseline to 4-weeks in the HF diet group (p <.05) (Murphy et al., 2010). A significant proportional increase in Actinobacteria was seen in both the ob/ob and HF diet groups after the first four weeks (compared to baseline) but did not significantly increase in the following 4-week period (Murphy et al., 2010). Therefore, it appears that microbiota associated with genetically induced obesity flourish in the gastrointestinal tracts of wild type mice in the presence of HF diet, and these microbes persist or increase over time. While animal studies cannot be directly interpreted as relevant to humans because of different diets, habitats, and species adaptations, all the animals in the studies included in this review share similar microbial profiles to humans at the higher taxonomic levels except Felis catus, which are colonized almost entirely by Firmicutes on the lower fat, dry food diet and have a 25% relative abundance of Fusobacteria in the higher fat, higher carbohydrate, wet diet. Like humans, obese mouse, rat, and monkey dams have altered gut microbiota composition uniquely across studies compared to lean dams (Kirwan et al., 2002). Furthermore, humans, rats, mice, and sows have distinct microbial and metabolic signatures between pre-pregnancy, gestation, and lactation, strengthening translation of animal studies to human clinical practice (Zhou et al., 2019; Kimura et al., 2020; Zhou et al., 2020). Though most studies concentrate on the effects of maternal obesity and the obesogenic diet on the offspring microbiota and outcomes, several studies focus on the microbiota during gestation and lactation and the associated changes to the maternal metabolism in response to a HFHS diet, obesity, prebiotic supplementation, exercise, and non-caloric sweetener use.

OBESITY AND METABOLISM DURING GESTATION

The basal metabolic rate in healthy pregnant women increases by 4%, 10%, and 24% during the first, second, and third trimesters,

respectively, because of increases in tissue synthesis and body mass and increased cardiovascular, renal, and respiratory work effort (Butte, 2005). Glucose metabolism changes significantly throughout gestation. Fasting blood glucose levels and insulin sensitivity decrease as pregnancy progresses, even as hepatic glucose and insulin production increases, thus allowing euglycemia in the mother and glucose transport across the placenta to support fetal growth (Lain and Catalano, 2007). In late gestation, maternal insulin insensitivity is profound, allowing enhanced glucose availability and clearance by the placenta and the fetus (Sivan et al., 1999). In obese women, the basal glucose and insulin levels are further increased while hepatic glucose production remains unchanged, indicating an inability to regulate glucose production *via* insulin-dependent pathways in the liver. The physiologic mechanisms leading to insulin insensitivity during pregnancy are not well understood, but potential contributing pathways have been identified. Tumor necrosis factor alpha (TNFa), a cytokine and adipokine, is secreted from the placenta and is a candidate regulatory molecule in muscle and adipose tissue. TNFa promotes the phosphorylation of insulin receptor substrate 1, which interferes with insulin binding, leading to insulin insensitivity in muscle and adipose tissue (Kirwan et al., 2002). Maternal plasma-free fatty acid concentrations also play a role in reduced hepatic insulin sensitivity by downregulating insulin receptor signaling in hepatocytes, which allows gluconeogenesis and glycolysis to continue in the setting of elevated blood glucose (Hadden and McLaughlin, 2009). Increased insulin resistance and elevated free fatty acid concentrations lead to increased adipose tissue stores in healthy pregnant women providing an accessible calorie source for both mother and fetus and a reservoir of cytokines and inflammatory markers that contribute to dynamic maternal metabolism (Lain and Catalano, 2007).

Also noted is the length of high fat/high fat high sugar (HF/HFHS) nutritional exposure and its effect on metabolic processes over time. HF diet has been shown to induce marked glucose intolerance and compromised insulin response in mouse models after only one week (Winzell and Ahren, 2004). The longer the mouse is exposed to a HF diet, the more pronounced the effect on their metabolic function—increased weight, adiposity, and sustained hyperglycemia are typically seen within four weeks of the HF diet regimen, with further progression into obesity and related sequelae by 16 weeks (Wang and Liao, 2012). While the transition to pregnancy (first trimester) is marked by increases in ketone bodies, myo-inositol, butyrate, tryptophan, and phenylalanine, the transition from gestation to lactation is marked by decreases in branched-chain amino acids (BCAA) and urea cycle metabolites. Obese dams have decreased microbial abundance and poorer transitions between the different metabolic states. Physiologic insulin resistance seen in late gestation appears in early gestation in obese dams, contributing to increased maternal adiposity and insulin resistance at an earlier fetal developmental period (Paul et al., 2016; Paul et al., 2018). Obese metabolic profiles have altered fatty acid oxidation, amino acid metabolism, gut microbial metabolites, and phospholipid metabolism (Paul et al., 2016). Metabolites involved in glucose and fatty acid metabolism, methionine, and tryptophan metabolism to serotonin are altered in obese dams (Paul et al., 2018). However, while obese rat dams have higher weight gain during gestation and higher energy intake than lean rat dams on a standard diet (SD), both groups retained similar blood glucose and blood insulin levels during gestation (Paul et al., 2018). Maternal HF diet, however, also influences the methylation of genomic regions proximal to *PPARG* and *FGF-21* genes, pathways involved in fatty acid and glucose regulation (Ge et al., 2012; Wankhade et al., 2017). These changes in maternal metabolism may influence fetal metabolic programming.

Animal studies have examined the effect of prebiotics on microbial composition and metabolic functioning in obese dams. Compared to rat dams on an ad libitum HFHS diet, weight-managed or oligofructose (OFS)-supplemented dams have lower blood glucose and plasma insulin concentrations in response to an oral glucose tolerance test, lower fasting plasma leptin, and higher peptide-YY (PYY), a satiety hormone. Insulin sensitivity is improved during lactation across groups. Altering the microbiota by adding the prebiotic oligofructose (OFS) to the diet increases the relative abundance of the Actinobacteria, Bifidobacterium, which corrects some metabolic functions by increasing PYY and glucagon-like peptide-1 (GLP-1), satiety hormones which lead to normalized weight gain during gestation. Although there was no SD control group in this study, maternal HFHS diet dams without OFS have increased levels of precursors to ketone bodies, elevated metabolites involved in lipid metabolism, elevated branch chain amino acids, and elevated glucogenic amino acids compared to weight-managed dams also receiving the HFHS diet or those not weight-managed but supplemented with OFS (Paul et al., 2016). The ability to manipulate maternal metabolism, and possible fetal metabolic programming by means of altering the microbiota is a positive indication that such manipulations may be a possible avenue for human translational study.

OFFSPRING MICROBIOTA AND METABOLISM

Maternal obesity, HF, or HFHS diets during pregnancy do not appear to consistently impact the initial birth weight of offspring, even though fatty acids and sugars pass freely to the fetus through the placenta, but typically do influence the weight of offspring over their lifetime (Smith et al., 1992). Metabolic programming of the offspring is also impacted, as offspring exposed to HF/HFHS diets during gestation display insulin resistance, glucose intolerance, and altered lipid profiles (Zhou et al., 2019; Kimura et al., 2020; Zhou et al., 2020). In a study examining the effects of germ-free (GF) and specific pathogen-free (SPF) environments on pregnant mice and their progeny, offspring from both GF and low-fiber fed dams expressed obesogenic phenotypes (increased weight gain and glucose intolerance) when exposed to HF diets during adulthood (Kimura et al., 2020). Interestingly, when dams in these conditions were treated with short-chain fatty acids (SCFA)

during pregnancy, offspring were resistant to obesity *via* SCFA-specific axes when exposed to the same HF diet after weaning. SCFAs are essential to the normal development of metabolic and neural systems in the pre-and postnatal period, affecting the metabolic programming of offspring in the embryonic stage (Kimura et al., 2020). This supports the notion that a decrease in microbial organisms related to diet- or antibiotic-induced gut dysbiosis that produce these essential metabolites can promote susceptibility to metabolic dysfunction and obesity in offspring through disruption of normal postnatal metabolism.

Research examining the effect of maternal exercise during pregnancy has shown similar ameliorative effects related to SCFA production (Zhou et al., 2020). Compared to SD and HF non-exercise groups, pregnant mice fed HF diets with voluntary access to exercise wheel activity prior to and during pregnancy had improved insulin sensitivity and reduced metabolic dysfunction in offspring as they transitioned into adulthood (Zhou et al., 2020). This metabolic improvement was associated with the protection of SCFA-producing bacteria in the HF exercise group. These diets during lactation and initial microbial colonization impact growth as differences in body weight or body fat content are observed before weaning, in some instances, before the offspring are of age to trial solid foods (Fåk et al., 2012; Paul et al., 2016; Bruce-Keller et al., 2017; Paul et al., 2018). Offspring weaned to the HF/HFHS diet typically become obese over time, though there are potential sex differences noted in the literature. Germ-free mice colonized with the cecal microbiota from donor mice on a HF diet had significantly heavier female offspring than SD dam's offspring before weaning and had higher body fat by nine weeks of age even though all offspring were weaned to a SD (Bruce-Keller et al., 2017). Offspring exposed to overnutrition during gestation and then weaned to a SD do not always weigh significantly more than offspring maintained on a SD but exhibit more adiposity, insulin resistance, glucose intolerance, and lipid profile disorders in adulthood (Ma et al., 2014; Buffington et al., 2016; Wankhade et al., 2017). Offspring exposed to SD during gestation then weaned to the HF diet do surpass the weights of those maintained on the SD, though it took more time to match the weights of those fed the HF diet throughout gestation, lactation, and after weaning (Wankhade et al., 2017).

Overnutrition during pregnancy leads to decreased alpha diversity in offspring and distinct clustering in principal coordinate analyses of beta diversity, though drivers of diversity are different between studies. Most of the differences in relative abundance in the microbiota of offspring in the special diet groups occur at the lower levels of taxonomic classification within the Firmicutes phylum. This is perhaps not surprising as this phylum, along with the phylum Bacteroidetes, usually dominates the intestines of mammals (Jandhyala et al., 2015). Firmicutes populations are expanded while populations from Proteobacteria, Actinobacteria, and Bacteroidetes are reduced in over-nourished offspring. Sex differences exist in both the microbiota composition and the obesity phenotype of offspring but were inconsistent between studies (Bruce-Keller et al., 2017; Dennison et al., 2017). A HF diet is also associated with physical changes in the gastrointestinal tracts of offspring. For example, the pH of the stomach is increased, the protein concentration of the duodenum is decreased, sucrase and maltase enzymatic activity is decreased, and there is increased intestinal permeability in over-nourished offspring (Fåk et al., 2012). Dams that receive the prebiotic OFS have increased abundance of the Actinobacteria : Bifidobacterium and the Firmicutes : Clostridium, with altered SCFA production; and they have offspring with increased satiety hormones, PYY and GLP, leading to reductions in body fat (Paul et al., 2016). Offspring on the HFHS diet have higher glucose but similar insulin levels, higher leptin levels, and lower ghrelin levels compared to those on the HF diet alone. Offspring on the HF diet have normal glucose and triglyceride levels but higher cholesterol and non-esterified fatty acid levels leading to increased liver steatosis and fibrosis (Paul et al., 2016; Dennison et al., 2017; Wankhade et al., 2017). Offspring exposed to the HF diet have increased inflammation and inflammatory markers haptoglobin in serum and *LY6D* in the liver (Fåk et al., 2012; Wankhade et al., 2017).

Mechanisms by which changes in the microbiota influence the offspring's growth and development are investigated though the changes in microbial populations induced by gestational overnutrition are not the same between studies. Structural (intestinal hyperpermeability) and chemical (decreased stomach pH) changes occur in the gastrointestinal tract because of exposure to the HF diet, which may allow bacterial components or products to bypass the body's normal defenses (Fåk et al., 2012). Increases in the bacterial components and metabolites promote upregulation of inflammatory signaling pathways leading to liver steatosis and fibrosis (Wankhade et al., 2017). Changes in glucose, insulin, and fatty acid metabolism during gestation and lactation alter placental energy transactions and the availability of substrates for neurotransmitter formation (Wankhade et al., 2017; Dennison et al., 2017). Thus, alterations in normal colonization result in increased obesity and metabolic dysfunction by many different mechanisms (Ma et al., 2014; Bermingham et al., 2018).

DISCUSSION

The information presented in this review reaffirms the importance of considering animal studies in translational research for obese pregnant individuals. Diversity changes associated with adaptations to normal pregnancy are intensified in the setting of maternal obesity and overnutrition (Paul et al., 2018; Zhou and Xiao, 2018). Differences in microbial colonization patterns during gestation because of diet are more visible in animals than in human studies, perhaps because of the controls on genetics, environment, sample collection, and diet consumption. Metabolic profiles shift throughout normal gestation and lactation because of shifts in the hormonal, inflammatory, and nutritional environment, resulting in increased weight gain, blood glucose and blood insulin levels, and free fatty acids. In the context of maternal obesity and overnutrition, metabolic profiles shift more erratically, exposing developing offspring to increased levels of glucose, insulin, leptin,

and BCAA leading to altered placental energy transfer. Obesity-induced methionine deficiency may interfere with methylation pathways involved in normal fetal development. Additionally, decreased tryptophan to serotonin metabolism may impact neurodevelopment and behavior in offspring (Paul et al., 2018).

Some studies reported no significant differences in offspring birthweight between dams fed HF or SD; instead, they highlighted the more frequent presence of differences in metabolic programming between these offspring and their susceptibility to obesity in adulthood. This is interesting, given previous associations between high maternal BMI and babies born large for gestational age, and may reflect the importance of the timing of maternal obesity and overnutrition to impact fetal developmental programming, or the effects of maternal microbial ecology (Jones et al., 2009). Although there is some evidence that the fetus begins acquiring its microbiota even before birth (Perez-Muñoz et al., 2017), the process of acquiring a stable adult-like microbiota takes up to 3 years in humans (Wankhade et al., 2017). Like germ-free mice, the nearly sterile fetus may not have the microbiota necessary to respond to HF diet exposure until after birth and colonization with a microbiota skewed toward obesity. Perhaps the maternal microbiota composition affects fetal metabolic programming independent of nutrition. More research is needed on maternal microbiota composition on fetal developmental programming. The bacterial constituents important for inducing obesity are challenging to determine as transplanted microbes from both obese and lean mice can induce obesity in HF diet germ-free mice (Rabot et al., 2016). Accordingly, offspring weaned to SD maintain normal weights despite their pre-weaning diet while offspring weaned to HF diet have increased weights even if they had been exposed to SD before weaning and during gestation (Buffington et al., 2016; Wankhade et al., 2017). Mice never exposed to the HF diet, and only to the microbiota of HF-fed mice, have increased body weight and fat content in a sex-dependent manner (Bruce-Keller et al., 2017). Several studies report that there are responders and non-responders to HF diet feeding among genetically identical test subjects, indicating that not all bacterial communities interact with host genetics to produce the obese phenotype (Ma et al., 2014).

Diet changes elicit rapid shifts in gut microbial communities (David et al., 2014). As the fetus does not always respond to the maternal HF diet with increased body weight before birth but does respond before consuming food other than breastmilk, it appears that the diet induces changes to the maternal microbiota, which then shifts and induces metabolic alterations in the mother and the fetus. The lack of obesity when HF-exposed offspring are weaned to a standard diet, but continued glucose intolerance, insulin resistance, and serum fatty acid elevation point to the influence of the microbiota in fetal metabolic programming. Delayed onset of obesity when SD-exposed offspring are weaned to the HF diet and increased growth rates of offspring of germ-free mice when the pregnant dams are exposed to microbiota transplant indicate that the diet-induced changes to the microbiota during development have lasting metabolic impact on offspring. Exposure to the HF diet *in utero* may alter the offspring's metabolic programming as changes in glucose tolerance, insulin resistance, lipid profiles, and satiety hormones are observed in adult offspring even though increased body weight may not be observed (Zhou et al., 2019; Zhou et al., 2020). The proposed mechanism by which the microbiota regulate offspring metabolic programming includes SCFA production and free fatty acid receptor signaling in the murine fetal sympathetic nervous system, adipose tissue, and pancreas that disrupts energy homeostasis (Kimura et al., 2020). Changes to the maternal microbiome through exercise, non-nutritive sweeteners, or fiber supplementation result in changes to the maternal microbial milieu and maternal and offspring metabolism (Zhou et al., 2019; Zhou et al., 2020). The occurrence of HF diet non-responders seems to indicate that there is also an interaction between host genetics and microbial inhabitants that influence the metabolic changes that lead to obesity. Fåk et al. (2012) explored this hypothesis by inducing increased obesity over HF-induced obesity by adding Proteobacteria and *Escherichia coli* to the drinking water of dams and offspring receiving a HF diet (Fåk et al., 2012).

Synthesis of results is challenging because of the different diets used, experimental species and conditions tested, taxonomic levels reported, and time points evaluated. However, variations between animal studies are fewer than those that occur between human studies because of the ability to exert stringent controls. There are important limitations to be considered when translating research in animal models to humans, including overarching biological differences between human and rodent subjects, housing and environmental conditions, and the ability to strictly control the diet in animal models to the degree that is irreplicable in human samples. Additionally, certain neuroendocrinological developmental processes occur at different times in the developing rodent versus human. The HPA axis and appetite regulatory network in rodents develops during the postnatal period, in contrast to developing during the third trimester in humans hindering comparisons between the stress response and eating behaviors (Rinaudo and Wang, 2012). Human pancreatic cells continue to go through remodeling until around age 4; in rats, the pancreas is developed later in pregnancy and experiences important remodeling around the time that offspring are weaned (Rinaudo and Wang, 2012). Data regarding comparisons in insulin production in the context of offspring of obese mothers should be evaluated with this fact in mind. As mentioned previously, in many animal studies, the methodology utilized would be unethical to replicate in human subjects, especially those who are pregnant. However, the ability to manipulate the nutrients, environment, genetics, and microbiota of animals, in addition to the ability to study the effects of changes *in vivo* and *in vitro*, is an important adjunct to human research to fully understand the mechanisms by which the microbiota interacts with the host to support the developing fetus and neonate for optimal health. Animal studies are also invaluable in closely evaluating the impact of obesity and overnutrition during gestation, lactation, and microbial colonization. The microbiota is an exciting target for influencing maternal and fetal metabolism, particularly as it is

easily manipulated and may provide an option for therapy that confers a life-long benefit. Future studies should examine the moderating effect of probiotic supplementations and physical exercise in obese, pregnant dams and the microbial colonization, metabolic and neural outcomes in offspring over their lifetime.

CONCLUSION

Studies across species reveal how changes in the maternal microbiota during pregnancy are necessary to support the normal physiologic changes during pregnancy and metabolic programming of offspring. Changes in maternal microbiota induced by various methods can lead to long-lasting changes in maternal and offspring metabolic health, with mechanisms such as molecular signaling by metabolites and inflammatory cytokines or the alteration in gut epithelium allowing bacterial invasion. More research is required to determine the mechanisms by which the intestinal microbes participate in or respond to changes in the gastrointestinal tract that result from maternal overnutrition in humans, but that they have a role in the outcomes associated with maternal overnutrition by altering the substrates available for maternal, placental, and fetal metabolic processes are evident.

AUTHOR CONTRIBUTIONS

CD and SP conceptualized the manuscript. SP and CC performed the search of the literature. CD and CC created Table 1 based on the search results. CD and SP drafted the manuscript with all authors (CC, AG, WH, HM) providing substantial revisions. All authors contributed to the article and approved the submitted version.

REFERENCES

Bäckhed, F., Manchester, J. K., Semenkovich, C. F., and Gordon, J. I. (2007). Mechanisms Underlying the Resistance to Diet-Induced Obesity in Germ-Free Mice. *Proc. Natl. Acad. Sci.* 104 (3), 979–984. doi: 10.1073/pnas.0605374104

Bermingham, E. N., Young, W., Butowski, C. F., Moon, C. D., Maclean, P. H., Rosendale, D., et al. (2018). The Fecal Microbiota in the Domestic Cat (Felis Catus) Is Influenced by Interactions Between Age and Diet; A Five Year Longitudinal Study. *Front. Microbiol.* 9, 1231. doi: 10.3389/fmicb.2018.01231

Bruce-Keller, A. J., Fernandez-Kim, S.-O., Townsend, R. L., Kruger, C., Carmouche, R., Newman, S., et al. (2017). Maternal Obese-Type Gut Microbiota Differentially Impact Cognition, Anxiety and Compulsive Behavior in Male and Female Offspring in Mice. *PloS One* 12 (4), e0175577. doi: 10.1371/journal.pone.0175577

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., and Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* 165 (7), 1762–1775. doi: 10.1016/j.cell.2016.06.001

Butte, N. F. (2005). Energy Requirements During Pregnancy and Consequences of Deviations From Requirement on Fetal Outcome. *Nestle Nutr. Workshop Ser. Paediatr. Program.* 55, 49–67; discussion 67-71. doi: 10.1159/000082593

Cheng, C., Wei, H., Yu, H., Xu, C., Jiang, S., and Peng, J. (2018). Metabolic Syndrome During Perinatal Period in Sows and the Link With Gut Microbiota and Metabolites. *Front. Microbiol.* 9, 1989. doi: 10.3389/fmicb.2018.01989

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. *Nature* 505 (7484), 559–563. doi: 10.1038/nature12820

Dennison, C. A., Eslinger, A. J., and Reimer, R. A. (2017). Preconception Prebiotic and Sitagliptin Treatment in Obese Rats Affects Pregnancy Outcomes and Offspring Microbiota, Adiposity, and Glycemia. *Front. Endocrinol.* 8, 301. doi: 10.3389/fendo.2017.00301

Edwards, S. M., Cunningham, S. A., Dunlop, A. L., and Corwin, E. J. (2017). The Maternal Gut Microbiome During Pregnancy. *MCN Am. J. Matern. Child Nurs.* 42 (6), 310–317. doi: 10.1097/NMC.0000000000000372

Fåk, F., Karlsson, C. L. J., Ahrné, S., Molin, G., and Weström, B. (2012). Effects of a High-Fat Diet During Pregnancy and Lactation are Modulated by E. Coli in Rat Offspring. *Int. J. Obes. 2005* 36 (5), 744–751. doi: 10.1038/ijo.2011.118

Gao, B., Zhong, M., Shen, Q., Wu, Y., Cao, M., Ju, S., et al. (2020). Gut Microbiota in Early Pregnancy Among Women With Hyperglycaemia vs. Normal Blood Glucose. BMC Pregnancy and Childbirth. *BMC Pregnancy Childbirth* 20 (1). doi: 10.1186/s12884-020-02961-5

Ge, X., Wang, Y., Lam, K. S., and Xu, A. (2012). Metabolic Actions of FGF21: Molecular Mechanisms and Therapeutic Implications. *Acta Pharm. Sin. B.* 2 (4), 350–357. doi: 10.1016/j.apsb.2012.06.011

Gomez-Arango, L. F., Barrett, H. L., Mcintyre, H. D., Callaway, L. K., Morrison, M., and Dekker Nitert, M. (2016). Connections Between the Gut Microbiome and Metabolic Hormones in Early Pregnancy in Overweight and Obese Women. *Diabetes* 65 (8), 2214–2223. doi: 10.2337/db16-0278

Guimarães, K. S. D. L., Braga, V. D. A., Noronha, SISRD, Costa, W. K. A. D., Makki, K., Cruz, J. D. C., et al. (2020). Lactiplantibacillus Plantarum WJL Administration During Pregnancy and Lactation Improves Lipid Profile, Insulin Sensitivity and Gut Microbiota Diversity in Dyslipidemic Dams and Protects Male Offspring Against Cardiovascular Dysfunction in Later Life. *Food Funct.* 11 (10), 8939–8950. doi: 10.1039/D0FO01718C

Hadden, D. R., and McLaughlin, C. (2009). Normal and Abnormal Maternal Metabolism During Pregnancy. *Semin. Fetal Neonatal Med.* 14 (2), 66–71. doi: 10.1016/j.siny.2008.09.004

Houttu, N., Mokkala, K., and Laitinen, K. (2018). Overweight and Obesity Status in Pregnant Women are Related to Intestinal Microbiota and Serum Metabolic and Inflammatory Profiles. *Clin. Nutr.* 37 (6 Pt A), 1955–1966. doi: 10.1016/j.clnu.2017.12.013

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., and Reddy, D. N. (2015). Role of the Normal Gut Microbiota. *World J. Gastroenterol. WJG* 21 (29), 8787–8803. doi: 10.3748/wjg.v21.i29.8787

Jones, H. N., Woollett, L. A., Barbour, N., Prasad, P. D., Powell, T. L., and Jansson, T. (2009). High-Fat Diet Before and During Pregnancy Causes Marked Up-Regulation of Placental Nutrient Transport and Fetal Overgrowth in C57/BL6 Mice. *FASEB J.* 23 (1), 271–278. doi: 10.1096/fj.08-116889

Kim, S., Kim, H., Yim, Y. S., Ha, S., Atarashi, K., Tan, T. G., et al. (2017). Maternal Gut Bacteria Promote Neurodevelopmental Abnormalities in Mouse Offspring. *Nature* 549 (7673), 528–532. doi: 10.1038/nature23910

Kimura, I., Miyamoto, J., Ohue-Kitano, R., Watanabe, K., Yamada, T., Onuki, M., et al (2020). Maternal Gut Microbiota in Pregnancy Influences Offspring Metabolic Phenotype in Mice. *Science* 367 (6481), eaaw8429. doi: 10.1126/science.aaw8429

Kirwan, J. P., Hauguel-De Mouzon, S., Lepercq, J., Challier, J. -C., Huston-Presley, L., Friedman, J., et al. (2002). TNF-Alpha is a Predictor of Insulin Resistance in Human Pregnancy. *Diabetes* 51 (7), 2207–2213. doi: 10.2337/diabetes.51.7.2207

Klancic, T., and Reimer, R. A. (2020). Prebiotic Oligofructose Prevents Antibiotic-Induced Obesity Risk and Improves Metabolic and Gut Microbiota Profiles in Rat Dams and Offspring. *Mol. Nutr. Food Res.* 64 (16), e2000288. doi: 10.1002/mnfr.202000288

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150 (3), 470–480. doi: 10.1016/j.cell.2012.07.008

Lain, K. Y., and Catalano, P. M. (2007). Metabolic Changes in Pregnancy. *Clin. Obstet. Gynecol.* 50 (4), 938–948. doi: 10.1097/GRF.0b013e31815a5494

Ma, J., Prince, A. L., Bader, D., Hu, M., Ganu, R., Baquero, K., et al. (2014). High-Fat Maternal Diet During Pregnancy Persistently Alters the Offspring Microbiome in a Primate Model. *Nat. Commun.* 5 (1), 3889. doi: 10.1038/ncomms4889

Murphy, E. F., Cotter, P. D., Healy, S., Marques, T. M., O'Sullivan, O., Fouhy, F., et al. (2010). Composition and Energy Harvesting Capacity of the Gut Microbiota: Relationship to Diet, Obesity and Time in Mouse Models. *Gut* 59 (12), 1635–1642. doi: 10.1136/gut.2010.215665

Nuriel-Ohayon, M., Neuman, H., and Koren, O. (2016). Microbial Changes During Pregnancy, Birth, and Infancy. *Front. Microbiol.* 7, 1031. doi: 10.3389/fmicb.2016.01031

Paul, H. A., Bomhof, M. R., Vogel, H. J., and Reimer, R. A. (2016). Diet-Induced Changes in Maternal Gut Microbiota and Metabolomic Profiles Influence Programming of Offspring Obesity Risk in Rats. *Sci. Rep.* 6 (1):20683. doi: 10.1038/srep20683

Paul, H. A., Collins, K. H., Bomhof, M. R., Vogel, H. J., and Reimer, R. A. (2018). Potential Impact of Metabolic and Gut Microbial Response to Pregnancy and Lactation in Lean and Diet-Induced Obese Rats on Offspring Obesity Risk. *Mol. Nutr. Food Res.* 62 (4):1700820. doi: 10.1002/mnfr.201700820

Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E., and Walter, J. (2017). A Critical Assessment of the "Sterile Womb" and "*In Utero* Colonization" Hypotheses: Implications for Research on the Pioneer Infant Microbiome. *Microbiome* 5 (1), 48. doi: 10.1186/s40168-017-0268-4

Rabot, S., Membrez, M., Blancher, F., Berger, B., Moine, D., Krause, L., et al (2016). High Fat Diet Drives Obesity Regardless the Composition of Gut Microbiota in Mice. *Sci. Rep.* 6. doi: 10.1038/srep32484

Rinaudo, P., and Wang, E. (2012). Fetal Programming and Metabolic Syndrome. *Annu. Rev. Physiol.* 74 (1), 107–130. doi: 10.1146/annurev-physiol-020911-153245

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G., Gasbarrini, A., et al. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem Across Age, Environment, Diet, and Diseases. *Microorganisms* 7 (1), 14. doi: 10.3390/microorganisms7010014

Singh, R. P., Halaka, D. A., Hayouka, Z., and Tirosh, O. (2020). High-Fat Diet Induced Alteration of Mice Microbiota and the Functional Ability to Utilize Fructooligosaccharide for Ethanol Production. *Front. Cell Infect. Microbiol.* 10 (376). doi: 10.3389/fcimb.2020.00376

Sivan, E., Homko, C. J., Chen, X., Reece, E. A., and Boden, G. (1999). Effect of Insulin on Fat Metabolism During and After Normal Pregnancy. *Diabetes* 48 (4), 834–838. doi: 10.2337/diabetes.48.4.834

Smith, C. H., Moe, A. J., and Ganapathy, V. (1992). Nutrient Transport Pathways Across the Epithelium of the Placenta. *Annu. Rev. Nutr.* 12 (1), 183–206. doi: 10.1146/annurev.nu.12.070192.001151

Wang, C.-Y., and Liao, J. K. (2012). A Mouse Model of Diet-Induced Obesity and Insulin Resistance. *Microb. Syst. Biol.* 821, 421–433. doi: 10.1007/978-1-61779-430-8_27

Wankhade, U. D., Zhong, Y., Kang, P., Alfaro, M., Chintapalli, S. V., Thakali, K. M., et al. (2017). Enhanced Offspring Predisposition to Steatohepatitis With Maternal High-Fat Diet is Associated With Epigenetic and Microbiome Alterations. *PloS One* 12 (4), e0175675. doi: 10.1371/journal.pone.0175675

Winzell, M. S., and Ahren, B. (2004). The High-Fat Diet-Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. *Diabetes* 53 (Supplement 3), S215–S219. doi: 10.2337/diabetes.53.suppl_3.S215

World Health Organization (WHO) (2021) *Obesity and Overweight* (World Health Organization). Available at: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (Accessed June 17, 2021).

Zhou, L., and Xiao, X. (2018). The Role of Gut Microbiota in the Effects of Maternal Obesity During Pregnancy on Offspring Metabolism. *Biosci. Rep.* 38 (2). doi: 10.1042/BSR20171234

Zhou, L., Xiao, X., Li, M., Zhang, Q., Yu, M., Zheng, J., et al. (2020). Maternal Exercise Improves High-Fat Diet-Induced Metabolic Abnormalities and Gut Microbiota Profiles in Mouse Dams and Offspring. *Front. Cell Infect. Microbiol.* 10:292. doi: 10.3389/fcimb.2020.00292

Zhou, L., Xiao, X., Zhang, Q., Zheng, J., and Deng, M. (2019). Maternal Genistein Intake Mitigates the Deleterious Effects of High-Fat Diet on Glucose and Lipid Metabolism and Modulates Gut Microbiota in Adult Life of Male Mice. *Front. Physiol.* 10, 985. doi: 10.3389/fphys.2019.00985

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 02 February 2022
doi: 10.3389/fcimb.2022.838491

Preliminary Identification of the Aerobic Cervicovaginal Microbiota in Mexican Women With Cervical Cancer as the First Step Towards Metagenomic Studies

*Gauddy Lizeth Manzanares-Leal[1], Jaime Alberto Coronel-Martínez[2], Miguel Rodríguez-Morales[3], Iván Rangel-Cuevas[4], Lilia Patricia Bustamante-Montes[5], Horacio Sandoval-Trujillo[6] and Ninfa Ramírez-Durán[1]**

[1] Laboratory of Medical and Environmental Microbiology, School of Medicine, Universidad Autonoma del Estado de Mexico, Toluca, Mexico, [2] Departments of Clinical Research and Medical Oncology, Instituto Nacional de Cancerologia, Mexico City, Mexico, [3] Genomics Laboratory, Instituto Nacional de Cancerologia, Mexico City, Mexico, [4] Gynecology Department, Maternal and Child Hospital, Instituto de Seguridad Social del Estado de México y Municipios, Toluca, Mexico, [5] Deanship of Health Sciences, Universidad Autonoma de Guadalajara, Guadalajara, Jalisco, Mexico, [6] Department of Biological Systems, Universidad Autonoma Metropolitana-Xochimilco, Mexico City, Mexico

Cervical cancer (CC) is considered a public health problem. Recent studies have evaluated the possible relationship between the cervicovaginal microbiome and gynecologic cancer but have not studied the relationship between aerobic bacterial communities and neoplasia. The study aimed to identify the cultivable aerobic bacterial microbiota in women with cervical cancer as a preliminary approach to the metagenomic study of the cervicovaginal microbiome associated with cervical cancer in Mexican women. An observational cross-sectional study was conducted, including 120 women aged 21-71 years, divided into two study groups, women with locally advanced CC (n=60) and women without CC (n=60). Sociodemographic, gynecological-obstetric, sexual, and habit data were collected. Cervicovaginal samples were collected by swabbing, from which standard microbiological methods obtained culturable bacteria. The strains were genetically characterized by PCR-RFLP of the 16S *r*RNA gene and subsequently identified by sequencing the same gene. Variables regularly reported as risk factors for the disease were found in women with CC. Differences were found in the prevalence and number of species isolated in each study group. Bacteria commonly reported in women with aerobic vaginitis were identified. There were 12 species in women with CC, mainly *Corynebacterium* spp. and *Staphylococcus* spp.; we found 13 bacterial species in the group without cancer, mainly *Enterococcus* spp. and *Escherichia* spp. The advanced stages presented a more significant number of isolates and species. This study provided a preliminary test for cervicovaginal metagenomic analysis, demonstrating the presence of aerobic cervicovaginal dysbiosis in women with CC and the need for more in-depth studies.

Keywords: cervical cancer, aerobic vaginitis, aerobic bacteria, human microbiome, cervicovaginal microbiome

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
António Machado, Universidad San Francisco de Quito, Ecuador
Andrew Maltez Thomas, University of Trento, Italy

***Correspondence:**
Ninfa Ramírez-Durán
ninfard@hotmail.com

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *17 December 2021*
Accepted: *13 January 2022*
Published: *02 February 2022*

Citation:
Manzanares-Leal GL, Coronel-Martínez JA, Rodríguez-Morales M, Rangel-Cuevas I, Bustamante-Montes LP, Sandoval-Trujillo H and Ramírez-Durán N (2022) Preliminary Identification of the Aerobic Cervicovaginal Microbiota in Mexican Women With Cervical Cancer as the First Step Towards Metagenomic Studies. Front. Cell. Infect. Microbiol. 12:838491. doi: 10.3389/fcimb.2022.838491

INTRODUCTION

Cervical cancer (CC) is one of the significant public health problems in the world. Globally, it represents the seventh most common cancer in humans and the fourth most common cancer in women, but it reaches second place in incidence and mortality in settings with a low human development index (Bray et al., 2018). In Mexico, this condition is aggravated because the diagnosis mainly occurs in locally advanced stages (IB-2 to IV-A), placing the pathology as the second leading cause of cancer death in women (Baezconde-Garbanati et al., 2019; Martínez-Rodríguez et al., 2021).

Recent studies have evaluated the relationship between the cervicovaginal microbiome and gynecological cancer to elucidate the involvement of bacterial communities in the establishment, progression, or cure of the disease. Dysbiosis due to the presence of bacterial vaginosis is the most studied entity, found to have a possible association with the induction of local inflammation and lactic acid depletion (Anahtar et al., 2015; Łaniewski et al., 2018), persistent high-risk Human Papillomavirus (HPV-Hr) infection (Jørgensen et al., 2009; Gao et al., 2013; Piyathilake et al., 2016; Ilhan et al., 2019; Norenhag et al., 2020; Wei et al., 2020), Cervical Intraepithelial Neoplasia (CIN) (Gillet et al., 2012; Huang et al., 2020) and the establishment of cervical cancer (Kwasniewski et al., 2018; Huang et al., 2020; Kang et al., 2021).

Among the main bacterial genera reported so far concerning CC are *Gardnerella, Prevotella, Dialister, Slackia, Actinomyces, Porphyromonas, Peptoniphilus, Anaerococcus, Peptostreptococcus, Streptococcus, Ureaplasma, Megasphaera, Mycoplasma, Sneathia, Mobiluncus, Fusobacterium,* and *Atopobium* (Anahtar et al., 2015; Di Paola et al., 2017; Wei et al., 2020), all of which are facultative anaerobic or strict anaerobic bacteria.

In 2002, Donders et al. (2002) reported the existence of a pathological entity called aerobic vaginitis, characterized by a high abundance and diversity of aerobic, enteric bacteria and inflammatory symptoms. In 2019, Wang et al. (2020) evaluated the bacterial profiles of aerobic vaginitis by both culture-dependent and molecular methods. They found no single profile to describe the vaginal bacteriome in patients with aerobic vaginitis. In 2021, Plisko et al. (2021) published a paper whose objective was to analyze the association between the vaginal microbiota of women with aerobic vaginitis and the presence of cervical intraepithelial neoplasia. In this study, aerobic microbiota was detected by wet-mount microscopy. The authors concluded that there might be a relationship between aerobic dysbiotic cervicovaginal microbiota and the development of cervical intraepithelial lesions.

Currently, to our knowledge, the aerobic microbiota present in women with established cervical cancer has not been evaluated. Therefore, this study aimed to identify the culturable aerobic bacterial microbiota in women with locally advanced cervical cancer as an approach before the metagenomic study of the cervicovaginal microbiome associated with cervical cancer in Mexican women.

METHOD

Study Design and Ethical Aspects

A cross-sectional, descriptive, observational study was carried out. Cervicovaginal fluid samples were collected by swabbing. Sample collection was developed from February 2016 to February 2018. The study protocol was approved by the National Cancer Institute of Mexico (INCan) with the code 016/11/ICI-ICI-CEI/1016. All procedures were carried out under proper bioethical standards. All women who decided to participate in the study signed the corresponding informed consent form.

Study Groups

Two study groups were included, according to the following characteristics: Study group 1 (CC): women older than 18 years, newly diagnosed with locally advanced cervical cancer, according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer (Saleh et al., 2020), and histologically confirmed (n=60); Study group 2 (Non-CC): women older than 18 years, without cervical cancer, confirmed with negative cervical cytology, companions (family members or neighbors) of women in study group 1 or who attended screening appointments (n=60).

The following exclusion criteria were applied: pregnant women, women who used systemic and local antimicrobials and antifungals 30 days before sampling, women who had sexual intercourse 48 hours before sampling, women who used vaginal douching, and who were menstruating on the day of sampling.

Study Population Characteristics

A questionnaire was applied to all participants to obtain sociodemographic data (age, education level, and marital status); gynecological-obstetric data (number of vaginal deliveries); sexual data (start of active sexual life, number of sexual partners, and use of hormonal contraceptive methods); habits (smoking); and disease data collected from clinical histories (clinical stage of the disease, histological type of tumor).

Sample Processing

Cervicovaginal fluid samples were inoculated in Petri dishes with Brain Heart Infusion (BHI) agar (Becton Dickinson, Cat. No. 214700) and in Petri dishes with blood agar (Becton Dickinson, Cat. No. 220150). The Petri dishes were incubated at 37°C for 48 hr under aerobic conditions. Once the incubation time had elapsed, the predominant colonies were isolated and purified.

DNA Extraction

The isolated and purified colonies were inoculated in BHI broth to obtain biomass, incubated at 37°C for 48 hr. Biomass recovery was performed by centrifugation at 10000 rpm for 10 minutes. Subsequently, the manufacturer's instructions performed DNA extraction using the Promega Wizard® Genomic kit (Cat. No. A1120).

Amplification of the 16S *r*RNA Gene

The 16S *r*RNA gene was amplified with the DNA obtained by Polymerase Chain Reaction (PCR). Taq DNA Polymerase (My Taq, Bioline. Cat. No. BIO-21105) was used and the following nucleotide sequences were used as universal primers: 27f: 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492r: 5'- TACGGYTACC TTGTTACGACTT-3'; 1492r: 5'- TACGGYTACCTTGTTA CGACTT-3'. The conditions used in thermal cycling for gene amplification were as follows: Initial denaturation, a 5-minute cycle at 94°C, followed by 29 cycles of denaturation for 1 minute at 94°C, annealing 30 seconds at 59°C, extension 1minute at 72°C. The final extension was 10 minutes at 72°C.

The amplified fragments were observed by 1% agarose gel electrophoresis (Conda Pronadisa, cat. no. 8100.10), stained with ethidium bromide (SIGMA cat. no. E7637-1G). The electrophoresis conditions were: 120 V for 40 min, in TAE buffer (TAE buffer 1X Invitrogen, Cat. No. 24710-030) as a running buffer.

Genomic Characterization

The amplicons of the 16S *r*RNA gene of the isolated strains were used to type the strains obtained to determine the genotypic differences of the isolates. For this purpose, PCR-RFLP ribotyping of the 16S *r*RNA gene with MSPI (Promega, Cat. No. R6401) and RSAI (Promega, Cat. No. R6371) enzymes were used. For both enzymes, the restriction reaction was carried out for 1 h at 37°C, inactivated by heating 15 min at 72°C, according to the manufacturer's instructions.

Restriction products were observed by 1.5% agarose gel electrophoresis (Conda Pronadisa, Cat. No. 8100.10), stained with ethidium bromide (SIGMA Cat. No. E7637-1G), under the following conditions: 120V for 80 minutes with TAE buffer (TAE buffer 1X Invitrogen, Cat. No. 24710-030) and 1 kb DNA molecular weight marker (Thermo Scientific, Cat. No. 5M0311).

The restriction patterns generated were analyzed according to the number of bands and size concerning the molecular weight marker used. A "ribotype" was defined as a group of strains with identical enzymatic restriction profiles

Genetic Identification

For genetic identification, between 15% and 50% of the strains from the ribotypes formed were selected. Once the representative strains were chosen, a second amplification of the 16S *r*RNA gene was performed by PCR, following the methodology described above. The amplification products were purified with the Amicon Ultra filter® kit (Millipore, Cat. No. UFC500308) and sent to the Macrogen USA sequencing service (Macrogen Sequenciation Service, Maryland, USA).

The sequences were analyzed and corrected with the following programs: ChromasPro version 2.6.4 (Technelysium Pty Ltd, South Brisbane, Australia) and BioEdit version 5.0.9 (Hall, 1999). Consensus sequences were constructed and compared with sequences deposited in the GenBank of the National Center for Biotechnology Information (NCBI) using the BLAST (Basic Local Alignment Search Tool) program (Altschul et al., 1990) as well as in the EzBiocloud public database (Yoon et al., 2017).

Statistical Analysis

Sociodemographic, gynecological-obstetric, sexual, habit, and disease characteristics and the number of isolated strains were analyzed using descriptive statistics. The Student's t-test compared the mean age of the participants. The Chi2 test was used to compare the proportions of the remaining variables and the number of isolates per culture medium. The Cochran-Mantel-Haenszel test was applied to rule out the possibility of sociodemographic variables acting as confounding variables in the presence or absence of isolated bacteria. For women with CC, analysis of the proportion of strains obtained by subgroup, stratified by parametrial and non-parametrial invasion, and tumor type was performed using Fisher's exact test. A significance level of $p<0.05$ was considered. Stata version 15 was used for the analysis of all data evaluated.

RESULTS

Sociodemographic, Gyneco-Obstetric, Sexual, and Habit Characteristics

Regarding the characteristics of the study population, the groups did not differ in age. Still, statistically significant differences were found in the level of education ($p=0.01$), vaginal deliveries ($p<0.01$), beginning of active sexual life ($p<0.01$), and use of contraceptive methods ($p=0.02$) (**Table 1**). For the women with CC, the clinical stage of the disease most frequently was IIB (50%), and squamous cell carcinoma accounted for 85% of the cases (**Table 1**). Statistically, no variable in this item acted as a confounder.

Strains Isolated

From study group 1 (CC), 116 strains were isolated; 52 on BHI agar (44.8%) and 64 on blood agar (55.2%). In group 2 (Non-CC), 67 strains were isolated; 21 on BHI agar (31.3%) and 46 on blood agar (68.7%). It can be observed that the group of women with CC presented a superior number of isolated strains; however, when comparing the proportions by type of culture medium, this result was not statistically significant ($p=0.07$).

Genomic Characterization

The 16S rRNA gene of the isolated strains was characterized using restriction enzymes. The gene fragments generated by the enzymes could be visualized and adequately separated by agarose gel electrophoresis; this generated bands that varied in number, size, and arrangement, which showed that the strains were genetically distinct and allowed their classification by grouping patterns of similar bands or ribotypes. Thus, 12 ribotypes were found in the group of women with CC, and 13 ribotypes were found in Study Group 2 (Non-CC) (**Table 2**). Using two restriction enzymes (MSPI and RSAI), we corroborated that the strains clustered as described, and the groups were genetically distinct.

TABLE 1 | Characteristics of the study population.

Characteristic	Study group 1 (CC) (n=60)	Study group 2 (Non-CC) (n=60)	Significance
	Sociodemographic characteristics		
Age	Mean (range, S.D.)	Mean (range, S.D.)	
	46 (22-68, 11.9)	45 (21-71, 11.7)	0.57
Education level	Frequency (%)	Frequency (%)	
None	12 (20.0)	5 (8.3)	0.01*
Elementary	20 (33.3)	13 (21.6)	
Secondary	19 (31.6)	19 (31.6)	
High school	8 (13.3)	15 (25.0)	
University and postgraduate	3 (5.0)	8 (13.3)	
Marital Status			
Married/free union	31 (51.6)	36 (60.0)	0.16
Widowed	6 (10.0)	3 (5.0)	
Single	23 (38.3)	21 (35.0)	
	Obstetrical and gynecological characteristics		
Deliveries	Frequency (%)	Frequency (%)	
≤ 3	38 (63.3)	56 (93.3)	<0.01*
>3	22 (36.6)	4 (6.6)	
	Sexual characteristics		
Beginning of active sexual life	Frequency (%)	Frequency (%)	
≤ 18 years	38 (63.3)	27 (45.0)	<0.01*
>18 years	22 (36.6)	33 (55.0)	
Number of sexual partners			
≤ 3	53 (88.3)	49 (81.6)	0.44
>3	7 (11.6)	11 (18.3)	
Contraceptive method use			
None	31 (51.6)	28 (46.6)	0.02*
Hormonal	19 (31.6)	10 (16.6)	
Non- hormonal	10 (16.6)	22 (36.6)	
	Habits		
Smoking habit	Frequency (%)	Frequency (%)	
Yes	11 (18.3)	18 (30.0)	0.66
No	49 (81.6)	42 (70.0)	
	Disease		
Clinical stage	Frequency (%)	Frequency (%)	
IB2	9 (15.0)	N/A	N/A
IIA	3 (5.0)	N/A	N/A
IIA1	1 (1.6)	N/A	N/A
IIA2	2 (3.3)	N/A	N/A
IIB	30 (50.0)	N/A	N/A
IIIA	2 (3.3)	N/A	N/A
IIIB	8 (13.3)	N/A	N/A
IVA	5 (8.3)	N/A	N/A
Tumor histological type			
Squamous cell carcinoma	51 (85.0)	N/A	N/A
Adenocarcinoma	9 (15.0)	N/A	N/A

*S.D, Standard deviation, %, percentage, N/A, not applicable. *Significance level p < 0.05,Student's t-test or chi2 test.*

Genetic Identification

Identification by 16S *r*RNA gene sequencing was performed on 40 strains representative of the 12 ribotypes of study group 1 (CC). Consensus sequences between 1311bp and 1472bp were obtained, with similarity percentages between 97% and 100%. Seven genera and 12 bacterial species were identified. The species corresponded to each of the ribotypes established in this study group (**Table 2**).

From Study Group 2 (Non-CC), 30 strains of 13 ribotypes were chosen. Consensus sequences between 1391bp and 1436bp were obtained with 97% and 100% similarity percentages. 10 genera and 13 bacterial species were identified. The species correspond to the ribotypes established in this group (**Table 2**).

The obtained sequences were deposited in the GenBank of the National Center for Biotechnology Information with access numbers MH108987–MH109026 (Group 1) and MH158254-MH158283 (Group 2).

Both women with CC and those without the disease presented mostly facultative aerobic and enteric bacteria. *Staphylococcus epidermidis*, *Streptococcus agalactiae, Enterococcus faecalis, Escherichia fergusonii*, and *Corynebacterium amycolatum* species were identified in both study groups, with percentages between 5% and 30% of presence in the group of women with CC and between 6% and 25% in women without CC.

The women with CC presented a specific bacterial community, which could not be isolated in women without

TABLE 2 | Molecularly identified strains, divided by ribotype and study group.

Ribotype No	Group 1 (CC)					Group 2 (Non-CC)				
	Strains by ribotype	Strains identified by 16S rRNA (%)	Species identified	BLAST %	EzBiocloud %	Strains by ribotype	Strains identified by 16S rRNA (%)	Species identified	BLAST %	EzBiocloud %
1	35	9 (25.7)	*Staphylococcus epidermidis*	98-99	98-99	17	8 (47.05)	*Enterococcus faecalis*	99-100	97-99
2	24	9 (37.5)	*Corynebacterium amycolatum*	97-100	97-99	13	6 (46.15)	*Escherichia fergusonii*	99-100	99
3	14	5 (35.7)	*Enterococcus faecalis*	97-100	98-99	6	2 (33.3)	*Facklamia hominis*	97-99	99
4	8	4 (50.0)	*Escherichia fergusonii*	97-100	99	5	3 (60.0)	*Staphylococcus epidermidis*	97-98	99-100
5	6	3 (50.0)	*Corynebacterium jeikeium*	98-100	97-99	5	2 (40.0)	*Staphylococcus auricularis*	97-99	98
6	6	3 (50.0)	*Streptococcus agalactiae*	98-100	98	4	2 (50.0)	*Corynebacterium amycolatum*	99-100	98
7	6	1 (16.6)	*Bacillus safensis*	98	99	4	1 (25.0)	*Brevibacterium masiliense*	99	99
8	5	1 (20.0)	*Lactobacillus rhamnosus*	97	100	3	1 (33.3)	*Streptococcus agalactiae*	99	99
9	4	2 (50.0)	*Streptococcus urinalis*	100	97-99	3	1 (33.3)	*Klebsiella oxytoca*	99	99
10	4	1 (25.0)	*Bacillus malikii*	97	99	2	1 (50.0)	*Staphylococcus pasteuri*	100	99
11	3	1 (33.3)	*Escherichia coli*	97	99	2	1 (50.0)	Paenibacillus urinalis	100	99
12	1	1 (100)	*Corynebacterium striatum*	97	99	2	1 (50.0)	*Staphylococcus capitis*	98	99
13	–	–	-	–	–	1	1 (100)	*Pseudocitrobacter faecalis*	100	98
Total	116	40 (34.5)	12 species			67	30 (44.7)	13 species		

CC, consisting of seven species, *Streptococcus urinalis, Escherichia coli, Bacillus safensis, Bacillus malikii, Corynebacterium jeikeium, Corynebacterium striatum*, and *Lactobacillus rhamnosus*. Likewise, the group of women without CC presented eight species not found in those with CC, consisting of *Staphylococcus pasteuri, Staphylococcus auricularis, Staphylococcus capitis* subsp. *capitis*, *Facklamia hominis, Paenibacillus urinalis, Pseudocitrobacter faecalis, Brevibacterium masiliense* and *Klebsiella oxytoca*.

Clinical stages of the disease were divided for analysis according to the presence or absence of parametrial invasion. Stages IB2 to IIA2 (without parametrial invasion) presented 8 bacterial species. The women with parametrial invasion (stages IIB to IVA) presented the 12 species identified. The species with the highest predominance was *Staphylococcus epidermidis*, as it was present in 100% of the patients without parametrial invasion, compared to 44.4% found in women with parametrial invasion; this result is statistically significant ($p<0.01$) (**Table 3**).

Regarding the species found according to the histological type of tumor, women with squamous cell carcinoma presented 11 of the 12 species identified (91.6%), except for *Corynebacterium striatum*. Women with adenocarcinoma presented 9 of the 12 species (75%). The difference in proportions between both groups for all bacteria only showed statistically significant differences in the presence of *C. amycolatum* ($p<0.01$), with a higher proportion in women with adenocarcinoma (**Table 3**).

DISCUSSION

Cervical cancer is one of the leading health problems in the world; there are multiple reports about the risk factors associated with HPV infection, its persistence, and cancer development. We have detected four risk factors in the Mexican population analyzed in this study. Education level, which can be related to socioeconomic status, was lower in the group of women with cervical cancer, coinciding with previous studies showing that detection of the disease is later as schooling level decreases (Chen et al., 2012; Thulaseedharan et al., 2012; Akinlotan et al., 2017; Nessa et al., 2020). The onset of active sexual life was at an earlier age in women with CC, and it has been reported that early age at first intercourse may predispose women to HPV infection (Creatsas and Deligeoroglou, 2012; Dahiya et al., 2017). Parity, which was higher in women with CC. This risk factor may be related to hormonal changes and epithelial modifications caused by repeated cervical trauma (Thulaseedharan et al., 2012; Nessa et al., 2020). And finally, the use of contraceptive methods, in which the higher use of hormonal methods by women with CC is observed.

It has been commonly reported that the composition of "healthy" or "normal" cervicovaginal bacterial communities is based on the presence or absence of lactobacilli. Ravel et al. (2011) have classified it into multiple categories or "clusters" (I-V) based on the dominant *Lactobacillus* species and the ethnic group in which they are found. Bacterial

TABLE 3 | Difference in proportions of bacterial species found according to the clinical stage of the disease and histological type of tumor.

Identified species	Clinical stage		Significance	Tumor histological type		Significance
	IB2, IIA, IIA1, IIA2 (Without parametrial invasion) n = 15	IIB, IIIA, IIIB, IVA (With parametrial invasion) n = 45		Squamous cell carcinoma = 51	Adenocarcinoma n = 9	
	% (Frequency)	% (Frequency)		% (Frequency)	% (Frequency)	
Staphylococcus epidermidis	100 (15)	44.4 (20)	<0.01*	56.8 (29)	66.6 (6)	0.72
Corynebacterium amycolatum	40.0 (6)	40.0 (18)	1.00	31.3 (16)	88.8 (8)	<0.01*
Enterococcus faecalis	26.0 (4)	22.2 (10)	0.73	21.5 (11)	33.3 (3)	0.18
Escherichia fergusonii	6.6 (1)	15.5 (7)	0.66	15.6 (8)	0.0 (0)	0.33
Corynebacterium jeikeium	6.6 (1)	11.1 (5)	1.00	11.7 (6)	0.0 (0)	0.57
Streptococcus agalactiae	0.0 (0)	13.3 (6)	0.32	9.8 (5)	11.1 (1)	1.00
Bacillus safensis	13.3 (2)	8.8 (4)	0.63	9.8 (5)	0.0 (0)	1.00
Lactobacillus rhamnosus	13.3 (2)	6.6 (3)	0.59	7.8 (4)	11.1 (1)	0.57
Streptococcus urinalis	0.0 (0)	8.8 (4)	0.56	3.9 (2)	11.1 (1)	0.39
Bacillus malikii	6.6 (1)	6.6 (3)	1.00	3.9 (2)	22.2 (2)	0.10
Escherichia coli	0.0 (0)	6.6 (3)	0.56	3.9 (2)	11.1 (1)	0.39
Corynebacterium striatum	0.0 (0)	2.2 (1)	1.00	0.0 (0)	11.1 (1)	0.15

*%, percentage; *significance level $p < 0.05$, Fisher's exact test.*

communities I, II III and V, are dominated by *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively, and were found more frequently in Asian and white women. Bacterial community IV is not dominated by any lactobacillus species and has a high presence of anaerobic bacteria. This group was overrepresented in Hispanic and black women, concluding that vaginal bacterial communities not dominated by *Lactobacillus* species are common and appear normal in these ethnic groups (Ravel et al., 2011; Kroon et al., 2018). However, according to Martínez-Peña et al. (Martínez-Peña et al., 2013), the healthy cervicovaginal microbiota of Mexican women is mainly composed of *L. acidophilus* group lactobacilli, namely *L. gasseri*, *L. fermentum*, *L. rhamnosus*, *L. jensenii*, *L. crispatus* and *L. brevis*, which is in contrast to that described by Ravel et al. (Ravel et al., 2011). Likewise, our results also show the presence of critical aerobic bacteria in women without CC. We believe that further studies are needed to establish the normal parameters of this bacterial community and to determine with greater certainty the variation according to population, genotype, or the presence of disease.

Several studies have already exposed the link of cervicovaginal bacterial microbiota and CC during the natural history of the disease, where it is shown that from HPV infection and up to the presence of a cervical intraepithelial lesion, there is an association between cervical dysbiosis and disease (Mitra et al., 2015; Usyk et al., 2020; Onywera et al., 2021). The most studied entity is bacterial vaginosis, in which a higher diversity of strict anaerobic species and a considerable decrease in lactobacilli are observed (Sodhani et al., 2017; Brusselaers et al., 2019; Kovachev, 2020). However, current reports suggest that aerobic bacteria may also play an essential role in this pathology (Vieira-Baptista et al., 2016; Plisko et al., 2021).

This study aimed to detect aerobic bacteria related to the presence of CC. Our findings show that most isolates corresponded with facultative aerobic bacteria, which may demonstrate their high prevalence both in the bacterial communities present in women with CC and those without the pathology. According to Donders et al. (2017), one of the essential elements in aerobic vaginitis is colonization for *Escherichia coli, Staphylococcus aureus, S. epidermidis, Streptococcus agalactiae*, and *Enterococcus faecalis*. In our study, both groups presented bacteria typical of aerobic vaginitis. However, there were more strains isolated in women with cancer. This finding could contribute to the observation of Plisko et al. (Plisko et al., 2021), who concluded that changes in the microbiota associated with aerobic vaginitis are related to pre-invasive lesions. With our results, we can consider that these changes may also be related to cancer establishment.

Regarding the presence of this same group of bacteria in women without CC, although with lower strains isolated and fewer species, we can elucidate that this may represent infection or dysbiosis, but HPV infection is probably necessary for neoplastic changes to develop. According to Vieira-Baptista et al. (Vieira-Baptista et al., 2016), aerobic vaginitis is not associated with an increased risk of acquiring HPV, but it has been associated with the persistence of infection. It can therefore be considered a risk factor. The consequences of this and why it may or may not be related to the development of neoplasia should be studied under future designs and should also be studied considering the presence or not of HPV infection. These are two aspects that we did not consider in our study, representing points for improvement in subsequent projects.

Concerning the clinical stage of the disease, we found that more advanced stages presented a higher number of isolates and a more significant number of species coincident with aerobic vaginitis. On the contrary, less extended clinical stages showed a lower number of species and a more consistent bacterial community, with a better local ecology; this is consistent with previous studies (Audirac-Chalifour et al., 2016; García-García et al., 2017).

About the presence of these species and the severity of the disease, we know that aerobic vaginitis is an entity that by itself generates discomfort by producing a variable amount of inflammation and thinning of the vaginal epithelium

(Oerlemans et al., 2020). We can elucidate that this dysbiotic microbiota could drive the pathology by promoting immune evasion, favoring tumor cell survival. It could also act by producing an inflammatory environment that in the long term promotes tissue damage resulting in an ecosystem more conducive to disease aggravation. These two aspects have been previously addressed to relate carcinogenesis to aerobic vaginosis, but not to explain the severity of the disease (Vieira-Baptista et al., 2016; Donders et al., 2017; Plisko et al., 2021). We suggest studies whose aim is directed to the analysis of these variables.

No marked differences were observed in histological type for the microbiota associated with either of the two carcinomas found in this study group. In general, squamous cell carcinoma was related to a more significant bacterial presence, both in quantity and species number; this could be influenced by the anatomical characteristics of the tumor, which could provide a more favorable habitat for the installation of these species. A larger sample size regarding adenocarcinoma is likely to be necessary to obtain conclusive results.

Our study does not provide fundamentals to determine a causal association between the bacteria characteristic of aerobic vaginitis and cervicovaginal neoplasia; we do not have elements to clarify if aerobic vaginitis promotes carcinogenesis or if it is established as a consequence of cancer development due to the deprivation of the immune system when trying to counteract the neoplasia. But considering previous studies that have demonstrated a link between an altered vaginal microbiome and pre-invasive cervical disease, we propose that aerobic vaginitis and the inflammation with which it is accompanied are probably crucial for the progression of preneoplastic lesions to cancer (Vieira-Baptista et al., 2016; Plisko et al., 2021).

This cross-sectional study, developed as a preliminary experiment for further research, made it possible to explore the essential characteristics of the cervicovaginal microbiota associated with cervical cancer, the differences between women with and without the pathology, and the need for more in-depth studies. It also allowed us to establish variables for future analyses, funding needs, and sample collection and treatment methods.

Although the "omics" era has accelerated all aspects of biological research, it is advisable to integrate traditional microbiology methods to formulate optimal study designs. Conducting a research project depends primarily on the scientific objective and the availability of samples, the quality of the samples, and the cost of the experiment. Massive sequencing of 16S *r*RNA gene amplicons and metagenomic sequencing is used to obtain a broader view of bacterial communities and determine potential functions and genome recovery. However, it generally involves the development of large-scale research projects with sufficient funding. In developing countries, where financial resources in science and technology are scarce, it is essential to perform detailed designs that properly manage samples, variables, and financial resources, minimizing losses. Our preliminary identification of the microbiota as a preliminary approach to metagenomic studies can contribute to establishing this type of design.

We can conclude that we were able to establish the presence of bacteria typical of the entity known as aerobic vaginitis in women with CC and that it is crucial to carry out more in-depth studies. There is little information about this dysbiosis and its association with cervical cancer, so the reported findings are helpful to corroborate that there are bacterial communities that, although they may be abundant, so far have been little studied and whose influence on the establishment, development, or cure of CC are unknown. Our report shows that cross-sectional study designs and traditional microbiological methodology are helpful to determine whether there is a need for more complex studies and to know whether it is necessary to optimize the management of variables, samples, and financial resources.

Among the limitations of this study, we consider it necessary to report that we did not manage to extend the participants' last use of antimicrobials and antifungals, so we limited ourselves to 30 days as exclusion criteria. We know that this period is short for the microbiota to recover satisfactorily and return to "normal". However, we tried to lessen its impact by choosing a large sample size that balanced some of the participants' confounding results. Subsequent studies should address this issue. Likewise, we could not diagnose aerobic vaginitis or identify HPV; however, this is a precedent for future work to determine the association between aerobic vaginitis and HPV infection or persistence. Finally, we consider it a limitation to have performed a cross-sectional study. However, this is an antecedent for future studies to address longitudinal designs in which the relationship between aerobic vaginitis and cervical cancer can be evaluated more effectively.

DATA AVAILABILITY STATEMENT

The obtained sequences are publicly available in the GenBank of the National Center for Biotechnology Information with access numbers MH108987-MH109026 (Group 1) and MH158254-MH158283 (Group 2). All other data in the study are available on request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Research Committee of the National Cancer Institute of Mexico (INCan, 016/11/ICI-CEI/1016). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GM-L, JC-M, MR-M, LB-M, HS-T, and NR-D participated in the study design. JC-M, MR-M, and IR-C collected epidemiological data. GM-L, MR-M, and IR-C performed sample collection. GM-L, HS-T, and NR-D executed the experiment. GM-L and

LB-M performed the analysis of results. All authors contributed to the article and approved the version presented.

ACKNOWLEDGMENTS

We acknowledge the support provided by the Consejo Mexiquense de Ciencia y Tecnología (COMECYT) through the program "Cátedras COMECYT EDOMEX", folio CAT2021-0106.

The authors are grateful for the financial support of the Secretaría de Investigación y Estudios Avanzados de la Universidad Autónoma del Estado de México through the research project, "Estudio metagenómico del microbioma cervicovaginal presente en mujeres con cáncer cervicouterino y bajo tratamientos antineoplásicos" 6387/2021ESP.

REFERENCES

Akinlotan, M., Bolin, J. N., Helduser, J., Ojinnaka, C., Lichorad, A., and McClellan, D. (2017). Cervical Cancer Screening Barriers and Risk Factor Knowledge Among Uninsured Women. *J. Community Health* 42, 770–778. doi: 10.1007/s10900-017-0316-9

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic Local Alignment Search Tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., et al. (2015). Cervicovaginal Bacteria are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. *Immunity* 42, 965–976. doi: 10.1016/j.immuni.2015.04.019

Audirac-Chalifour, A., Torres-Poveda, K., Bahena-Román, M., Téllez-Sosa, J., Martínez-Barnetche, J., Cortina-Ceballos, B., et al. (2016). Cervical Microbiome and Cytokine Profile at Various Stages of Cervical Cancer: A Pilot Study. *PLoS One* 11, e0153274–e0153274. doi: 10.1371/journal.pone.0153274

Baezconde-Garbanati, L., Agurto, I., Gravitt, P. E., Luciani, S., Murphy, S., Ochoa, C., et al. (2019). Barriers and Innovative Interventions for Early Detection of Cervical Cancer. *Salud Publica Mex.* 61, 456–460. doi: 10.21149/10425

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Brusselaers, N., Shrestha, S., van de Wijgert, J., and Verstraelen, H. (2019). Vaginal Dysbiosis and the Risk of Human Papillomavirus and Cervical Cancer: Systematic Review and Meta-Analysis. *Am. J. Obstet. Gynecol.* 221, 9–18.e8. doi: 10.1016/j.ajog.2018.12.011

Chen, H.-Y., Kessler, C. L., Mori, N., and Chauhan, S. P. (2012). Cervical Cancer Screening in the United State–2010: Characteristics of Women Who are Never Screened. *J. women's Heal.* 21, 1132–1138. doi: 10.1089/jwh.2011.3418

Creatsas, G., and Deligeoroglou, E. (2012). Microbial Ecology of the Lower Genital Tract in Women With Sexually Transmitted Diseases. *J. Med. Microbiol.* 61, 1347–1351. doi: 10.1099/jmm.0.042507-0

Dahiya, N., Bachani, D., Acharya, A. S., Sharma, D. N., Gupta, S., and Haresh, K. P. (2017). Socio-Demographic, Reproductive and Clinical Profile of Women Diagnosed With Advanced Cervical Cancer in a Tertiary Care Institute of Delhi. *J. Obstet. Gynaecol. India* 67, 53–60. doi: 10.1007/s13224-016-0907-x

Di Paola, M., Sani, C., Clemente, A. M., Iossa, A., Perissi, E., Castronovo, G., et al. (2017). Characterization of Cervico-Vaginal Microbiota in Women Developing Persistent High-Risk Human Papillomavirus Infection. *Sci. Rep.* 7, 10200. doi: 10.1038/s41598-017-09842-6

Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., and Vieira-Baptista, P. (2017). Aerobic Vaginitis: No Longer a Stranger. *Res. Microbiol.* 168, 845–858. doi: 10.1016/j.resmic.2017.04.004

Donders, G. G. G., Vereecken, A., Bosmans, E., Dekeersmaecker, A., Salembier, G., and Spitz, B. (2002). Definition of a Type of Abnormal Vaginal Flora That is Distinct From Bacterial Vaginosis: Aerobic Vaginitis. *BJOG* 109, 34–43. doi: 10.1111/j.1471-0528.2002.00432.x

Gao, W., Weng, J., Gao, Y., and Chen, X. (2013). Comparison of the Vaginal Microbiota Diversity of Women With and Without Human Papillomavirus Infection: A Cross-Sectional Study. *BMC Infect. Dis.* 13, 271. doi: 10.1186/1471-2334-13-271

García-García, A., Coronel-Martínez, J., Leon, D. C., Romero-Figueroa, M. D. S., Caballero-Pantoja, Y. E., Manzanares-Leal, G. L., et al. (2017). Detection of Actinomyces Spp. In Cervical Exudates From Women With Cervical Intraepithelial Neoplasia or Cervical Cancer. *J. Med. Microbiol.* 66, 706–712. doi: 10.1099/jmm.0.000485

Gillet, E., Meys, J. F. A., Verstraelen, H., Verhelst, R., De Sutter, P., Temmerman, M., et al. (2012). Association Between Bacterial Vaginosis and Cervical Intraepithelial Neoplasia: Systematic Review and Meta-Analysis. *PLoS One* 7, e45201. doi: 10.1371/journal.pone.0045201

Hall, T. A. (1999). BioEdit : A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/Nt. *Nucleic Acids Symp. Ser.* 41, 95–98.

Huang, Y., Wu, X., Lin, Y., Li, W., Liu, J., and Song, B. (2020). Multiple Sexual Partners and Vaginal Microecological Disorder are Associated With HPV Infection and Cervical Carcinoma Development. *Oncol. Lett.* 20, 1915–1921. doi: 10.3892/ol.2020.11738

Ilhan, Z. E., Łaniewski, P., Thomas, N., Roe, D. J., Chase, D. M., and Herbst-Kralovetz, M. M. (2019). Deciphering the Complex Interplay Between Microbiota, HPV, Inflammation and Cancer Through Cervicovaginal Metabolic Profiling. *EBioMedicine* 44, 675–690. doi: 10.1016/j.ebiom.2019.04.028

Jørgensen, H., Fjaervik, E., Hakvåg, S., Bruheim, P., Bredholt, H., Klinkenberg, G., et al. (2009). Candicidin Biosynthesis Gene Cluster is Widely Distributed Among Streptomyces Spp. Isolated From the Sediments and the Neuston Layer of the Trondheim Fjord, Norway. *Appl. Environ. Microbiol.* 75, 3296–3303. doi: 10.1128/AEM.02730-08

Kang, G.-U., Jung, D.-R., Lee, Y. H., Jeon, S. Y., Han, H. S., Chong, G. O., et al. (2021). Potential Association Between Vaginal Microbiota and Cervical Carcinogenesis in Korean Women: A Cohort Study. *Microorganisms* 9, 294. doi: 10.3390/microorganisms9020294

Kovachev, S. M. (2020). Cervical Cancer and Vaginal Microbiota Changes. *Arch. Microbiol.* 202, 323–327. doi: 10.1007/s00203-019-01747-4

Kroon, S. J., Ravel, J., and Huston, W. M. (2018). Cervicovaginal Microbiota, Women's Health, and Reproductive Outcomes. *Fertil. Steril.* 110, 327–336. doi: 10.1016/j.fertnstert.2018.06.036

Kwasniewski, W., Wolun-Cholewa, M., Kotarski, J., Warchol, W., Kuzma, D., Kwasniewska, A., et al. (2018). Microbiota Dysbiosis is Associated With HPV-Induced Cervical Carcinogenesis. *Oncol. Lett.* 16, 7035–7047. doi: 10.3892/ol.2018.9509

Łaniewski, P., Barnes, D., Goulder, A., Cui, H., Roe, D. J., Chase, D. M., et al. (2018). Linking Cervicovaginal Immune Signatures, HPV and Microbiota Composition in Cervical Carcinogenesis in Non-Hispanic and Hispanic Women. *Sci. Rep.* 8, 7593. doi: 10.1038/s41598-018-25879-7

Martínez-Peña, M. D., Castro-Escarpulli, G., and Aguilera-Arreola, M. G. (2013). Lactobacillus Species Isolated From Vaginal Secretions of Healthy and Bacterial Vaginosis-Intermediate Mexican Women: A Prospective Study. *BMC Infect. Dis.* 13, 189. doi: 10.1186/1471-2334-13-189

Martínez-Rodríguez, F., Limones-González, J. E., Mendoza-Almanza, B., Esparza-Ibarra, E. L., Gallegos-Flores, P. I., Ayala-Luján, J. L., et al. (2021). Understanding Cervical Cancer Through Proteomics. *Cells* 10, 1854. doi: 10.3390/cells10081854

Mitra, A., MacIntyre, D. A., Lee, Y. S., Smith, A., Marchesi, J. R., Lehne, B., et al. (2015). Cervical Intraepithelial Neoplasia Disease Progression is Associated With Increased Vaginal Microbiome Diversity. *Sci. Rep.* 5, 16865. doi: 10.1038/srep16865

Nessa, A., Ara, R., Fatema, P., Nasrin, B., Chowdhury, A., Khan, K. H., et al. (2020). Influence of Demographic and Reproductive Factors on Cervical Pre-Cancer and Cancer in Bangladesh. *Asian Pac. J. Cancer Prev.* 21, 1883–1889. doi: 10.31557/APJCP.2020.21.7.1883

Norenhag, J., Du, J., Olovsson, M., Verstraelen, H., Engstrand, L., and Brusselaers, N. (2020). The Vaginal Microbiota, Human Papillomavirus and Cervical Dysplasia: A Systematic Review and Network Meta-Analysis. *BJOG Int. J. Obstet. Gynaecol.* 127, 171–180. doi: 10.1111/1471-0528.15854

Oerlemans, E. F. M., Wuyts, S., Bellen, G., Wittouck, S., De Boeck, I., Ruban, K., et al. (2020). The Dwindling Microbiota of Aerobic Vaginitis, an Inflammatory State Enriched in Pathobionts With Limited TLR Stimulation. *Diagnostics* 10, 879. doi: 10.3390/diagnostics10110879

Onywera, H., Anejo-Okopi, J., Mwapagha, L. M., Okendo, J., and Williamson, A.-L. (2021). Predictive Functional Analysis Reveals Inferred Features Unique to Cervicovaginal Microbiota of African Women With Bacterial Vaginosis and High-Risk Human Papillomavirus Infection. *PLoS One* 16, e0253218. doi: 10.1371/journal.pone.0253218

Piyathilake, C. J., Ollberding, N. J., Kumar, R., Macaluso, M., Alvarez, R. D., and Morrow, C. D. (2016). Cervical Microbiota Associated With Higher Grade Cervical Intraepithelial Neoplasia in Women Infected With High-Risk Human Papillomaviruses. *Cancer Prev. Res. (Phila).* 9, 357–366. doi: 10.1158/1940-6207.CAPR-15-0350

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic Vaginitis—Underestimated Risk Factor for Cervical Intraepithelial Neoplasia. *Diagnostics* 11, 97. doi: 10.3390/diagnostics11010097

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., et al. (2011). Vaginal Microbiome of Reproductive-Age Women. *Proc. Natl. Acad. Sci.* 108, 4680 LP – 4687. doi: 10.1073/pnas.1002611107

Saleh, M., Virarkar, M., Javadi, S., Elsherif, S. B., de Castro Faria, S., and Bhosale, P. (2020). Cervical Cancer: 2018 Revised International Federation of Gynecology and Obstetrics Staging System and the Role of Imaging. *AJR. Am. J. Roentgenol.* 214, 1182–1195. doi: 10.2214/AJR.19.21819

Sodhani, P., Gupta, S., Gupta, R., and Mehrotra, R. (2017). Bacterial Vaginosis and Cervical Intraepithelial Neoplasia: Is There an Association or Is Co-Existence Incidental? *Asian Pac. J. Cancer Prev.* 18, 1289–1292. doi: 10.22034/APJCP.2017.18.5.1289

Thulaseedharan, J. V., Malila, N., Hakama, M., Esmy, P. O., Cheriyan, M., Swaminathan, R., et al. (2012). Socio Demographic and Reproductive Risk Factors for Cervical Cancer - A Large Prospective Cohort Study From Rural India. *Asian Pac. J. Cancer Prev.* 13, 2991–2995. doi: 10.7314/apjcp.2012.13.6.2991

Usyk, M., Zolnik, C. P., Castle, P. E., Porras, C., Herrero, R., Gradissimo, A., et al. (2020). Cervicovaginal Microbiome and Natural History of HPV in a Longitudinal Study. *PLoS Pathog.* 16, e1008376. doi: 10.1371/journal.ppat.1008376

Vieira-Baptista, P., Lima-Silva, J., Pinto, C., Saldanha, C., Beires, J., Martinez-de-Oliveira, J., et al. (2016). Bacterial Vaginosis, Aerobic Vaginitis, Vaginal Inflammation and Major Pap Smear Abnormalities. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 657–664. doi: 10.1007/s10096-016-2584-1

Wang, C., Fan, A., Li, H., Yan, Y., Qi, W., Wang, Y., et al. (2020). Vaginal Bacterial Profiles of Aerobic Vaginitis: A Case–Control Study. *Diagn. Microbiol. Infect. Dis.* 96, 114981. doi: 10.1016/j.diagmicrobio.2019.114981

Wei, Z.-T., Chen, H.-L., Wang, C.-F., Yang, G.-L., Han, S.-M., and Zhang, S.-L. (2020). Depiction of Vaginal Microbiota in Women With High-Risk Human Papillomavirus Infection. *Front. Public Heal.* 8. doi: 10.3389/fpubh.2020.587298

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: A Taxonomically United Database of 16S rRNA Gene Sequences and Whole-Genome Assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. doi: 10.1099/ijsem.0.001755

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.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 21 February 2022
doi: 10.3389/fcimb.2022.824925

Physiological Changes and Interactions Between Microbiome and the Host During Pregnancy

*Zain Zaki Zakaria[1,2], Shouq Al-Rumaihi[1], Rana S. Al-Absi[3], Huda Farah[1], Muram Elamin[1], Rahaf Nader[1], Salma Bouabidi[1], Sara Elgaili Suleiman[1], Shahd Nasr[1] and Maha Al-Asmakh[1,2]**

[1] Department of Biomedical Sciences, College of Health Sciences, Qatar University (QU) Health, Qatar University, Doha, Qatar, [2] Biomedical Research Center, Qatar University (QU), Doha, Qatar, [3] Department of Biological and Environmental Sciences, College of Arts and Sciences, Qatar University (QU), Doha, Qatar

In recent years, it has become clear that microbiome play a variety of essential roles in human metabolism, immunity, and overall health and that the composition of these microbiome is influenced by our environment, diet, weight, hormones, and other factors. Indeed, numerous physiological and pathological conditions, including obesity and metabolic syndrome, are associated with changes in our microbiome, referred to as dysbiosis. As a result, it is not surprising that such changes occur during pregnancy, which includes substantial weight gain and significant changes in metabolism and immune defenses. The present review relates physiological changes during pregnancy to alterations in the microbial composition at various sites, including the gut, oral cavity, and vagina. Pregnancy has been linked to such microbial changes, and we believe that, in contrast to certain disease states, these microbial changes are vital for a healthy pregnancy, probably through their influence on the mother's immunological, endocrinological, and metabolic status.

Keywords: microbiome, pregnancy, oral microbiota, gut microbiota, vaginal microbiota, probiotics, physiological changes

OPEN ACCESS

Edited by:
Maria De Lourdes Pereira, University of Aveiro, Portugal

Reviewed by:
Sonia M. Rodrigues Oliveira, University of Aveiro, Portugal
Karma Gurmey Dolma, Sikkim Manipal University, India
Ana Cristina Esteves, University of Aveiro, Portugal

***Correspondence:**
Maha Al-Asmakh
maha.alasmakh@qu.edu.qa

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *29 November 2021*
Accepted: *26 January 2022*
Published: *21 February 2022*

Citation:
Zakaria ZZ, Al-Rumaihi S, Al-Absi RS, Farah H, Elamin M, Nader R, Bouabidi S, Suleiman SE, Nasr S and Al-Asmakh M (2022) Physiological Changes and Interactions Between Microbiome and the Host During Pregnancy. Front. Cell. Infect. Microbiol. 12:824925. doi: 10.3389/fcimb.2022.824925

INTRODUCTION

To ensure a healthy environment for fetal development, pregnancy is associated with pronounced changes in metabolism, hormonal status and immunological defenses, all of which may be influenced by microbiota resident in the gut, vagina, placenta and oral cavity (Nuriel-Ohayon et al., 2016). For example, changes in the endocrine system in response to maternal factors such as diet and usage of antibiotics influence the compositions of the gut and oral microbiome (Nuriel-Ohayon et al., 2019, Amir et al., 2020). Moreover, progesterone can increase the abundance of bifidobacterium in the gut (Nuriel-Ohayon et al., 2019; Amir et al., 2020).

Dysbiosis of the gut microbiome during pregnancy is associated with gestational diabetes, preeclampsia and restricted fetal growth. In addition, certain oral bacteria are pathogenic and can exert deleterious effects on the health and maturation of the fetus. Periodontal infections that are transmitted to other locations can lead to premature birth, low birth weight, and preeclampsia (Figuero et al., 2020) so that virtually all pregnant women require periodontal care (Rapone et al., 2020).

Here, we summarize recent research findings regarding alterations in microbiome during pregnancy.

PHYSIOLOGICAL CHANGES DURING PREGNANCY

To support the healthy growth and development of the fetus, pregnant women undergo changes in their endocrine, cardiovascular, respiratory, renal, and digestive systems.

Changes in the Endocrine System

Already upon conception, the levels of certain hormones in a woman increase. Upon successful implantation of a fertilized egg into the uterine wall, placental trophoblasts begin to produce human chronic gonadotrophin (hCG) (Miller et al., 2020), the level of which rises during the first few weeks of pregnancy until it reaches its peak level of approximately 20000 mIU/mL during weeks 10-12, and thereafter, and at the end of the first trimester, falls steadily (Hendriks et al., 2019). hCG stimulates cells in the corpus luteum to start producing progesterone and estrogen, the levels of which increase as pregnancy progresses and the placenta grows, reaching their peaks during the third trimester (Sykes and Bennett, 2018). In addition, the hCG hormone is involved in the formation of vessels and the placenta, the differentiation of fetal cells and growth of fetal organs and preventing premature contractions of the uterus musculature (Barjaktarovic et al., 2017).

The many processes mediated by progesterone include the adaptation of the cervix for implantation of the fetus and differentiation of stromal cells into decidual cells. Furthermore, increasing progesterone levels prevent uterine contractions both by diminishing the levels of receptors for prostaglandin and oxytocin and directly inhibiting the contraction of resident smooth muscle cells (Sykes and Bennett, 2018). Rising estrogen levels are responsible for neoangiogenesis and the formation of tissues that become the placenta and support lactation (Noyola-Martínez et al., 2019). These hormonal changes cause the typical fatigue, nausea, constipation, and headaches associated with early pregnancy (Fuhler, 2020). Moreover, the growing placenta also produces hormones, including human placental lactogen (HPL), relaxin and human chronic gonadotrophin.

In addition, the thyroid gland of pregnant women may become more active, leading to hyperthyroidism (Kobaly and Mandel, 2019). Placental hormones such as lactogen promote insulin resistance, which is often accompanied by elevated production of insulin. Therefore, to avoid excessive plasma concentrations of glucose, pregnant women are often advised to pay attention to regulating their intake of carbohydrates (Mockridge and Maclennan, 2019)

Circulatory and Cardiovascular Changes

A major cause of the cardiovascular changes associated with pregnancy is relaxation of the vascular smooth muscle in response to the increased circulating levels of estrogens, progesterone, and prostaglandins (Morton, 2021). Moreover, these hormonal changes lower the resistance of the pulmonary and systemic vessels and, thereby, blood pressure, which in most cases eventually returns to the non-pregnant value by the third trimester. Furthermore, the diastolic and stroke volumes and heart rate are elevated during pregnancy (Chen and Basso, 2007), resulting in a continuous increase in cardiac output, especially during the second and third trimesters (Morton, 2020). This increase, which can be as much as 50% during the third trimester, is targeted primarily to the placenta and uterus to nourish the growing fetus and the breasts in preparation for breastfeeding the newborn infant (Elkus and Popovich, 1992).

Furthermore, during the early phase of pregnancy, activation of the renin-angiotensin-aldosterone system causes sodium and water retention. Ultimately, this could expand the plasma volume and dilute the number of red blood cells (Claros et al., 2020), leading to anemia. In this context, the requirement for iron increases two- to three-fold, and many women experience iron deficiency during the second trimester of their pregnancy (Scholl and Reilly, 2000). The fetus requires this additional iron to synthesize hemoglobin and certain other enzymatic functions. In addition, capillary wedge and oncotic pressure reductions make pregnant women susceptible to pulmonary edema (Jarvis and Nelson-Piercy, 2020).

Respiratory Changes

To fulfill the requirement of the developing fetus for oxygen, the respiratory system undergoes several physiological and anatomical changes, including elevated tidal volume, ventilation, and respiratory rate (Lemos et al., 2010; Hegewald and Crapo, 2011). At the same time, the expiratory reserve volume, total pulmonary capacity, and functional residual capacity all decline during gestation (Lemos et al., 2010), mainly due to alterations in the flexibility of ligaments in response to the higher levels of progesterone. In addition, the rise in intra-abdominal pressure as the uterus grows contributes considerably to these respiratory effects (Lee et al., 2017).

Moreover, this increase in ventilation causes the partial pressures of oxygen (paO_2) and carbon dioxide (paO_2) to be augmented and attenuated, respectively. These changes should, in turn, facilitate gas exchange across the placenta.

Changes in the Renal System

During pregnancy, the renal system undergoes several physiological and anatomical adaptations designed to support the development of the fetus. One major change of this sort is enlargement and increased weight of the kidneys due to a larger interstitial volume and more extensive vasculature (Cheung and Lafayette, 2013). Moreover, due to the tremendous pressure exerted by the growing fetus (Cheung and Lafayette, 2013), the capacity of the mother's bladder decreases, causing more frequent urination, one of the most common symptoms associated with pregnancy. In addition, renal blood flow may be elevated, increasing the glomerular filtration rate by as much as 50% and thereby reducing serum levels of creatinine (Park et al., 2017). Finally, both relaxation of smooth muscles in response to elevated progesterone levels and mechanical

compression by the enlarging uterus can lead to retention of urine and hydronephrosis (Suarez et al., 2019).

As a result of the changes in the renal system during pregnancy this can also lead to deficiencies and raised levels of certain solutes. One change is hyponatremia which is caused by the high levels of hCG in pregnancy (Abbassi-Ghanavati et al., 2009). Another change is proteinuria due to protein excretion in urine being higher than normal. This is believed to be a result of multiple factors such as the increased Glomerular filtration rate (GFR), increased protein transport across the glomerular barrier as well as the decreased reabsorption of filtered protein (Kattah et al., 2017). Glucosuria is also another possible outcome, seen in around 50% of pregnant patients. It is mainly caused by decreased absorption of glucose in the proximal tubule (Alto, 2005).

Changes in the Gastrointestinal System

Nausea, vomiting, heartburn, and constipation, the most common symptoms associated with pregnancy, are caused by changes in the gastrointestinal tract as the uterus enlarges (Herrero et al., 2001; Body and Christie, 2016). In addition, rising progesterone levels relax the lower esophageal sphincter, promoting reflux into the gastro-esophagus and, ultimately, heartburn (Jarvis and Nelson-Piercy, 2020). At the same time, pregnancy leads to constipation by slowing down gastric emptying. Furthermore, pregnant women are more prone to develop gallstones due to their elevated progesterone levels, which relaxes muscles, inhibits the release of cholecystokinin, and may thereby result in biliary stasis (Mockridge and Maclennan, 2019).

Changes in the Hematologic System

As with every other system in the body, the hematologic system also experiences changes during pregnancy. The most significant changes include expanded plasma volume accompanied with a higher red blood cell mass that leads to physiologic anemia as mentioned earlier (Bernstein et al., 2001). Additionally, during the first trimester white blood cells increase due to the physiological stress that pregnancy places on the body. This is especially evident with neutrophils. The most likely reason for this is the lack of neutrophilic apoptosis ability during pregnancy as a result of an increase in inhibitory factors in the serum. In addition, there is an increase in the number of myelocytes and metamyelocytes which indicate the increased level of activity in the bone marrow and erythropoiesis during pregnancy. In the first and second trimesters there is also a decrease in the lymphocyte count, compared to an increase in the third trimester (Chandra et al., 2012; Al-Shafei et al., 2020). Studies have also shown that platelet counts decrease in pregnancy, mainly in the third trimester. This is known as gestational thrombocytopenia, which occurs as a result of the hemodilution present in pregnancy accompanied with increased platelet activation and clearance. Pregnancy is also associated with an increase in the levels of coagulation factors due to the increase in estrogen levels during this time, resulting in a prothrombotic state, especially in the third trimester (Patel and Balanchivadze, 2021). **Table 1** and **Figure 1** summarize the physiological changes associated with pregnancy.

CHANGES IN MICROBIOME ASSOCIATED WITH PREGNANCY

The Gut Microbiome

The gastrointestinal tract is colonized by various microorganisms, including bacteria, protozoa, viruses and archaean and the composition of this microbiome varies with age. In adult humans, approximately 80% of this microbiome consists of bacteria belonging to the phyla Actinobacteria, Firmicutes and Bacteroidetes (Baldassarre et al., 2018). In the case of the neonate, colonization of the gut, which occurs during and after birth, is affected by mode of delivery and breastfeeding. The neonatal gut is colonized immediately for the most part by *Enterococci*, *Staphylococci*, and *Enterobacteria*; during the first days of post-natal life, *Lactobacillus*, *Clostridium*, *Bifidobacterium*, and *Bacteroides* take up residence; and the gut composition of bacteria become similar to that of adults at one year of age (Baldassarre et al., 2018).

Most pregnancies progress without incident. But approximately 8 percent of all pregnancies involves deleterious fetal and maternal health complications, the most common being preterm birth, diminished intrauterine growth, preeclampsia, and eclampsia (Maresh, 1990). The gut microbiota associated with normal and complicated pregnancies differ and the physiological changes, including the rise in the progesterone level, affect the composition of the maternal microbiome. More specifically, the microbiome in pregnant women contains a larger proportion of, in particular, *Bifidobacterium*, but also of *Proteobacteria* and *Actinobacteria* (Koren et al., 2012). During the third trimester alpha diversity is reduced, while both beta diversity and the abundance of opportunistic pathogens are elevated.

Some researchers have concluded that these changes are temporary and of no significance (DiGiulio et al., 2015; Yang et al., 2020), whereas others have found that insertion of human gut microbes from the third trimester into mice leads to weight gain and more pronounced low-grade inflammation (Turjeman et al., 2021). Furthermore, all alterations in the gut microbiome directly influence maternal metabolism (Paul et al., 2018), which, in turn, impacts the development and growth of fetal organs (Jiang et al., 2021).

In addition, the composition of the gut microbiome may be altered by poor dental health or inflammatory bowel disease in a manner linked to an increased risk for spontaneous premature delivery. In one investigation, the beta diversity of the maternal gut microbiota was found to differ between those who delivered preterm and normal deliveries (Hiltunen et al., 2021). In another case, mothers who underwent spontaneous preterm delivery exhibited lower diversity in their gut microbiome, particularly with respect to *Bifidobacterium* and *Streptococcus* (Dahl et al., 2017).

Moreover, gut microbes can give rise to intrauterine infection, as reflected in the presence of these microbes in the amniotic

TABLE 1 | Physiological changes during pregnancy.

Cardiovascular system	• Decrease peripheral vascular resistance • Increased heart rate • Decreased arterial pressure • Increased cardiac output • Increase in total body water, capillary hydrostatic pressure, and blood volume	(Sanghavi and Rutherford, 2014; Furfaro et al., 2018)
Respiratory system	• Mucosal changes in the upper airway include edema, hyperemia, leakage of plasma into the stroma, glandular hypersecretion, increased mucopolysaccharide content and increased phagocytic activity • Increased tidal volume • Decreased residual volume • Increased minute-ventilation by 30-40% increased respiratory center simulation → increased respiratory rate • Decreased PaCO2	(Hegewald and Crapo, 2011; Cordioli et al., 2013)
Gastrointestinal system	• Decreased muscle tone across the digestive tract • Delayed gastric emptying and diaphragm elevation by the pregnant womb • Increase in gastric PH and reduced gastrointestinal motility • Increased production of pro-inflammatory cytokines by Kupffer cells • Changes in bile composition	(Cordioli et al., 2013; Furfaro et al., 2018; Gomes et al., 2018)
Renal system	• The glomerular filtration rate increases by 50% • Decrease in serum urea, creatinine, and uric acid values • Ureteropelvic dilation and decreased ureteral pressure due to smooth muscle relaxation • Increased intravesical pressure due to the pregnant uterus weight • Increased renal plasma flow and vesicoureteral reflux • Asymptomatic bacteriuria • Flaccid bladder	(Cordioli et al., 2013; Cheung and Lafayette, 2013)
Genital system	• Decreased vaginal Ph • Increased glycogen in vaginal epithelium • Increased uterine blood flow and the vascular bed proliferates • Uterus increases in size to contain the growing fetus	(Parry and Vodstrcil, 2007; Cordioli et al., 2013)
Hematologic system	• Increases factors VII, VIII, IX, X, XII, Von Willebrand and fibrinogen • Decreased fibrinolytic activity • Decreased protein S • Increased plasma and red cell volume • Anemia	(Cordioli et al., 2013)

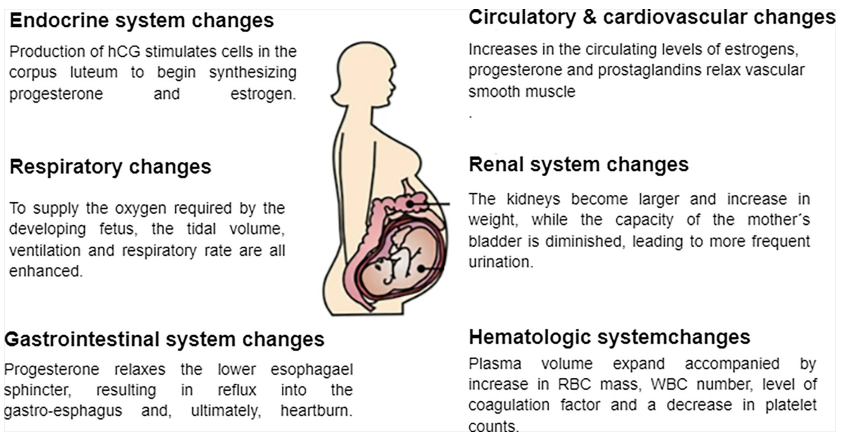


FIGURE 1 | Physiological changes associated with pregnancy.

fluid of women who experienced premature rupture of membranes (Edwards et al., 2017). The mechanism by which gut microbes move to the uterus is not yet known, but two possible mechanisms have been proposed: the first is that gram-negative bacteria, which express lipopolysaccharide that elicits the production of prostaglandins and other mediators of inflammation, ascend *via* the vagina; and the second, that the content of the gut leaks into the uterus or placenta (Edwards et al., 2017).

The Oral Microbiome

The healthy human oral cavity contains approximately 50-100 million bacteria belonging to 700 species (Kilian et al., 2016),

including *Lactobacilli, Staphylococci, Streptococcus* (Dewhirst et al., 2010; Kumar et al., 2013; Saadaoui et al., 2021). The composition of this complex community is affected by several factors, such as nutrition, oxygen levels, and pH (Saadaoui et al., 2021). Imbalances in the oral microbiome have been found to be associated with certain diseases, as well as with pregnancy. Imbalances in the oral microbiota, particularly during pregnancy, have been linked to a variety of disorders (Farrell et al., 2012). In fact, the oral microbiome of a healthy pregnant woman and a pregnant woman with certain diseases, e.g., gestational diabetes, differ (Li et al., 2021).

The changes in microbiome that occur in connection with pregnancy include the microbiome in the oral cavity (Saadaoui et al., 2021; Turjeman et al., 2021). For instance, the microbiome detected in saliva differs between pregnant and non-pregnant women, with the former showing an abundance of, e.g., *Porphyromonas*, *Treponema* and *Neisseria*, while in the latter, *Veillonella* and *Streptococcus* were overrepresented (Lin et al., 2018). The oral microbiome of pregnant women contain high numbers of bacteria, mainly during the first trimester (Fujiwara et al., 2017) including *Porphyromonas, Neisseria*, and *Treponema* (Lin et al., 2018) and certain pathogenic bacteria (Chong et al., 2018). Moreover, certain specific species of bacteria, such as *Staphylococci, Streptococci, Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*, are more abundant in the oral microbiome during the first and second trimesters of pregnancy. During pregnancy, the proliferation and growth of *Streptococcus, Lactobacillus, Escherichia coli*, and *Bifidobacterium* species vary (Pelzer et al., 2012).

Furthermore, the hormonal changes that pregnant women undergo promote the formation of bacterial plaque, thereby resulting in gingivitis, especially during the second to third trimesters (de Souza Massoni et al., 2019), which causes complications of pregnancy such as preeclampsia, preterm birth (PTB), low birth weight, and miscarriage (Cobb et al., 2017). The amniotic fluid of a woman who went into preterm labor contained *Fusobacterium nucleatum*, suggesting that oral bacteria can translocate to the placenta (Nuriel-Ohayon et al., 2019; Amir et al., 2020). In another woman who suffered from gingivitis and gave an unusual full-term stillbirth, *Fusobacterium nucleatum* was detected in both the placenta and newborn infant, indicating that this bacterium originated from the maternal subgingival plaque (Nuriel-Ohayon et al., 2019; Amir et al., 2020). It appears possible that the environments in the oral cavity and placenta contain similar factors that promote colonization and growth of *Fusobacterium nucleatum* (Nuriel-Ohayon et al., 2019; Amir et al., 2020).

In addition, a positive correlation between the presence of a periodontopathogen (*Porphyromonas gingivalis*) and progesterone levels in the first trimester of pregnancy was observed (de Souza Massoni et al., 2019). Other studies confirmed the growth of certain gram-negative anaerobic bacteria, including *Prevotella nigrescens*, *Campylobacter rectus* (Yokoyama et al., 2008; Gürsoy et al., 2009), and *Prevotella intermedia* (Muramatsu and Takaesu, 1994), which is promoted by the hormonal changes that occur during pregnancy. Moreover, high estrogen levels promote infection by *Candida* (Kumar et al., 2013; Fujiwara et al., 2017).

Willmott and co-workers (2020) demonstrated that the composition of the oral microbiome accurately reflects the dietary content of nitrate and the healthy regulation of blood pressure (Willmott et al., 2020). Bacteria in the oral cavity, located primarily on the tongue's surface, reduce nitrate enzymatically, resulting in the presence of nitrite in the saliva, which is subsequently transformed in the stomach into nitric acid and then reduced to nitric oxide (NO). This process is related to blood pressure in two different ways: the plasma level of nitrate is related inversely to blood pressure, and, at the same time, NO is a key signal molecule in connection with processes that regulate the circulatory system (Willmott et al., 2020).

Nitric Oxide (NO) is one of the reaction products produced by nitric oxide synthase (NOS) enzymes that catalyze NADPH and tetrahydrobiopterin (BH4)-dependent oxidation of L-arginine to L-citrulline (Ghimire et al., 2017). Nitric oxide serves in a wide aspect of human physiology and it takes part in vasodilation, endothelial function, mitochondrial function, prevention of platelets aggregation, neurotransmission, immune defense, and metabolism (Hezel and Weitzberg, 2015). In research from Walker et al., (2018) it was indicated that The oral microbiome play an important role in the production of the nitric oxide (NO) through nitrate-nitrite-NO pathway. The oral microbiota is deficient in the NO enzymes responsible for yielding the catalyzed NO from L-arginine (Qu et al., 2016; Willmott et al., 2020). According to Walker et al., (2018), the production of nitric oxide occurs in the human body by two methods: dependent and independent. Furthermore, the microbiome in the human body produces NO in an independent way through fermentation depending on food intake by the consumption of nitrate ($NO3^-$) and nitrite ($NO2^-$). In addition, oral microbiota has a major role in cardiovascular system wellbeing and in the regulation of blood pressure, including in pregnancy (Willmott et al., 2020). A case-control study in a tertiary facility in Ghana (Darkwa et al., 2018) found that the level of serum nitric oxide rises rapidly during pregnancy and peaks during the third trimester of a healthy pregnancy. However, other research found that the progressive rise in serum nitric oxide levels during pregnancy is not significant. According to (Zullino et al., 2018), nitric oxide is the principal vasodilator in the placenta, making it crucial to several physiological functions of an uncomplicated pregnancy. Placental perfusion, platelet adhesion and aggregation in the intervillous space, and fetoplacental vascular response are all regulated by NO during implantation, early embryonic development, and feto-placental vascular reaction (Zullino et al., 2018).

The Vaginal Microbiome

The composition of the vaginal microbiome, which plays an essential role in both maternal and fetal health (Nelson et al., 2016), can be altered by many different factors, including hormones, sexual practices, pregnancy, hygiene, urogenital infections and pharmacological treatments (Kroon et al., 2018; Noyes et al., 2018; Parolin et al., 2018). Typically, *Lactobacilli* predominate and, together with other bacterial species, maintain a pH of 3.8-4.5

(Mendling, 2016). The vaginal environment changes dramatically during pregnancy, resulting in an even greater abundance of *Lactobacillus* spp. and pronounced changes in the metabolic profiles of the bacteria present (Marangoni et al., 2021). Complicated pregnancies and preterm birth are associated with less *Lactobacilli* and a greater variety of bacteria (Di Simone et al., 2020).

Several investigations into a potential link between the composition of the vaginal microbiome and miscarriage have revealed that miscarriages during the first trimester appear to associate with lower levels of *Lactobacillus* spp. and more pronounced alpha diversity. The presence of pathogenic microorganisms raises the risk for infections such as bacterial vaginosis, which has been linked to premature rupture of membranes and preterm birth. A meta-analysis concluded that, even after controlling for other major risk factors, the risk of preterm delivery in women with bacterial vaginosis caused by, e.g., *Prevotella bivia*, *Peptostreptococcus*, and/or *G. vaginalis* increased more than two-fold (Leitich et al., 2003). Moreover, a higher risk for preterm birth rate was correlated with the presence of specific vaginal fungi such as *Candida albicans* (Neuman and Koren, 2017) and variations in the vaginal pH caused by changes in the microbiome (Mysorekar and Cao, 2014).

In addition to the factors mentioned above, the composition of the vaginal microbiota is influenced by ethnic background and genetic polymorphisms that affect the ability to produce anti- or pro-microbial substances. Such polymorphisms are present in the genes that encode the antagonist of the interleukin 1 (IL-1) receptor and the Toll-like receptor (TLR) 4, which acts in the innate recognition of Gram-negative bacteria and can influence individual susceptibility to complications during pregnancy (Mendling, 2016). Furthermore, women who have embryonic miscarriages exhibit higher vaginal levels of interleukin 2 (IL-2) and lower levels of interleukin 10 (IL-10) than control subjects (Xu et al., 2020). **Table 2** summarize the change in the maternal microbiome during pregnancy. And **Figure 2** shows the potential mechanisms of crosstalk between maternal microbiota and offspring immunity.

THE EFFECT OF PROBIOTICS ON MICROBIOME DURING PREGNANCY

The World Health Organization (WHO) defines probiotics as live microorganisms that, upon administration through the diet or as a

TABLE 2 | List of organisms and their associated effects during pregnancy.

Causative microbiome	Effects on pregnancy	Reference
The gut microbiome		
Bifidobacteria	Atopic eczema and asthma	(Baldassarre et al., 2018)
Enterobacteriaceae	Preterm newborns	(Baldassarre et al., 2018)
Enterococcaceae		
Proteobacteria	Metabolic syndrome such as weight gain, hyperglycemia,	(Koren et al., 2012)
Actinobacteria	insulin resistance.	
Prevotella	Protection against food allergies and Gestational diabetes	(Koren et al., 2012; Turjeman et al., 2021)
Staphylococcus spp,	Weight gain	(Turjeman et al., 2021)
Escherichia coli		
Ruminococcaceae, and *Collinsella*	Gestational diabetes	(Turjeman et al., 2021)
Enterobacteriaceae, *DesulfovibrioParabacteroides distasonis*		
Bulleidia moorei,	Risk of Preeclampsia	(Turjeman et al., 2021)
Clostridium perfringens,		
Fusobacterium and Veillonella		
Planococcaceae, Lactobacillaceae and	Preterm neonates	(Hiltunen et al., 2021)
Enterobacteriaceae		
Bifidobacterium	Preterm neonates	(Dahl et al., 2018)
Streptococcus		
Listeria monocytogenes	Still birth	(Edwards et al., 2017)
The oral microbiome		
Pg and intrauterine Bergeyella	Delivered prematurely	(Saadaoui et al., 2021)
Lautropia and *Neisseria*	Saliva and dental plaque	(Li et al., 2021)
Porphyromonas gingivalis	Periodontal inflammation, placentas of patients with preeclampsia	(Cobb et al., 2017; Lin et al., 2018)
Filifactor alocis	Placentas of patients with preeclampsia	(Cobb et al., 2017)
Fusobacterium nucleatum	Preterm birth, periodontal disease and adverse pregnancy complications	(Cobb et al., 2017; Amir et al., 2020; Saadaoui et al., 2021)
Prevotella nigrescens	Pregnancy gingivitis	(Gürsoy et al., 2009)
Genera Rothia and Staphylococcus	Oral nitrate reduction	(Willmott et al., 2020)
Prevotella	Oral nitrate reduction	(Willmott et al., 2020)
The vaginal microbiome		
Lactobacilli	Pregnancy-related complications and preterm birth	(Di Simone et al., 2020)
Prevotella bivia, Peptostreptococcus, and/or *G. vaginalis*	Preterm delivery	(Leitich et al., 2003)
Candida albicans	Preterm delivery	(Neuman and Koren, 2017)

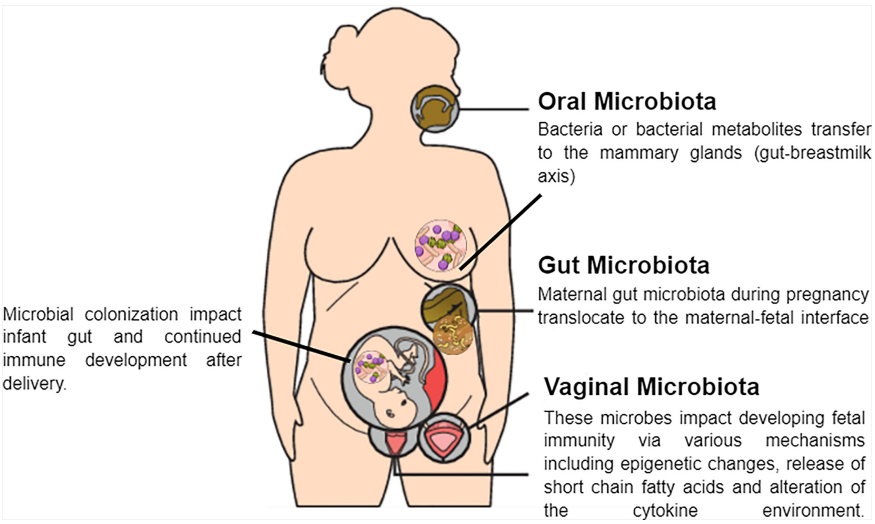


FIGURE 2 | Potential mechanisms of crosstalk between maternal microbiota and the fetus.

supplement, have beneficial effects on the host (Buggio et al., 2019). A variety of studies indicate that by regulating the gut and vaginal microflora, the right combination of probiotics (Baldassarre et al., 2018) can lessen the risk of pregnancy complications such as preterm birth (Arango et al., 2015). It was proposed recently that probiotics prevent preterm birth by altering the composition and function of the gut microbiome to improve maternal glucose metabolism (Buggio et al., 2019).

However, findings in this area are controversial. For instance, one study that included 4098 women found that probiotics can either decrease or increase the risk for birth between weeks 34-37 of pregnancy (Buggio et al., 2019), supporting the proposal that more detailed investigations on the types of probiotic, the characteristics of individual women and length of administration are required (Jarde et al., 2018). In another case, probiotics provided no protection and were shown to be capable of initiating the inflammatory cascade associated with preterm birth (Othman et al., 2007; Arango et al., 2015). In contrast, dietary supplementation with probiotics has been reported to reduce the abundance of *Bifidobacterium* and attenuate the increase in *Atopobium vaginae* that is linked with more than 70% of cases of bacterial vaginosis, which causes preterm birth (Baldassarre et al., 2019). At the same time, it appears that following antibiotic treatment, supplementary probiotics can lower the vaginal pH to an optimal value hence promoting the restoration of vaginal microbiota, thereby preventing the reduction in levels of anti-inflammatory cytokines (Baldassarre et al., 2019).

Moreover, the administration of probiotics during pregnancy has been shown to raise the levels of anti-inflammatory molecules such as IL-10 and TGF-B in breast milk, which aids in the maturation of the infant's gut by stimulating the secretion of IgA and oral tolerance (Baldassarre et al., 2018). Furthermore, probiotics can effectively prevent allergic reactions. The lymphoid system of the newborn is not fully developed, with a limited Th1 response, and the microbiota plays an essential role in bridging this gap between Th1 and Th2 responses. Accordingly, alteration of the gut microbiota that results in loss of this modulation of inflammatory cytokines can augment the risk for development of atopic eczema (Baldassarre et al., 2018).

More than 15% of young infants suffer from gestational disorders such as infantile colic. Probiotics can influence the pathogenesis of such disorders by altering the composition of the gut microbiome, which is involved in direct bidirectional relationships with the brain. The three mechanisms proposed as explanations for this influence by probiotics include alterations in the secretion of cytokines and chemokines, involvement of microbiota in neural pathways, and stimulation of the intestinal neuroendocrine pathway. Interestingly, the usage of a mixture of probiotics has been found to reduce crying by infants with colic during breastfeeding (Baldassarre et al., 2018), and probiotics appear to significantly impact the composition of the neonatal microbiota. It has been suggested that probiotics such as *Lactobacillus* can interact directly with the host *via* pattern recognition receptors (PRR), for which the peptidoglycan in the wall of gram-positive bacteria and lipopolysaccharide of gram-negative bacteria serve as ligands (Devi et al., 2021). The direct interaction involves the binding of probiotic bacteria to these receptors on the host's dendritic and intestinal epithelial cells, thereby preventing cytokine-induced apoptosis and production of defensins and mucus (Devi et al., 2021).

Another proposed mechanism involves the generation of toxic or antimicrobial compounds such as bacteriocin by probiotics (Oktaviyani et al., 2021). For example, *Lactobacillus crispatus* F177 and *Lactobacillus paracasei* F2 and F28 produce hydrogen peroxide, which suppresses the growth of *Staphylococcus aureus*. In addition, probiotics compete with pathogenic species for adhesion to the surface of intestinal epithelial cells. Adhesion molecules such as a mucus-binding protein on the surface of probiotics facilitate

their interaction with host dendritic cells, thus enhancing the phagocytic capacity of these cells (Devi et al., 2021).

CONCLUSIONS

In this review, we describe the changes in the compositions of the gut, oral and vaginal microbiome that occur in connection with pregnancy. The hormonal, immunological and metabolic changes that pregnant women undergo influence these compositions and vice-versa, appropriate adaptation is required to support optimal fetal growth and development.

Imbalances in the microbiota can lead to complications of pregnancy such gestational diabetes, preterm delivery and preeclampsia. Manipulating microbiome composition during pregnancy through probiotics could result in improved maternal health and pregnancy outcomes. Maternal micorbioem and fetal interaction during pregancy is critical for fetal developemt.

The mechanisms by which these microbiome and the host interact during pregnancy and regulation of these interactions require elucidation.

AUTHOR CONTRIBUTIONS

Conceptualization, ZZ & MA-A; writing—original draft preparation, SA-R, RA, HF, ME, RN, SB, SS, and SN; Figure design and tables, ZZ; writing—review and editing ZZ & MA-A; funding acquisition, MA-A. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by Qatar National Research Fund (QNRF), grant number UREP26-104-3-044.

REFERENCES

Abbassi-Ghanavati, M., Greer, L. G., and Cunningham, F. G. (2009). Pregnancy and Laboratory Studies: A Reference Table for Clinicians. *Obstet. Gynecol.* 114 (6), 1326–1331. doi: 10.1097/AOG.0b013e3181c2bde8

Al-Shafei, A. I., Musa, S. M., Rayis, D. A., Lutfi, M. F., El-Gendy, O. A., and Adam, I.. (2020). Heart Rate Variability and Hematological Parameters in Pregnant Women. *J. Clin. Lab. Anal.* 34 (6), e23250. doi: 10.1002/jcla.23250

Alto, W. A. (2005). No Need for Glycosuria/Proteinuria Screen in Pregnant Women. *J. Fam. Pract.* 54 (11), 978–983.

Amir, M., Brown, J. A., Rager, S. L., Sanidad, K. Z., Ananthanarayanan, A., and Zeng, M. Y.. (2020). Maternal Microbiome and Infections in Pregnancy. *Microorganisms* 8 (12), 1996. doi: 10.3390/microorganisms8121996

Arango, L. F. G., Barrett, H. L., Callaway, L. K., and Nitert, M. D.. (2015). Probiotics and Pregnancy. *Curr. Diabetes Rep.* 15 (1), 567. doi: 10.1007/s11892-014-0567-0

Staying-Healthy-During-Pregnancy: 4 Common Pregnancy Complications. Available at: www.hopkinsmedicine.orghttps://www.hopkinsmedicine.org/health/conditions-and-diseases/staying-healthy-during-pregnancy/4-common-pregnancy-complications.

Baldassarre, M. E., Di Mauro, A., Capozza, M., Rizzo, V., Schettini, F., Panza, R., et al. (2018). Rationale of Probiotic Supplementation During Pregnancy and Neonatal Period. *Nutrients* 10 (11), 1693. doi: 10.3390/nu10111693

Baldassarre, M. E., Palladino, V., Amoruso, A., Pindinelli, S., Mastromarino, P., Fanelli, M., et al. (2019). Dysbiosis and Prematurity: Is There a Role for Probiotics? *Nutrients* 11 (6), 1273. doi: 10.3390/nu11061273

Barjaktarovic, M., Korevaar, T. I., Jaddoe, V. W., de Rijke, Y. B., Visser, T. J., Peeters, R. P., et al. (2017). Human Chorionic Gonadotropin (hCG) Concentrations During the Late First Trimester Are Associated With Fetal Growth in a Fetal Sex-Specific Manner. *Eur. J. Epidemiol.* 32 (2), 135–144. doi: 10.1007/s10654-016-0201-3

Bernstein, I. M., Ziegler, W., and Badger, G. J. (2001). Plasma Volume Expansion in Early Pregnancy. *Obstet. Gynecol.* 97 (5 Pt 1), 669–672. doi: 10.1016/s0029-7844(00)01222-9

Body, C., and Christie, J. A. (2016). Gastrointestinal Diseases in Pregnancy: Nausea, Vomiting, Hyperemesis Gravidarum, Gastroesophageal Reflux Disease, Constipation, and Diarrhea. *Gastroenterol. Clin* 45 (2), 267–283. doi: 10.1016/j.gtc.2016.02.005

Buggio, L., Somigliana, E., Borghi, A., and Vercellini, P. (2019). Probiotics and Vaginal Microecology: Fact or Fancy? *BMC Women's Health* 19 (1), 1–6. doi: 10.1186/s12905-019-0723-4

Chandra, S., Tripathi, A. K., Mishra, S., Amzarul, M., and Vaish, A. K. (2012). Physiological Changes in Hematological Parameters During Pregnancy. *Indian J. Hematol. Blood Transfus.* 28 (3), 144–146. doi: 10.1007/s12288-012-0175-6

Chen, A., and Basso, O. (2007). Does Low Maternal Blood Pressure During Pregnancy Increase the Risk of Perinatal Death? *Epidemiol. (Cambridge. Mass.)* 18 (5), 619. doi: 10.1097/ede.0b013e31812713e6

Cheung, K. L., and Lafayette, R. A. (2013). Renal Physiology of Pregnancy. *Adv. Chronic. Kidney Dis.* 20 (3), 209–214. doi: 10.1053/j.ackd.2013.01.012

Chong, C. Y. L., Bloomfield, F. H., and O'Sullivan, J. M. (2018). Factors Affecting Gastrointestinal Microbiome Development in Neonates. *Nutrients* 10 (3), 274. doi: 10.3390/nu10030274

Claros, N. M., de la Fuente, J. A., Cardiel, M. A., Donaire, J. G., and Herráiz, M. (2020). Papel Del Sistema Renina-Angiotensina En El Embarazo Y La Preeclampsia. *Hipertensión. y. Riesgo. Vasc.* 37 (2), 72–77. doi: 10.1016/j.hipert.2020.02.003

Cobb, C. M., Kelly, P. J., Williams, K. B., Babbar, S., Angolkar, M., and Derman, R. J. (2017). The Oral Microbiome and Adverse Pregnancy Outcomes. *Int. J. Women's Health* 9, 551. doi: 10.2147/IJWH.S142730

Cordioli, R. L., Kelly, P. J., Williams, K. B., Babbar, S., Angolkar, M., and Derman, R. J. (2013). Sepsis and Pregnancy: Do We Know How to Treat This Situation? *Rev. Bras. Ter. Intensiva.* 25 (4), 334–344. doi: 10.5935/0103-507X.20130056

Dahl, C., Stanislawski, M., Iszatt, N., Mandal, S., Lozupone, C., Clemente, J. C., et al. (2017). Gut Microbiome of Mothers Delivering Prematurely Shows Reduced Diversity and Lower Relative Abundance of Bifidobacterium and Streptococcus. *PloS One* 12 (10), e0184336. doi: 10.1371/journal.pone.0184336

Dahl, C., Stigum, H., Valeur, J., Iszatt, N., Lenters, V., Peddada, S., et al. (2018). Preterm Infants Have Distinct Microbiomes Not Explained by Mode of Delivery, Breastfeeding Duration or Antibiotic Exposure. *Int. J. Epidemiol.* 47 (5), 1658–1669. doi: 10.1093/ije/dyy064

Darkwa, E. O., Djagbletey, R., Sottie, D., Owoo, C., Vanderpuye, N. M., Essuman, R., et al. (2018). Serum Nitric Oxide Levels in Healthy Pregnant Women: A Case-Control Study in a Tertiary Facility in Ghana. *Matern Health. Neonatol. Perinatol.* 4 (1), 1–5. doi: 10.1186/s40748-017-0072-y

de Souza Massoni, R. S., Aranha, A. M. F., Matos, F. Z., Guedes, O. A., Borges, Á. H., Miotto, M., et al. (2019). Correlation of Periodontal and Microbiological Evaluations, With Serum Levels of Estradiol and Progesterone, During Different Trimesters of Gestation. *Sci. Rep.* 9 (1), 1–9. doi: 10.1038/s41598-019-48288-w

Devi, P., Nanaji, Y., Khanna, N., Yadav, A. K., and Pawar, S. V.. (2021). Animal Models for Probiotic Interventions Under Gut Inflammatory Conditions. In Kaur I. P., Pawar S. V and Rishi P. (eds) *Probiotic Research in Therapeutics*. Springer, Singapore 85–121. doi: 10.1007/978-981-33-6236-9_4

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W.-H., et al. (2010). The Human Oral Microbiome. *J. Bacteriol.* 192 (19), 5002–5017. doi: 10.1128/JB.00542-10

DiGiulio, D. B., and Callahan, B. J.. (2015). Temporal and Spatial Variation of the Human Microbiota During Pregnancy. *Proc. Natl. Acad. Sci. U. S. A.* 112 (35), 11060–11065. doi: 10.1073/pnas.1502875112

Di Simone, N., Santamaria Ortiz, A., Specchia, M., Tersigni, C., Villa, P., Gasbarrini, A., et al. (2020). Recent Insights on the Maternal Microbiota: Impact on Pregnancy Outcomes. *Front. Immunol.* 11, 2372. doi: 10.3389/fimmu.2020.528202

Edwards, S. M., Cunningham, S. A., Dunlop, A. L., and Corwin, E. J.. (2017). The Maternal Gut Microbiome During Pregnancy. *MCN. Am. J. Matern Child Nurs.* 42 (6), 310. doi: 10.1097/NMC.0000000000000372

Elkus, R., and Popovich, J.Jr (1992). Respiratory Physiology in Pregnancy. *Clin Chest. Med.* 13 (4), 555–565. doi: 10.1016/S0272-5231(21)01125-4

Farrell, J. J., Zhang, L., Zhou, H., Chia, D., Elashoff, D., Akin, D., et al. (2012). Variations of Oral Microbiota Are Associated With Pancreatic Diseases Including Pancreatic Cancer. *Gut* 61 (4), 582–588. doi: 10.1136/gutjnl-2011-300784

Figuero, E., Han, Y. W., and Furuichi, Y. (2020). Periodontal Diseases and Adverse Pregnancy Outcomes: Mechanisms. *Periodontol. 2000.* 83 (1), 175–188. doi: 10.1111/prd.12295

Fuhler, G. M. (2020). The Immune System and Microbiome in Pregnancy. *Best Pract. Res. Clin. Gastroenterol.* 44-45, 101671. doi: 10.1016/j.bpg.2020.101671

Fujiwara, N., Tsuruda, K., Iwamoto, Y., Kato, F., Odaki, T., Yamane, N., et al. (2017). Significant Increase of Oral Bacteria in the Early Pregnancy Period in Japanese Women. *J. Invest. Clin. Dentist* 8 (1), e12189. doi: 10.1111/jicd.12189

Furfaro, L. L., Chang, B. J., and Payne, M. S. (2018). Applications for Bacteriophage Therapy During Pregnancy and the Perinatal Period. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.02660

Ghimire, K., Altmann, H. M., Straub, A. C., and Isenberg, J. S.. (2017). Nitric Oxide: What's New to NO? *Am. J. Physiol. Cell Physiol.* 312 (3), C254–c262. doi: 10.1152/ajpcell.00315

Gomes, C. F., Sousa, M., Lourenço, I., Martins, D., and Torres, J.. (2018). Gastrointestinal Diseases During Pregnancy: What Does the Gastroenterologist Need to Know? *Ann. Gastroenterol.* 31 (4), 385–394. doi: 10.20524/aog.2018.0264

Gürsoy, M., Haraldsson, G., Hyvönen, M., Sorsa, T., Pajukanta, R., and Könönen, E.. (2009). Does the Frequency of Prevotella Intermedia Increase During Pregnancy? *Oral. Microbiol. Immunol.* 24 (4), 299–303.doi: 10.1111/j.1399-302X.2009.00509.x.

Hegewald, M. J., and Crapo, R. O. (2011). Respiratory Physiology in Pregnancy. *Clinics Chest. Med.* 32 (1), 1–13. doi: 10.1016/j.ccm.2010.11.001

Hendriks, E., MacNaughton, H., and MacKenzie, M. C. (2019). First Trimester Bleeding: Evaluation and Management. *Am. Fam. Phys.* 99 (3), 166–174.

Herrero, B. T., Martínez, M. R., Plaza, M. L., Gutiérrez, L., García, R. P., García, J. C., et al. (2001). Signos Y Síntomas Gastrointestinales Durante El Embarazo Y Puerperio En Una Muestra De Mujeres Españolas. *Atención. Primaria.* 28 (1), 53–58. doi: 10.1016/S0212-6567(01)78896-8

Hezel, M., and Weitzberg, E. (2015). The Oral Microbiome and Nitric Oxide Homoeostasis. *Oral. Dis.* 21 (1), 7–16. doi: 10.1111/odi.12157

Hiltunen, H., Collado, M. C., Ollila, H., Kolari, T., Tölkkö, S., Isolauri, E., et al. (2021). Spontaneous Preterm Delivery Is Reflected in Both Early Neonatal and Maternal Gut Microbiota. *Pediatr. Res.*, 1–8. doi: 10.1038/s41390-021-01663-8

Jarde, A., Lewis-Mikhael, A.-M., Moayyedi, P., Stearns, J. C., Collins, S. M., Beyene, J., et al. (2018). Pregnancy Outcomes in Women Taking Probiotics or Prebiotics: A Systematic Review and Meta-Analysis. *BMC Pregnancy. Childbirth.* 18 (1), 1–14. doi: 10.1186/s12884-017-1629-5

Jarvis, S., and Nelson-Piercy, C. (2020). Common Symptoms and Signs During Pregnancy. *Obstet. Gynaecol. Reprod. Med.* 24 (8), 245–249. doi: 10.1016/j.ogrm.2020.07.005

Jiang, G., Zhou, Z., Li, X., Qian, Y., and Wang, K. (2021). The Gut Microbiome During Pregnancy. *Matern Fetal. Med*. doi: 10.1097/FM9.0000000000000091

Kattah, A., Milic, N., White, W., and Garovic, V. (2017). Spot Urine Protein Measurements in Normotensive Pregnancies, Pregnancies With Isolated Proteinuria and Preeclampsia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 313 (4), R418–r424. doi: 10.1152/ajpregu.00508.2016

Kilian, M., Chapple, I., Hannig, M., Marsh, P., Meuric, V., Pedersen, A., et al. (2016). The Oral Microbiome–An Update for Oral Healthcare Professionals. *Br. Dental J.* 221 (10), 657–666. doi: 10.1038/sj.bdj.2016.865

Kobaly, K., and Mandel, S. J. (2019). Hyperthyroidism and Pregnancy. *Endocrinol. Metab. Clinics* 48 (3), 533–545. doi: 10.1016/j.ecl.2019.05.002

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150 (3), 470–480. doi: 10.1016/j.cell.2012.07.008

Kroon, S. J., Ravel, J., and Huston, W. M. (2018). Cervicovaginal Microbiota, Women's Health, and Reproductive Outcomes. *Fertil. Steril.* 110 (3), 327–336. doi: 10.1016/j.fertnstert.2018.06.036

Kumar, A., Basra, M., Begum, N., Rani, V., Prasad, S., Lamba, A. K., et al. (2013). Association of Maternal Periodontal Health With Adverse Pregnancy Outcome. *J. Obstet. Gynaecol. Res.* 39 (1), 40–45. doi: 10.1111/j.1447-0756.2012.01957.x

Lee, S.-Y., Chien, D.-K., Huang, C.-H., Shih, S.-C., Lee, W.-C., and Chang, W.-H.. (2017). Dyspnea in Pregnancy. *Taiwanese J. Obstet. Gynecol.* 56 (4), 432–436. doi: 10.1016/j.tjog.2017.04.035

Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C., and Husslein, P.. (2003). Bacterial Vaginosis as a Risk Factor for Preterm Delivery: A Meta-Analysis. *Am. J. Obstet. Gynecol.* 189 (1), 139–147. doi: 10.1067/mob.2003.339

Lemos, A., de Souza, A. I., Figueiroa, J. N., Cabral-Filho, J. E., and de Andrade, A. D.. (2010). Respiratory Muscle Strength in Pregnancy. *Respir. Med.* 104 (11), 1638–1644. doi: 10.1016/j.rmed.2010.05.020

Li, X., Zheng, J., Ma, X., Zhang, B., Zhang, J., Wang, W., et al. (2021). The Oral Microbiome of Pregnant Women Facilitates Gestational Diabetes Discrimination. *J. Genet. Genomics.* 48 (1), 32–39. doi: 10.1016/j.jgg.2020.11.006

Lin, W., Jiang, W., Hu, X., Gao, L., Ai, D., Pan, H., et al. (2018). Ecological Shifts of Supragingival Microbiota in Association With Pregnancy. *Front. Cell. Infect. Microbiol.* 8, 24. doi: 10.3389/fcimb.2018.00024

Marangoni, A., Laghi, L., Zagonari, S., Patuelli, G., Zhu, C., Foschi, C., et al. (2021). New Insights Into Vaginal Environment During Pregnancy. *Front. Mol. Biosci.* 8. doi: 10.3389/fmolb.2021.656844

Maresh, M. (1990). Medical Complications in Pregnancy. *Baillieres Clin. Obstet. Gynaecol* 4 (1), 129–147. doi: 10.1016/s0950-3552(05)80216-0

Mendling, W. (2016). Normal and Abnormal Vaginal Microbiota. *LaboratoriumsMedizin* 40 (4), 239–246. doi: 10.1515/labmed-2016-0011

Miller, C., McQuade, M., MacCallum, J., and Zhang, Y. V.. (2020). Persistently Elevated hCG in a Patient With No Clear Evidence of Pregnancy. *Clin. Chim. Acta* 510, 703–706. doi: 10.1016/j.cca.2020.09.004

Mockridge, A., and Maclennan, K. (2019). Physiology of Pregnancy. *Anaesthesia. Intensive Care Med.* 20 (7), 397–401. doi: 10.1016/j.mpaic.2019.05.001

Morton, A. (2020). Physiological Changes and Cardiovascular Investigations in Pregnancy. *Heart Lung Circ.* 30 (1), e6–e15.

Morton, A. (2021). Physiological Changes and Cardiovascular Investigations in Pregnancy. *Heart Lung Circ.* 30 (1), e6–e15.doi: 10.1016/j.hlc.2020.10.001

Muramatsu, Y., and Takaesu, Y. (1994). Oral Health Status Related to Subgingival Bacterial Flora and Sex Hormones in Saliva During Pregnancy. *Bull. Tokyo Dental Coll.* 35 (3), 139–151.

Mysorekar, I. U., and Cao, B. (2014). Microbiome in Parturition and Preterm Birth. *Semin. Reprod. Med.* 32 (1), 50–55. doi: 10.1055/s-0033-1361830.

Nelson, D. B., Rockwell, L. C., Prioleau, M. D., and Goetzl, L.. (2016). The Role of the Bacterial Microbiota on Reproductive and Pregnancy Health. *Anaerobe* 42, 67–73. doi: 10.1016/j.anaerobe.2016.09.001

Neuman, H., and Koren, O. (2017). The Pregnancy Microbiome. *Nestle. Nutr. Inst. Workshop Ser.* 88, 1–9. doi: 10.1159/000455207

Noyes, N., Cho, K.-C., Ravel, J., Forney, L. J., and Abdo, Z.. (2018). Associations Between Sexual Habits, Menstrual Hygiene Practices, Demographics and the Vaginal Microbiome as Revealed by Bayesian Network Analysis. *PloS One* 13 (1), e0191625. doi: 10.1371/journal.pone.0191625

Noyola-Martínez, N., Halhali, A., and Barrera, D. (2019). Steroid Hormones and Pregnancy. *Gynecol. Endocrinol.* 35 (5), 376–384. doi: 10.1080/09513590.2018.1564742

Nuriel-Ohayon, M., Neuman, H., Ziv, O., Belogolovski, A., Barsheshet, Y., Bloch, N., et al. (2019). Progesterone Increases Bifidobacterium Relative Abundance During Late Pregnancy. *Cell Rep.* 27 (3), 730–736.e3. doi: 10.1016/j.celrep.2019.03.075

Nuriel-Ohayon, M., Neuman, H., and Koren, O. (2016). Microbial Changes During Pregnancy, Birth, and Infancy. *Front. Microbiol.* 7, 1031. doi: 10.3389/fmicb.2016.01031

Oktaviyani, D., Alawiyyah, R. Z., Nusaiba, P., and Malik, A. (2021). A Review: Composition of Neonatal Meconium Microbiota and Its Role for Potential Probiotic. *Pharm. Sci. Res.* 8 (1), 6.

Othman, M., Alfirevic, Z., and Neilson, J. P. (2007). Probiotics for Preventing Preterm Labour. *Cochrane Database Syst. Rev.* (1), CD005941. doi: 10.1002/14651858.CD005941.pub2

Othman, M., Alfirevic, Z., and Neilson, J. P. (2007). Probiotics for Preventing Preterm Labour. *Cochrane Database Syst. Rev.* 2017 (1), CD005941. doi: 10.1002/14651858.CD005941.pub2.

Park, S., Lee, S. M., Park, J. S., Hong, J.-S., Chin, H. J., Na, K. Y., et al. (2017). Midterm eGFR and Adverse Pregnancy Outcomes: The Clinical Significance of Gestational Hyperfiltration. *Clin. J. Am. Soc. Nephrol.* 12 (7), 1048–1056. doi: 10.2215/CJN.12101116

Parolin, C., Foschi, C., Laghi, L., Zhu, C., Banzola, N., Gaspari, V., et al. (2018). Insights Into Vaginal Bacterial Communities and Metabolic Profiles of Chlamydia Trachomatis Infection: Positioning Between Eubiosis and Dysbiosis. *Front. Microbiol.* 9, 600. doi: 10.3389/fmicb.2018.00600

Parry, L. J., and Vodstrcil, L. A. (2007). Relaxin Physiology in the Female Reproductive Tract During Pregnancy. *Relaxin. Relat. Peptides.* p, 34–48. doi: 10.1007/978-0-387-74672-2_4

Paul, H. A., Collins, K. H., Bomhof, M. R., Vogel, H. J., and Reimer, R. A. (2018). Potential Impact of Metabolic and Gut Microbial Response to Pregnancy and Lactation in Lean and Diet-Induced Obese Rats on Offspring Obesity Risk. *Mol. Nutr. Food Res.* 62 (4). doi: 10.1002/mnfr.201700820

Patel, P., and Balanchivadze, N. (2021). Hematologic Findings in Pregnancy: A Guide for the Internist. *Cureus* 13 (5), e15149. doi: 10.7759/cureus.15149

Pelzer, E. S., Allan, J. A., Theodoropoulos, C., Ross, T., Beagley, K. W., and Knox, C. L.. (2012). Hormone-Dependent Bacterial Growth, Persistence and Biofilm Formation–a Pilot Study Investigating Human Follicular Fluid Collected During IVF Cycles. *PloS One* 7 (12), e49965. doi: 10.1371/journal.pone.0049965

Qu, X. M., Wu, Z. F., Pang, B. X., Jin, L. Y., Qin, L. Z., and Wang, S. L.. (2016). From Nitrate to Nitric Oxide: The Role of Salivary Glands and Oral Bacteria. *J. Dent. Res.* 95 (13), 1452–1456. doi: 10.1177/0022034516673019

Rapone, B., Ferrara, E., Montemurro, N., Converti, I., Loverro, M., Loverro, M. T., et al. (2020). Oral Microbiome and Preterm Birth: Correlation or Coincidence? A Narrative Review. *Open Access Macedonian. J. Med. Sci.* 8 (F), 123–132. doi: 10.3889/oamjms.2020.4444

Saadaoui, M., Singh, P., and Al Khodor, S. (2021). Oral Microbiome and Pregnancy: A Bidirectional Relationship. *J. Reprod. Immunol.* p, 103293. doi: 10.1016/j.jri.2021.103293

Sanghavi, M., and Rutherford, J. D. (2014). Cardiovascular Physiology of Pregnancy. *Circulation* 130 (12), 1003–1008. doi: 10.1161/CIRCULATIONAHA.114.009029

Scholl, T. O., and Reilly, T. (2000). Anemia, Iron and Pregnancy Outcome. *J. Nutr.* 130 (2), 443S–447S. doi: 10.1093/jn/130.2.443S

Suarez, M. L. G., Kattah, A., Grande, J. P., and Garovic, V.. (2019). Renal Disorders in Pregnancy: Core Curriculum 2019. *Am. J. Kidney Dis.* 73 (1), 119–130. doi: 10.1053/j.ajkd.2018.06.006

Sykes, L., and Bennett, P. R. (2018). Efficacy of Progesterone for Prevention of Preterm Birth. *Best Pract. Res. Clin. Obstet. Gynaecol.* 52, 126–136. doi: 10.1016/j.bpobgyn.2018.08.006

Turjeman, S., Collado, M. C., and Koren, O. (2021). The Gut Microbiome in Pregnancy and Pregnancy Complications. *Curr. Opin. Endocrine. Metab. Res.* 18, 133–138. doi: 10.1016/j.coemr.2021.03.004

Walker, M. Y., Pratap, S., Southerland, J. H., Farmer-Dixon, C. M., Lakshmyya, K., and Gangula, P. R.. (2018). Role of Oral and Gut Microbiome in Nitric Oxide-Mediated Colon Motility. *Nitric. Oxide* 73, 81–88. doi: 10.1016/j.niox.2017.06.003

Willmott, T., McBain, A. J., Humphreys, G. J., Myers, J., and Cottrell, E.. (2020). Does the Oral Microbiome Play a Role in Hypertensive Pregnancies? *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.00389

Xu, L., Huang, L., Lian, C., Xue, H., Lu, Y., Chen, X., et al. (2020). Vaginal Microbiota Diversity of Patients With Embryonic Miscarriage by Using 16s rDNA High-Throughput Sequencing. *Int. J. Genomics* 2020. doi: 10.1155/2020/1764959

Yang, H., Guo, R., Li, S., Liang, F., Tian, C., Zhao, X., et al. (2020). Systematic Analysis of Gut Microbiota in Pregnant Women and its Correlations With Individual Heterogeneity. *NPJ Biofilms Microbiomes* 6 (1), 1–12. doi: 10.1038/s41522-020-00142-y

Yokoyama, M., Hinode, D., Yoshioka, M., Fukui, M., Tanabe, S., Grenier, D., et al. (2008). Relationship Between Campylobacter Rectus and Periodontal Status During Pregnancy. *Oral. Microbiol. Immunol.* 23 (1), 55–59. doi: 10.1111/j.1399-302X.2007.00391.x

Zullino, S., Buzzella, F., and Simoncini, T. (2018). Nitric Oxide and the Biology of Pregnancy. *Vasc. Pharmacol.* 110, 71–74. doi: 10.1016/j.vph.2018.07.004

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.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 11 March 2022
doi: 10.3389/fcimb.2022.839526

A Gut Feeling in Amyotrophic Lateral Sclerosis: Microbiome of Mice and Men

Sarah Martin[1], *Carolina Battistini*[1] *and Jun Sun*[1,2,3,4]*

[1] Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois at Chicago, Chicago, IL, United States, [2] Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL, United States, [3] University of Illinois at Chicago (UIC) Cancer Center, University of Illinois at Chicago, Chicago, IL, United States, [4] Jesse Brown VA Medical Center, Chicago, IL, United States

Amyotrophic lateral sclerosis (ALS) is a severely debilitating disease characterized by progressive degeneration of motor neurons. ALS etiology and pathophysiology are not well understood. It could be the consequences of complex interactions among host factors, microbiome, and the environmental factors. Recent data suggest the novel roles of intestinal dysfunction and microbiota in ALS etiology and progression. Although microbiome may indeed play a critical role in ALS pathogenesis, studies implicating innate immunity and intestinal changes in early disease pathology are limited. The gastrointestinal symptoms in the ALS patients before their diagnosis are largely ignored in the current medical practice. This review aims to explore existing evidence of gastrointestinal symptoms and progress of microbiome in ALS pathogenesis from human and animal studies. We discuss dietary, metabolites, and possible therapeutic approaches by targeting intestinal function and microbiome. Finally, we evaluate existing evidence and identify gaps in the knowledge for future directions in ALS. It is essential to understanding the microbiome and intestinal pathogenesis that determine when, where, and whether microbiome and metabolites critical to ALS progression. These studies will help us to develop more accurate diagnosis and better treatment not only for this challenging disease, but also for other neurodegenerative diseases.

Keywords: amyotrophic lateral sclerosis (ALS), gastrointestinal digestion, enteric neuron, metabolite, microbiome, bacteria, CNS - central nervous system, motor neurodegeneration

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Yuishin Izumi, Tokushima University, Japan
Jessica Mandrioli, University of Modena and Reggio Emilia, Italy

***Correspondence:**
Jun Sun
junsun7@uic.edu

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *20 December 2021*
Accepted: *14 February 2022*
Published: *11 March 2022*

Citation:
Martin S, Battistini C and Sun J (2022) A Gut Feeling in Amyotrophic Lateral Sclerosis: Microbiome of Mice and Men. Front. Cell. Infect. Microbiol. 12:839526. doi: 10.3389/fcimb.2022.839526

INTRODUCTION

ALS is a highly fatal neuromuscular disease currently with no cure. ALS has a median incidence of about 2.8 cases per 100,000 persons per year and a median prevalence about 5.4 cases per 100,000 persons for a median age at 61.8 ± 3.8 years (Chio et al., 2013). Although relatively rare, this public health impact of ALS is significant due to its morbidity and mortality.

The causes of ALS are largely unknown, only 10–15% of ALS cases are familial amyotrophic lateral sclerosis (fALS), with the remainder considered sporadic ALS (sALS) (Turner et al., 2017). Monogenic mutations in over 30 genes are associated with about ALS cases, including C9orf72, SOD1, FUS, and TARDBP/TDP43 (Nicolas et al., 2018; Volk et al., 2018). However, these monogenic

forms explain only 15% of sporadic cases and 66% of familial cases (Turner et al., 2017). ALS pathogenesis is multifactorial and involves complex host-environment interactions. The risk factors include nutritional status, lower body mass index (BMI), smoking, high level of physical fitness, and military service (Wang et al., 2017). Environmental neurotoxins might contribute to ALS, e.g. β-Methylamino-L-alanine (BMAA) in people in Guam (Banack and Cox, 2003; Murch et al., 2004) and formaldehyde in factory workers (Weisskopf et al., 2009; Roberts et al., 2016).

Although ALS is a neurodegenerative disorder, progressive loss of motor neurons. Little is known about the gastrointestinal (GI) changes in ALS patients before and after the diagnosis, although autonomic dysfunction is reported in ALS. On one hand, in the literature, we see the comments that ALS patients do not complain of gastrointestinal symptoms and there is no GI symptom in ALS. On the other hand, there are cases that celiac disease with neurologic manifestations was misdiagnosed as ALS (Turner et al., 2007; Brown et al., 2010; Ham et al., 2017). There is report that gastrointestinal motor dysfunction occurred in ALS with delayed gastric emptying and delayed colonic transit times in patients (Wingate, 1999). Pancreatic and parotid deficiencies were observed in ALS patients (Charchaflie et al., 1974). There is a possible link between ALS and sensitivity to gluten based on an Israel study (Gadoth et al., 2015). In certain cases, an ALS syndrome might be associated with autoimmunity and gluten sensitivity. These reports suggest that ALS patients show GI symptoms, maybe at the early stage of their disease or before their diagnoses.

Growing evidence indicates that gut microbiome may actively contribute to ALS pathogenesis. 'Microbiome' includes all the microorganisms living (e.g., bacteria, virus, fungi) in the body. The definition of microbiome proposed by Whipps et al. is a broad one which describes microbiome as a "characteristic microbial community" in a "reasonably well-defined habitat which has distinct physio-chemical properties" as their "theatre of activity" (Whipps et al., 1988). Microbes inhabit the human body mediating key metabolic, physiological, and immune functions (Baquero and Nombela, 2012) and can be considered as another human organ. The last decade has witnessed a rebirth in interest in the microbes because of their novel and potential roles in disease pathogenesis and treatment. We are the first to report the elevated intestinal inflammation, reduced beneficial bacteria, and shift of microbiome profile in ALS (Wu et al., 2015; Rowin et al., 2017; Zhang et al., 2017; Zhang et al., 2021). Later, studies in human ALS and experimental animal models also reported the altered microbiome in the ALS (Fang, 2016; Labarre et al., 2017; Blacher et al., 2019; Figueroa-Romero et al., 2019; Burberry et al., 2020). Antibiotic usage, especially repeated use of antibiotics, are reported to be associated with a higher subsequent risk of ALS (Sun et al., 2019). The novel impact of gut microbiota on ALS development could explain some of the disease features beyond the influence of the genetic factor. However, microbiome studies in ALS are still very limited.

In the current review, we summarize the altered GI functions and roles of the gut microbiota in ALS, based on human data and experimental models. We discuss possible therapeutic approaches by targeting intestinal function and microbiome. Our goal is to evaluate existing evidence and identify gaps in the knowledge for future directions in ALS and other neurodegenerative diseases.

GASTROINTESTINAL (GI) DISORDERS IN HUMAN ALS

Pancreatic function was studied in ALS patients. In a 1967 study (Quick and Greer, 1967) that examined the pancreatic function of 5 ALS patients and 5 patients with non-ALS neurological diseases, they found that all 5 ALS patients displayed elevations in leucine aminopeptidase. 4 of the 5 ALS patients had abnormal results to tolbutamide testing. In the 4 ALS patients that underwent secretin testing, there were abnormalities in the response the secretin. The results of this study suggested that there is a disturbance of the function of the exocrine gland in patients with ALS, a finding that was consistent across patients. This is believed to be the first study that observed changes with this gland, as previous studies had only observed alterations of the endocrine gland. This study raised the question if there is a direct relationship between ALS and the pancreas or if alterations of the pancreas are due to secondary complications.

Utterback et al. (1970) analyzed the pancreatic function of 10 ALS patients and 1 patient with chronic pancreatitis. They found that the patients experienced a variety of intestinal symptoms. One patient experienced small bowel abnormalities which consisted of segmentation and small bowel coarsening. Another patient underwent rapid transit time of the small bowel. A third patient experienced small sliding hernia while another had a potential duodenal ulcer. Yet another patient had varices and a traction diverticulum of the esophagus. The authors determined that the only system with abnormalities was endocrine function. They concluded that abnormalities with glucose function resulted in malnutrition, diminished physical activity, and decreased muscle mass. In another study looking at the pancreatic function of ALS patients, Charchaflie et al. (1974), found that ALS patients experienced an insufficient pancreatic exocrine response. Additionally, there was no pathological structural changes observed, specifically organic lesions. The ALS patients experienced a marked decrease in amylase concentration. Post stimulation with citric acid, the parotid secretory response was significantly lower in the ALS patients, compared to the control group (Charchaflie et al., 1974). Additionally, the bicarbonate concentration in the saliva was significantly lower in the ALS patients. There was no difference in plasma osmolarity between ALS patients and normal controls. These results suggest a functional impairment of the exocrine gland. The pancreatic deficiencies were potentially a result of modifications of neuroendocrine mechanisms. They also raised the question whether ALS pathogenesis could be a result of digestive gland functional insufficiencies. However, there is a report on no abnormal pancreatic, but abnormal in glucose utilization in ALS. The authors believed host factors (e.g., age,

malnutrition, diminished physical activity, and decreased muscle mass) may explain the observed abnormal glucose utilization.

Wingate (1999) reported GI motor dysfunction with delayed gastric emptying and colonic transit times in patients with ALS (Wingate, 1999). Nubling et al. investigated disease severity, intestinal dysfunction, and lower urinary tract symptoms (LUTS) in 43 patients with ALS (Nubling et al., 2014). Urinary incontinence was increased in patients with ALS aged ≥ 60 years compared to the EPIC cohort. Intake of muscle relaxants and anticholinergics was associated with both urinary incontinence and severity of symptoms (Nubling et al., 2014). Furthermore, a high prevalence of constipation (46%), but stool incontinence was only reported in 9% of the group. Overall, the increased prevalence of urge incontinence and high GI symptom burden imply in patients with ALS. There are cases that celiac disease with neurologic manifestations was misdiagnosed as ALS (Turner et al., 2007; Brown et al., 2010; Ham et al., 2017). An ALS syndrome may be associated with autoimmunity and gluten sensitivity (Group, 2016).

In 1987, Nakano et al., found an increase in the size of pale hepatocytes, mild fatty infiltration, and variability in the nuclear size of these hepatocytes in ALS patients (Nakano et al., 1987). Additionally, fibrosis around the hepatocytes was present, as well as abnormalities of the hepatocellular mitochondria. This was one of the first studies to observe this fibrosis and was found in 76.2% of ALS patients versus 42.3% of controls. However, there was no relationship between these abnormal findings in the livers of ALS patients with the progression of the disease. This study suggest that abnormalities of the liver potentially occur in ALS and that the presence of these abnormalities would not be secondary to disease symptoms.

Jonsson et al., 2008 looked at the liver pathology from a single ALS patient in a case report. They found granular mutant SOD1 inclusions in the hepatocytes and kidney tubular epithelium and the absolute levels of G127X SOD1 were greater in the liver than in the temporal lobe. Staining of the hepatocytes was more widespread and uneven. The liver had inactive micronodular cirrhosis with some steatosis. Blood plasma levels indicated a moderate form of liver disease. From these results, the authors asked the question whether G127X SOD1 could contribute to these alterations to the liver.

The enteric nervous system (ENS) provides neural control in the gastrointestinal tract. When analyzing human ALS patient tissue, Luesma et al., 2014 (Luesma et al., 2014) found that there was evidence of the presence of adult enteric neurons stained with c-Ret (a RET kinase inhibitor) in the myenteric and submucosal ganglia of the adult duodenum. It suggested that c-Ret was necessary for the human mature ENS. Further studies would be needed to look at this connection with neurodegenerative diseases such as ALS.

Our study (Rowin et al., 2017) evaluated infection and markers of intestinal inflammation and the human gut microbiome in stool samples from ALS patients. A majority of patients had signs of intestinal inflammation. This is the first comprehensive examination of inflammatory markers in the stool of ALS patients. A previous study (Zhang et al., 2009) reported that the level of plasma lipopolysaccharides (LPS), a bacterial endotoxin, significant increased and had a positive correlation with activation of blood monocyte/macrophage in sALS groups, and LPS was most elevated in patients with advanced sALS disease (Zhang et al., 2009). Circulating endotoxin and systemic immune activation in sALS suggested intestinal leakage and local inflammation in these sALS patients.

Clearly, there are GI disorders in patients with ALS. We summarized the current literature in **Table 1**. These reports suggest that ALS patients show GI symptoms, maybe at the early stage of their disease or before their diagnoses. However, GI symptoms for pre-diagnosis of ALS are largely ignored. It is challenging to perform systemic studies before the diagnoses.

GUT PATHOPHYSIOLOGY IN ALS MOUSE MODELS

Transgenic animals are expected to imitate the key features of ALS. There are different ALS experimental models, with their advantages and disadvantages (Philips and Rothstein, 2015). In patients with ALS, there are spinal cord and muscle pathology. About 10% of familial ALS cases are attributed to mutations in Cu, Zn superoxide dismutase (SOD). Normal SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. The ALS pathology can be modeled in $hSOD1^{G93A}$ mice, which carry the human point mutant $SOD1^{G93A}$.

Similar to patients with ALS, the $hSOD1^{G93A}$ mice showed liver problems, e.g., hepatic abnormalities and lymphocytic infiltration (Lee and Yang, 2018). Lee and Yang (2018) investigated the liver of $hSOD1^{G93A}$ mice transgenic mice, changes in liver protein expression over the course of disease progression were found. This included a significant increase in inflammatory cytokines (TNF-α, IL-1β, and CD11b), oxidative stress-related proteins (NQ01 and ferritin), and regulators of cell death (Bax and pJNK). For pre-symptomatic mice $hSOD1^{G93A}$ mice, there was a significant increase in fibrosis-related proteins (HDAC4, GADD45α, and PDGFb) in the liver. These findings suggested that liver dysfunction may lead to liver fibrosis in $hSOD1^{G93A}$ mice. Additionally, liver dysfunction in $hSOD1^{G93A}$ mice could potentially be mediated by increased inflammation and oxidative stress, in addition to the upregulation of fibrosis-related proteins.

We found the damaged tight junctions (TJs) and increased intestinal permeability in the $hSOD1^{G93A}$ mice (Wu et al., 2015). TJs act as barriers in the intestinal epithelial cells to protect host from pathogens, inflammation, and other injuries (Zhang et al., 2013; Zhang et al., 2019). We studied $hSOD1^{G93A}$ and age matched wild type mice and found that TJ ZO-1 protein expression was significantly decreased in the intestine of G93A, compared to WT mice at 2-month-old. The G93A mice do not show ALS symptoms until 3 months old. The adherent junction protein E-cadherin was significantly decreased in the intestines of G93A mice, compared to the age-matched WT mice. ZO-1 was a tight junction protein restricted to cellular borders in a

TABLE 1 | Involved organs of GI tract in patients with ALS.

Organs	Symptoms	Stage of disease	Sample size	References
Pancreas and parotid glands	Parotid flow rate and bicarbonate concentration from ALS patients were found to be decreased by about 66% and 70% respectively, when compared with controls.		17 patients	https://pubmed.ncbi.nlm.nih.gov/4852110/
Pancreas		Symptomatic	5 patients with ALS and 5 additional patients with neurologic disease other than ALS	https://pubmed.ncbi.nlm.nih.gov/6066871/
Pancreas/ Small Intestine	There were varices and a traction diverticulum of esophagus in one patient, a small sliding hernia in another, and a possible duodenal ulcer. There was some segmentation and coarsening of the small bowel in one patient and a rapid transit time in another.	Symptomatic	10 patients with ALS and 1 patient with chronic pancreatitis	https://pubmed.ncbi.nlm.nih.gov/5505684/
ENS	c-Ret immunoreactivity in myenteric and submucosal ganglia, GFAP-ir labeled cells appeared within myenteric ganglia while c-Kit labelled cells appeared around myenteric ganglia. Propose the ENS marker for the ALS patients		12 human sample No direct data from ALS patients	https://pubmed.ncbi.nlm.nih.gov/24868525/
Intestine	Intestinal inflammatory markers in stool samples	Symptomatic	5 patients with ALS	(Rowin et al., 2017) https://pubmed.ncbi.nlm.nih.gov/28947596/
GI motor dysfunction	delayed gastric emptying and colonic transit times	Symptomatic		(Wingate, 1999)
GI motor dysfunction/ urinary tract	investigated disease severity, lower urinary tract symptoms (LUTS) and intestinal dysfunction.	Symptomatic	43 patients with ALS	(Nubling et al., 2014)
Liver	Granular G127X SOD1 inclusions were seen in hepatocytes in the liver, and the staining was more widespread. Granular G127X SOD1 inclusions were also found in kidney tubular epithelium. There was presence of inactive micronodular cirrhosis with some steatosis of the liver. There were some elevated liver disease markers.	Postmortem	1 patient suffered from rapidly progressing ALS and died of respiratory insufficiency and 5 age-matched patients who died of non-neurological diseases	https://pubmed.ncbi.nlm.nih.gov/18409196/
Liver	Increased size of pale hepatocytes, mild fatty infiltration	Symptomatic	21 patients with ALS and control specimens randomly chosen from 215 patients with various liver diseases	https://pubmed.ncbi.nlm.nih.gov/3800708/
Systemic	Increased LPS, Inflammation	Symptomatic	sALS	(Zhang et al., 2009)

smooth and organized pattern at the apical side of the healthy colons. We identified abnormal and disrupted ZO-1 distribution in the membrane of intestinal epithelial cells in ALS mice at the age of 2 months. We also showed a significant increase in abnormal Paneth cells and increased inflammatory cytokine IL-17 in hSOD1^{G93A} mice (Wu et al., 2015). As the changes in intestine function occurs early, it suggests the possibility that altered intestinal homeostasis may take place before the decline of neuromuscular function in ALS mice. This study demonstrated that impaired gut-neuromuscular crosstalk could potentially contribute to the progression of ALS.

Intestinal mobility is a key physiologic parameter governing digestion and absorption of nutrients affected by the ENS, microbiome, and host genetics (Rao and Gershon, 2016; Niesler et al., 2021). Few studies in general have been conducted looking at the connection between the ENS in the SOD1G39A ALS model. Our recent study (Zhang et al., 2021) reported the dysfunction of ENS in SOD1^{G39A}. Before ALS onset, 2-month-old G93A mice had significant lower intestinal motility, decreased grip strength, and reduced time in the rotarod. We observed increased GFAP, a marker for ENS, and decreased SMMHC expression, a marker for smooth muscle (Zhang et al., 2021). These ENS changes are significantly correlated with increased aggregation of hSOD1^{G93A} in the spinal cord, small intestine, and colon (Zhang et al., 2021).

In a 2011 study by Finkelstein et al, the authors discovered that the liver parenchyma underwent significant atrophy and lipid redistribution, while an increase in natural killer T cells in both the lymphoid organs and spinal cord of the mSOD1 mice compared to WT mice was also observed. While the liver itself decreased in size as the disease progressed, the proportions of natural killer T cells in the liver increased by 4-fold at the end-stage of the disease. In mSOD1 mice with the C57B1 background, the natural killer T cells had a similar fate. T cells were significantly enhanced in the spinal cord and spleen of mSOD1 mice. Additionally, natural killer cells were significantly enhanced in the spinal cord of mSOD1 mice, but not in the spleen and liver. This study showed that changes to liver are present during disease progression in transgenic mice and therapeutic interventions that target these changes are needed.

Some animal models may not always exhibit similar co-morbidity phenotypes to human patients with ALS. The transgenic mice overexpressing a mutant human TDP43 gene (Hatzipetros et al., 2014), the Prp-TDP43^{A315T} mice, exhibited decreased survival rates that may be associated with neurodegeneration, occurring in the myenteric plexus of the colon. In these Prp-TDP43A315T mice,

death coincided with severe GI pathology. In the lower GI tract, distal to the cecum, there were pathological changes, such as swelling, intraintestinal coagulated blood, and necrotic tissue. It is most likely a result of overexpression of the transgene in the lower GI tract, as opposed to the upper GI where there is no expression of the transgene and no neurodegeneration. The neurodegeneration of the lower GI tract is a feature that is unique to the Prp-TDP43^{A315T} mice. Similar findings have been reported by others (Guo et al., 2012). TDP43 ^{A315T} mice exhibited not only a reduction in food ingestion and defecation, but also a thinned colon and swollen small intestine. The swollen small intestine and thinned large bowel also indicate peristaltic malfunction in the TDP43 ^{A315T} mice. In the colon, the accumulated TDP43 ^{A315T} in the myenteric nerve plexus and myenteric neuron degeneration could result in phenotypes similar to hypoganglionosis. These results, in addition to no limb paralysis present at the end stage of disease progression, suggested that intestinal dysfunction contributed more to their death than severe muscle weakness in the TDP43 ^{A315T} mice. The authors believed that while the TDP43 ^{A315T} model can be used to analyze GI tract degeneration, it did not display a phenotype that was acceptable to study ALS therapeutics.

Herdewyn et al. (2014) found a significant reduction in the ability of the transgenic TDP43 ^{A315T} mice to generate propulsive contractions. There was degeneration of nitric oxide synthase (NOS) (Fleck et al., 2021) neurons in the enteric nervous system, but no difference in choligeneric neurons. Because NOS and excitatory neurons both regulate intestinal peristalsis, lack of both excitation and inhibition would result in a failure to generate contractile force and the prevention of peristalsis. The degenerated NOS neurons suggested the loss of inhibitory control that led to abnormal intestinal propulsion, dysmotility and pseudo-obstruction, and sudden death (Herdewyn et al., 2014). There was downregulation of endogenous TDP-43 in the spinal cord and brain of the TDP43 ^{A315T} mice prior to the observation of neurodegeneration. The feeding of jellified food prevented intestinal obstruction, allowing the motor phenotype to develop, and significantly extending survival. These results suggested that the degeneration of NOS neurons in the myenteric plexus resulted in intestinal dysmotility in the TDP43 ^{A315T} mice. In yet another study using the TDP43 ^{A315T} model (Esmaeili et al., 2013), the authors found intestinal dilation with the caecum and lower ileum mildly to moderately dilated and contained dry, firm fecal material. One animal had a fibrous band at the ileocecocolic junction. The stomachs of the animals were all empty. The authors concluded that the animals were dying due to a reduction in motility that originates in the ileocaecal area of the GI tract. A limitation of this study is a general limit of the TDP43 ^{A315T} animal model itself –lacking lower motor neuron degeneration in the ventral horn of the spinal cord. Toxicity from TDP43 overexpression may lead to a reduction in GI motility, though it is still not fully understood how TDP-43 overexpression leads to GI tract abnormalities in the TDP43 ^{A315T} mice. These studies suggest that gastrointestinal complications may have been the cause of death for TDP43 ^{A315T} mice and not spinal motor neuron loss.

Studies in the animal models have shown the GI petrophysical changes before the motor neuron degeneration was observed. We summarized these findings in **Table 2**. Although the transgenic animal models cannot sufficiently represent the development of human ALS, especially the sALS, studies in these models still provide valuable data and mechanisms during the disease progression.

ALTERED GUT MICROBIOME IN ALS MOUSE MODELS

There are known active host-microbiome interactions in the GI tract. Our 2015 study has demonstrated the altered bacterial abundance, reduced levels of butyrate-producing bacteria in the ALS SOD1^{G93A} animals (Wu et al., 2015). The intestinal microbiome was altered as butyrate producing bacteria (e.g., *Butyrivibrio Fibrisolvens*) were reduced in ALS mice at the age of 2 months before the onset of disease. The cellular and physiological changes mirror the population and function changes in the gut microbiome of the SOD1^{G93A} mice. With an interventional study attempting to alleviate symptoms through treatment with sodium butyrate (Zhang et al., 2017). SOD1G93A mice chronically treated with sodium butyrate fared better than those not given the compound, showing improved intestinal barrier function and delays in both weight loss and death of 50 and 38 days respectively. Further longitudinal studies (Zhang et al., 2021) demonstrated the early stage microbial changes in the mice at 1-month old. The altered bacterial community is related to autoimmunity *(e.g., Clostridium* sp. *ASF502, Lachnospiraceae bacterium A4)*, inflammation (e.g., *Enterohabdus Muris*), and metabolism (e.g., *Desulfovibrio fairfieldensis*) at 1- and 2-month-old SOD1^{G93A} mice, suggesting the early microbial contribution to the ALS pathological changes (Zhang et al., 2021).

A 2019 study (Blacher et al., 2019) confirmed the significant differences in the fecal microbiome composition of the SOD1^{G93A} mice, compared with wild-type littermate controls. In the SOD1-Tg mice (generally known as SOD1^{G93A} mice), antibiotic treatment was associated with a significant exacerbation of motor abnormalities in the mice throughout the progression of the disease. Motor neuron cell death increased after chronic exposure to antibiotics. In antibiotic treated SOD1^{G93A} mice, the addition of *P. distastonis* and *R. torques* exacerbated disease progression. Treatment with *L. gasseri* and *P. melaninogenica* displayed disease promoting effects in a some but not all behavioral tests. None of the 11 bacterial strains that were tested affected motor abilities in the wild type mice. There was a slow reduction in the levels of *A. muciniphila* in SOD1^{G93A} mice with progression of the disease, while its levels remained constant across time in the wild-type mice. *A. muciniphila* treatment significantly and substantially prolonged the lifespan of SOD1^{G93A} mice, compared to vehicle-treated mice or SOD1^{G93A} mice treated with other commensal microbiome species. *A. muciniphila* favorably modulated the course and severity of the disease in mice, whereas *P. distastonis*, *R. torques*, and possibly *L. gasseri*, *P. melaninogenica* adversely modulated the course and severity of ALS in mice. Overall, these results confirm the involvement of the gut microbiome in SOD1^{G93A} mice.

TABLE 2 | Involved organs of GI tract in ALS animal models.

Organs	Symptoms	Stage of disease	Sample size	References
Colon	There was swelling, intraintestinal coagulated blood and necrotic tissue in the lower GI tract, distal to the cecum, versus no pathology in age-matched wild-type mice. The slowing down of gut motility was significantly different in TDP43 mice compared to WT mice.	Presymptomatic and symptomatic	Over 650 transgenic C57BL/6J Prp-TDP43A315T male and female mice	https://pubmed.ncbi.nlm.nih.gov/24141148/
Colon	Transgenic mice experienced gradually reduced food ingestion and defecation compared to WT controls. They also found thinned colon and swollen small intestine in the TDP-43 A315T mice.	Presymptomatic and symptomatic	12 transgenic mice with the TDP43A315T mutation and 12 non-transgenic litter mates	https://pubmed.ncbi.nlm.nih.gov/22578468/
Intestine	Intestinal dilation, mildly dilated cecum and lower ileum, contained dry firm fecal material, jejunum and duodenum contained watery material, stomachs were empty, compared to age-matched wild-type controls.	Prior to disease development	15 male, 15 female, TDP43A315T transgenic mice 15 non-transgenic control	https://pubmed.ncbi.nlm.nih.gov/23317354/
ENS/Colon/ Small Intestine/ Cecum	Degeneration of NOS neurons in the ENS and a high transgene expression of human mutant TDP-43 in the nuclei of neurons of the myenteric plexus. Mice had an extremely rigid abdomen (intestinal obstruction), a thinned colon, enlarged cecum and distension of the small intestines. For the degeneration of NOS neurons, the number of neurons was decreased and the neurons that remained were enlarged. Nitric oxide synthase expressing neurons were severely affected in terminal ileum and colon.	Presymptomatic and symptomatic	Numbers vary for each comparison. TDP43A315T) mice and NTG C57BL/6 J mice	https://pubmed.ncbi.nlm.nih.gov/24938805/
Colon/ Small Intestine	LPS, Inflammatory cytokines, leaky gut	Presymptomatic and symptomatic	Wild-type and hSOD1 ^{G93A} mice, from 3-15 mice per comparison	(Wu et al., 2015)
Colon/ Small Intestine	Butyrate treatment to reduce the GI symptoms	Presymptomatic and symptomatic	Wild-type and hSOD1 ^{G93A} mice, from 3-15 mice per comparison	(Zhang et al., 2017)
ENS/Colon/ Small Intestine	Slower bowel movement, abnormal ENS	Presymptomatic and symptomatic	Wild-type and mSOD1 ^{G93A} mice, from 6-20 mice per comparison	(Zhang et al., 2021)
Metabolites/ Inflammation/ inter-organ communications	Changes in carbohydrate levels, amino acid metabolism, and formation of gamma-glutamyl amino acids in the ALS mice. Shifts in several microbially-contributed catabolites of aromatic amino acids agree with butyrate-induced changes in composition of gut microbiome. Butyrate treatment significantly suppressed the IBA1 level in the microglia of SOD1^{G93A} mice. The serum IL-17 and LPS were significantly reduced in the butyrate treated SOD1^{G93A} mice.	Presymptomatic and symptomatic	Wild-type and mSOD1 ^{G93A} mice with or without butyrate treatment, untargeted and target metabolomic studies	https://biorxiv.org/cgi/content/short/2022.01.15.476456v1
Liver	The liver had the most prominent increase in the proportion of NKT cells compared to WT mice. The liver decreased in size as the disease progressed. T cell abundance was significantly lover in mSOD1 relative to WT. There was also atrophy of hepatocytes and lipid aggregation in the liver. Liver parenchyma also had significant atrophy.	Pre-symptomatic and symptomatic	Wild-type and mSOD1 ^{G93A} mice, anywhere from 3-14 mice per comparison	https://pubmed.ncbi.nlm.nih.gov/21829620/
Liver	Increase in inflammatory expression (TNF-alpha and IL-1B), increase in oxidative stress-related protein (NQO1 and ferritin), fibrosis-related proteins are upregulated (HDAC4, GADD45α and PDGFβ)	Presymptomatic and symptomatic	non-transgenic, pre-symptomatic (2-month-old) transgenic mice, symptomatic (4-month-old) transgenic mice	https://pubmed.ncbi.nlm.nih.gov/30130789/

Another study detected differences in the gut microbiome between SOD1^{G93A} mice and controls at multiple time points (Figueroa-Romero et al., 2019). Interestingly, they noted that the ALS phenotype in the mice, including survival, varied depending on the animal facility used to house the mice; the authors suggested this curious observation was a result of differences in gut microbiota (Figueroa-Romero et al., 2019).

Intestine acts as a barrier to harmful molecules. A range of dietary toxins has been implicated in the cause of ALS, and dysbiosis could facilitate their entry because dysbiosis leads to increased intestinal permeability, as we observed in the previous report (Wu et al., 2015).

The ALS C9orf72 mouse model harbors loss of function mutations in the orthologous gene C9orf72 (Philips and Rothstein, 2015). A 2020 study (Burberry et al., 2020) found that the murine norovirus, *Helicobacter* spp., *Pasteurella pneumotropica* and *Tritrichomonas muris* were significantly more abundant in *C9orf72*^{Harvard} mice than in C9orf72^{Broad} mice. The increase in *Helicobacter* spp. levels suggested that gut microbiota changes may be the underlying mechanism of increased rate of mortality and inflammatory phenotypes in *C9orf72*^{Harvard} mice. Treatment with antibiotics significantly decreased both the abundance and diversity of bacterial species without affecting the levels of murine norovirus, compared to vehicle treated controls. The bacterial species that decreased included *Helicobacter* spp. Administering lifetime antibiotics to the C9orf72^{Harvard} -/- resulted in the complete suppression of inflammatory or autoimmune phenotypes, suggesting that when C9orf72 function is decreased, gut bacteria signals and promote autoimmunity and inflammation. Acute administration of broad-spectrum antibiotics reduced inflammatory and autoimmune phenotypes and improved rotarod performance in the mutant mice.

We summarized the existing studies on gut microbiome in ALS models in **Table 3**. Different ALS experimental models show altered microbiome, and the pathophysiological changes could by modulated by bacterial metabolites or antibiotic treatment. These results suggested that gut microbiota and environment modify immunity, neuroinflammation, and motor deficits in the development of ALS.

HUMAN STUDIES OF THE GUT MICROBIOME IN ALS

When examining 6 ALS patients and 5 healthy people without ALS, a 2016 study (Fang et al., 2016) found microbiomes of ALS showed a significant difference in the global bacterial gene content compared to healthy controls. Phylum *Bacteroidetes*,

TABLE 3 | Microbiome studies in ALS experimental models.

Study	Key Bacterial changes	Sample size	Methods	Reference
Leaky intestine and impaired microbiome in SOD1 ^{G93A}	Reduced butyrate-producing bacteria (*Butyrivibrio Fibriosolvens*), *E. coli*, and *Fermicus* in ALS mice at the age of 2 months	SOD1 ^{G93A} mice and age-matched wild-type mice	Western blot analysis, immunofluorescence, ELISA, real-time qPCR, fetal microbiome sequencing	https://pubmed.ncbi.nlm.nih.gov/25847918/
Target Intestinal microbiota to alleviate disease progression in ALS	Butyrate treatment significantly increased *Butyrivibrio* species, also significantly increased *Bacteroide*, *Odoribacter*, *Eubacterium*, significantly depleted *Tannerella*	SOD1 ^{G93A} and age-matched wild-type mice	Western blot analysis, immunofluorescence, cell transfection and live cell imaging, real-time qPCR	https://pubmed.ncbi.nlm.nih.gov/28129947/
Effects of Intraoperative Vagal Nerve Stimulation (VNS) on the microbiome in SOD1 ^{G93A} mice	In all mice, the most abundant bacteria were in the *Bacteroidales* group and the bacterial families of *Rikenellaceae* and *Lachnospiraceae.* VNS did not change the microbiome in the SOD1 ^{G39A} mice.	30 SOD1^{G93A} mice and 30 age-matched WT controls	Vagal nerve surgical stimulation, fecal collection, DNA extraction, rRNA sequencing	https://pubmed.ncbi.nlm.nih.gov/30424824/
Microbiome, immune system and epigenome with disease progression in SOD1 ^{G93A} mice.	In the ileum, *Firmicutes* were more abundant than *Bacteroidetes* in SOD1^{G93A} mice versus WT mice. At 90 days, *Firmicutes* were less abundant than *Bacteroidetes* in SOD1 ^{G93A} mice versus WT mice. In feces, *Firmicutes* were more abundant in SOD1^{G93A} mice aged 37 and 60 days compared to WT mice. *Bacteroidetes* were more abundant in colon from the SOD1^{G93A} 60-day-old mice.	8 30-day-old SOD1^{G93A} mice and WT control littermates for each comparison	Motor function assessed using rotarod, collected fecal and intestinal content samples, isolated bacterial DNA for sequencing, blood, brain, spleen, and spinal cord leukocytes collected, bone marrow collected, flow cytometry	(Figueroa-Romero et al., 2019) https://pubmed.ncbi.nlm.nih.gov/31597644/
C9orf72 suppresses systemic and neural inflammation induced by bacteria	*Helicobacter*, *Pasteurella pneumotropica*, *Tritrichomonas muris* were significantly more common in C9orf72(Harvard) mice than in C9orf72(Broad) mice. *Helicobacter* were found in both pro-inflammatory environments but were not found in pro-survival environments.	Range between experiments from 10 to 114; C9orf72$^{+/+}$, C9orf72$^{+/-}$, C9orf72$^{-/-}$ mice	Motor behavior assessed using rotarod, fecal pellets and intestinal contents collected, immunofluorescence, flow cytometry, PCR	https://pubmed.ncbi.nlm.nih.gov/32483373/
Longitudinal studies of ENS, SOD1^{G93A} aggregation, and microbiome modulation by butyrate or antibiotics	Butyrate or antibiotic treatment resulted in a significantly longer latency to fall in the rotarod test, reduced SOD1^{G93A} aggregation, and enhanced enteric neuromuscular function. Feces from 2-month-old SOD1^{G93A} mice significantly enhanced SOD1^{G93A} aggregation in human colonoids transfected with a SOD1^{G93A}-GFP plasmid. Longitudinal studies of microbiome data showed the altered bacterial community related to autoimmunity *(e.g., Clostridium* sp. *ASF502, Lachnospiraceae bacterium A4)*, inflammation (e.g., *Enterohabdus Muris*), and metabolism (e.g., *Desulfovibrio fairfieldensis*) at 1- and 2-month-old SOD1^{G93A} mice, suggesting the early microbial contribution to the pathological changes.	Presymptomatic and symptomatic	Wild-type and mSOD1 ^{G93A} mice with butyrate or antibiotic treatment, from 6-20 mice per comparison Human colonoids transfected with a SOD1^{G93A}-GFP plasmid	(Zhang et al., 2021)
Gut microbiome and metabolites in modulating ALS in mice	Addition of *E.coli* nadA-/- decreased hanging-wire grip test performance and worsened neurological score in SOD1 mice compared to WT mice. Adding *P. distasonis* and *R. torques* exacerbated progression of the disease. *A. muciniphila* improves motor neuron degeneration and increases lifespan in SOD1 mice. Mice given *A. muciniphila* accumulate nicotinamide in the central nervous system. Nicotinamide alone had a limited protective role to slow the disease progression. Facility-dependent changes of microbiome were found, suggesting that genetic susceptibility to ALS and a locally prevalent microbial signature drive early dysbiosis.	For mice, varies with experiment. Pooled results = ~10-30 mice	For mice, depleting the microbiome and quantifying motor abilities, DNA sequencing, mono-inoculating 11 strains of bacteria into mice, untargeted metabolomic profiling.	https://pubmed.ncbi.nlm.nih.gov/31330533/

class *Bacteroidia*), order *Bacteriodales*, and genus *Dorea* were significantly higher in ALS patients than in the healthy controls. *Lachnospiraceae* (at family level), *Firmicutes* (at phylum level) and *Clostridia* (at class level), *Oscillibacter* (at genus level), *Family XII* (at family level), *Anaerostipes* (at genus level), *Lachnospiraceae* (at genus level), and *Clostridiales* (at order level) were significantly higher in the healthy controls than in the ALS patients. In the healthy controls, the 3 dominant phyla in 80% of the fecal microbiomes was *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. There was a significant decrease of *Oscillibacter*, *Anaerostipes*, and *Lachnospiraceae* in ALS patients.

The gastrointestinal complaints of the ALS patients predated neurological symptoms. In 2017 (Rowin et al., 2017), we studied the medical tests of 5 patients with ALS. The GI symptoms included gastroesophageal reflux, disease, chronic constipation, intermittent diarrhea, and abdominal pain and bloating. All ALS patients showed dysbiosis indicated by a decreased diversity of the microbiome compared to the healthy individuals. Patients 1, 3, and 5 had reduced benefit bacteria at the phylum level. Three patients (#1, 3 and 5) had a low Firmicutes/Bacteroidetes (F/B) ratio. Low *Ruminococcus* spp. occurred in all three ALS patients with a low F/B ratio, while *Clostridium* spp. and *Roseburia* spp. were low in patient 1. Patient 5 had high *Bacteroides- Prevotella*, *Odoribacter* spp., *Barnesiella* spp. and *Bacteroides vulgatus*. Patient 3 had high *Bacteroides vulgatus*, which could be the cause of a low F/B ratio. Patient 2 did not have a low F/B ratio, but the patient was taking probiotic supplements at the time of stool sample collection. All the ALS patients had a change to their gut microbiome that was characterized by a low diversity of the microbiome compared to the healthy individuals who had intact abundance within their gut microbiome. Our results suggested a potential role of intestinal inflammation and microbiome in the development and/or progression of human ALS.

In an observational study of 37 human ALS patients versus 29 healthy BMI- and aged- matched family members as controls (Blacher et al., 2019), the microbiome composition of ALS patients was significantly different from those of the healthy controls. There was only a small significant difference in the abundance of certain bacterial species in ALS patients. The microbiomes of the ALS patients showed a significant difference in the global bacterial gene content (Blacher et al., 2019). Additionally, there was a significant reduction in the abundance of several genes that map to the *Akkermansia muciniphila* genome.

A 2017 study (Brenner et al., 2018) analyzed the microbiome of 25 human patients with ALS and 32 healthy age- and gender-matched controls. They primarily found different proportions of uncultured *Ruminococcaceae*, However, aside from this finding and a difference in the overall number of microbial species, there was no significant differences in the quantity, diversity, and relative abundance of the fecal microbiota of the ALS patients. The range of different types of species of the intestinal microbiota did not differ between the ALS patients and the healthy controls. The F/B did not differ significantly between the ALS patients and healthy controls. The authors raised the possibility that while the gut microbiota of ALS patients experienced a change in metabolite production, ALS may not be associated with a significantly altered gut microbiota composition. This conclusion may be because the authors used a strict selection of ALS patient that had a high revised ALS functional rating scale without bulbar or respiratory symptoms, in order to avoid confounding factors. They analyzed a larger group of patients that were clinically well characterized. However, when ALS patients are at late stages of the disease, they may suffer from a variety of conditions that can affect the composition of the gut microbiota, influencing results. Revisiting this study, we actually know that F/B at the phylum level is a not realizable readout to indicate the functional changes of microbiome and the changes of microbiome should be investigated in-depth. The different proportion of Ruminococcaceae was an indicator of altered microbiome in these ALS patients. The authors only selected patients that were at a high functional level and did not have any overt bulbar or respiratory symptoms, therefore producing small and possibly inconsequential significant results. To generate more meaningful correlation, studies that involve all stages of the disease would need to be conducted. A secondary limitation is that altered metabolite production may be present in ALS patients, further disrupting the gut microbiota.

A 2018 study (Mazzini et al., 2018) analyzed the microbiota in 50 ALS patients and 50 healthy controls that were matched for sex, age, and origin. They found that there was a clear division between the bacterial profiles of ALS patients and the healthy controls. There was a lower DNA concentration in ALS patients compared to the controls. There was a low abundance of *Clostridium* and yeast, and there was a higher abundance of *E. coli* and *Enterobacteria*. The results of this study confirmed the ongoing hypothesis that the gut microbiota is altered in ALS patients and could potentially play a role in the pathogenesis of ALS.

In a 2020 study (Zeng et al., 2020) that examined the gut microbiota associated with ALS in 20 human patients and 20 age-matched healthy controls, there was a significant increase in the relative abundance of Bacteroidetes at the phylum level, *Kineothrix*, *Parabacteroides*, *Odoribacter*, *Sporobacter*, *Eisenbergiella*, *Mannheimia*, *Anaerotruncus*, and *unclassified Porphyromonadaceae* at the genus level and a significant reduction in *Firmicutes* at the phylum level and *Megamonas* at the genus level. Additionally, Bacteria at the domain level, Bacteroidetes at the phylum level, Bacteroidia at the class level, Bacteroidales at the order level, and Porphyromonadaceae at the family level were higher in ALS patients compared to controls. Microbes at the species level were significantly higher in ALS patients, including *Sulfuricurvum kujiense*, *Cyanothece* sp. and *Haladaptatus paucihalophilus*, while *Enterococcus columbae* was significantly decreased. These results indicated that the community diversity and species composition of the fecal microbiota is changed in patients with ALS. Limitations of this study include the fact that the authors could not determine whether the correlation between the intestinal dysbacteriosis and ALS occurred before or after the onset of ALS, so it still cannot be determined whether a causal relationship exists.

Another 2020 study (Di Gioia et al., 2020) examined 50 human patients with probable or defined sporadic and 50 age-matched case controls. The longitudinal study addressed the microbiota in patients with ALS and the impact of a probiotic supplementation on the gut microbiota and ALS progression. There was a significantly lower amount of *Clostridium* cluster I and yeasts and a higher concentration of *E. coli* in the ALS patients. A lower DNA concentration was present in the ALS patients, while the number of total bacteria was not significantly different between the ALS patients and the control group. *Enterobacteriaceae* were higher in ALS patients. The more representative phyla were *Bacteroidetes* and *Firmicutes* in both groups, which showed an abundance of 40-45%. *Cyanobacteria* phylum members were significantly higher in ALS patients versus control patients. At the genus level, *Lactobacillus*, Citrobacter, *Coprococcus*, and *Ruminococcaceae* were more abundant in the ALS patient group. *Veillonellaceae* and *Lachnospiraceae* families, *Parasutterella*, *Ruminococcus* and *Subdogranulum* were reduced in ALS group, compared to the controls. Overall, these results suggested the existence of differences in the bacterial composition between healthy controls and patients with ALS. This study demonstrated the imbalance of intestinal bacteria that may play important roles in modulating the central nervous system (CNS). Limitations of this study include the restricted group size - 20 ALS patients were not able to complete the study and 1 died. Additionally, intra-individual variability was not incorporated into the study.

In a 2021 study (Niccolai et al., 2021), they looked at the gut microbiota and its SCFA composition and the intestinal and systemic inflammatory response in ALS patients compared to their healthy controls. In 19 ALS patients and 9 healthy family caregivers that were matched for sex and were close in age, there were significant differences in cytokine levels. When looking at the cytokine levels in the serum, they found that IL-8, IL-15, MCP-1 and VEGF-A levels were significantly lower in ALS patients compared to controls. When looking at the cytokine levels in the fecal samples, they found that IL-2 was higher in ALS patients compared to controls, although the difference was not significant. Additionally, IL-21 was lower in ALS patients that had a faster progressing form of the disease, compared to patients that has medium or slow progressions. Patients with a slower progression of ALS had lower microbial diversity than other variations of progression. Similar results were displayed for ALS patients with the classical form of the disease compared to patients with various clinical phenotypes. Control samples displayed a higher Firmicutes/Bacteroides ratio than the ALS patients with the Firmicutes and Bacteroides being the most represented phyla in all samples. ALS patients that had a medium progression form of the disease displayed a higher Firmicutes/Bacteroides ratio than both ALS patients with other forms of progression and healthy controls. However, these differences did not appear to be statistically significant. At the genus level, the healthy controls displayed an enrichment of *Erysipelotrichaceae_UCG-003*, *Fusicatenibacter*, and *Subdoligranulum* compared to the ALS patients. There appeared to be an increase of Streptococcus in the samples from ALS patients with a slow progression of the disease. From this study, the difference in serum profile between the ALS patients and healthy controls suggests that there may be an intestinal inflammatory aspect to ALS. Since IL-21 was expression more in patients with a slow form of ALS compared to a fast progression, it could potentially be developed into a prognostic biomarker. The study confirmed that there is dysregulation occurring in both a systemic and intestinal manner in ALS patients. Limitations of this study include small sample sizes and the presence of various confounding factors that can include diet and secondary disease effects.

A 2021 study (Niccolai et al., 2021) found the differences in 15 bacterial species between the ALS patients and healthy controls. This included butyrate-prodiucing bacteria *Roseburia intestinalis* and *Eubacterium rectale*. The total relative abundance of several butyrate producers was significantly lower in ALS patients compared to healthy controls. *Bilophila (unclassified)*, *Clostridiaceae bacterium JC1118*, *Coprobacter fastidiosus*, *Eubacterium eligens*, and *Ruminococcus sp 5 1 39 BFAA* was lower in ALS patients compared to controls, while the relative abundance of *Escherichia (unclassified)* and *Streptococcus salivarius* was higher in ALS patients. To avoid the potential changes that occur in late term ALS, they compared the samples from PALS that were collected within one year after diagnosis, the relative abundance of both *Roseburia intestinalis* and *Eubacterium rectale* were significantly lower in the ALS patients compared to healthy controls. The levels of total butyrate-producing bacteria were lower in the PALS patients compared to healthy controls, but the difference was not statistically significant. In patients that were not using Riluzole at the time of sample collection, they found that the results were similar – Roseburia intestinalis, Eubacterium rectale, and total abundance of butyrate-producing bacteria were lower in these ALS patients compared to healthy controls. The results were the same when excluding ALS patients that were using a gastrostomy tube. Overall, they found reduced levels of anti-inflammatory SCFA-producing bacteria in patients with ALS. A limitation of this study could be that patients unintentionally changed their diet after developing ALS, therefore altering the gut microbiota composition. Dietary differences were unfortunately not able to be accounted for. This study further indicates that gut microbiome could act as an integral component in the pathophysiology of ALS.

A 2022 study (Zhang et al., 2022) investigated the connection between the gut microbiota and ALS in a GWAS of ALS that involved 20,806 patients and 59,804 control individuals. They found that *OTU10032 unclassified Enterobacteriaceae* (2 independent SNPs) and *unclassified Acidaminococcaceae* (4 uncorrelated SNPs) were associated with a higher risk of ALS. Gamma-glutamyl amino acids demonstrated possible negative effects of the risk of developing ALS. Gamma-glutamylphenylalanine specifically displayed an increased risk of developing ALS that was significant. 1-arachidonoyl-GPI and 3-methyl-2-oxobutyrate, two metabolites, were connected with an increased risk of developing ALS. An increase in the levels of 4-acetamidobutanoate may lower the risk of developing ALS. Additionally, an increase in the relative abundance of *OTU4607_Sutterella* was associated with genetically

predicted ALS. An increase in the relative abundance of *Lactobacillalesorder* was associated with genetically predicted ALS. This study showed that a bidirectional relationship between the gut microbiota and ALS. Limitations of this study include weak instrument bias and population stratification, though this bias was somewhat reduced by restricting the dataset to individuals of European ancestry.

A 2022 study (Hertzberg et al., 2022) analyzed the gut microbiota of ALS patients while using the patients' spouses as the healthy controls. The authors found that the ALS patients did not have any butanoate metabolic pathway enzymes. They also displayed lower amounts of enzymes that are utilized in various metabolic pathways. These include pathways involving response regulators and carbon metabolism pathways. When comparing the 25 stool and inflammatory markers, including IL-2, IL-4, tumor necrosis factor, and interferon, there were no statistically significant differences between the ALS patients and the healthy controls. Additionally, ALS patients did not have amplicon sequence variants of the genus *Prevotella*, specifically *P. timonensis*, compared to the healthy controls. This specific result was consistent with other studies that suggest that gut microbiome dysbiosis is associated with neurodegenerative diseases. The authors suggested that the difference in P. timonensis in ALS patients versus the controls may have contributed to the lack of differences in pro-inflammatory cytokine levels. In contrast to previous studies, they did not find a lack of butyrate-producing microbes. However, they found a decrease in the enzymes associated with butyrate metabolism, which is consistent with previous studies, suggesting an association between gut butyrate availability and progression of ALS. Further studies looking at this association would be needed. Limitations of this study include the small sample size and the lack of diet information. To overcome the small sample size, larger scaled studies would need to be conducted.

In a 2019 study (Zhai et al., 2019) looking at 8 patients with ALS and 8 healthy controls, they found that the richness and evenness of bacterial and archaeal communities of healthy individuals were healthier than those of the ALS patients. They also found that the average relative abundance of *Firmicutes* in ALS patients was 4.7% higher than the abundance in healthy controls. On the class level, the relative abundance of *Negativicutes* and *Bacili* were decreased compared to healthy controls. The relative abundance of phylum Euryarchaeota, class Methanobacteria, and genus *Methanobrevibacter* were all significantly increased in ALS patients compared to the healthy controls. While there were no significant differences in metabolites between ALS patients and healthy controls, there was an increase in SCFA, NO_2-N/NO_3N, and γ-aminobutyric acid in ALS patients compared to healthy controls. The results of this study suggested that the biodiversity and composition of intestinal microflora in ALS patients is of lower quality than that of controls. The increase in the Firmicutes/Bacteroidetes ratio and *Methanobrevibacter* indicates that there is a gut flora composition imbalance in patients with ALS. One limitation of this study was that metagenomic sequencing was not performed in order to analyze microbial function variation. Another limitation would be the need for microbial community acclimation in order to better understand the composition of the microbiota in ALS patients.

In a 2020 study (Ngo et al., 2020) that looked at the relationship of the fecal microbiota and prognosis of ALS, they found that ALS patients with a higher richness and evenness of their fecal microbiome had a decreased survival from their onset of symptoms than the patients who had less richness and evenness. There was no significant difference in *Proteobacteria* between ALS patients and controls. There were also no significant differences in the proportional abundance in the two most abundant phyla, *Firmicutes* and *Bacteriodetes*, between ALS patients and controls. Overall, they found the fecal microbiota in a group of ALS patients is not significantly different than those of healthy controls. They believe that clinical and metabolic features of ALS are not connected with the fecal microbiome composition. However, the diversity and richness of the fecal microbiome may be associated with an increased risk of an earlier death. The results suggest that the fecal microbiota of ALS patients does not differ from those of healthy individuals. One limitation of this study is that they included both patients who were newly diagnosed and patients who had already progressed and were attending clinics. Other limitations would be a small sample size and the amplifying of variability due to the patients' dietary habits.

A potential proxy for the effect of the gut microbiome on disease is the effect of oral antibiotics which significantly modify the balance of gut microbial species. A 2019 study demonstrated that any use of antibiotics, especially repeated use, was associated with increased risk of ALS in the native Swedish population (2006-2013 period) (Sun et al., 2019). Similarly, $SOD1^{G93A}$ mice repeatedly exposed to antibiotics develop a more severe motor phenotype and increased motor neuron loss (Blacher et al., 2019),

We summarized the studies on gut microbiome in ALS patients in **Table 4**. These results suggested novel roles of intestinal microbiome in the development of ALS and the potential benefits of decreasing the number of pathogens and increasing probiotics. The challenge of early studies of microbiome in ALS lies in the following aspects: 1) limited sample size; 2) preliminary data analysis; and 3) reluctance from neurologists to pay attention the intestinal changes in the early stage of ALS. In the earlier time of microbiome research, a F/B ratio was used. Although it indicated the evidence of dysbiosis in patients with ALS. The F/B ratio only reflect the relative abundance of bacteria at the phylum level and may miss the changes at the species, thus, it is not a reliable readout for the in-depth analysis and advanced studies of microbiome data.

INTESTINAL INFLAMMATION IN ALS

There is abundant evidence many substances involved in the promotion of inflammatory processes are present in the CNS of patients. Previous reviews have discussed the role of inflammation in ALS and the possibility of treating ALS by immune modulation (McCombe and Henderson, 2011; Kwon

TABLE 4 | Microbiome studies in patients with ALS.

Study	Key Bacterial changes	Sample size	Methods	Reference
Microbial diversity in ALS	*Bacteroidetes*, *bacteroidia*, *bacteroidales*, and *dorea* were significantly higher in ALS patients. A significant decrease of *Oscillibacter*, *Anaerostipes*, and *Lachnospiraceae* in ALS patients compared to controls.	Six ALS patients and five healthy people without ALS	Extracted genomic DNA and high-throughput sequencing.	https://pubmed.ncbi.nlm.nih.gov/27703453/
The alteration of gut microbiome and metabolism in ALS patients	Increases in *Bacteriodetes*, *Kineothrix*, *Parabacteroides*, *Odoribacter*, *Sporobacter*, *Eisenbergiella*, *Mannheimia*, *Anaerotruncus*, unclassified *Porphyromonadaceae* in ALS patients. Significant reduction in *Firmicutes* and *Megamonas* in ALS patients compared to control patients.	20 patients with probable or definite ALS and 20 healthy controls	16s rRNA sequencing; Shotgun sequencing (consisting of 10 ALS patients and 10 controls; Metabolomics analysis.	https://pubmed.ncbi.nlm.nih.gov/32747678/
The fecal microbiome of ALS patients	Different proportions of *Ruminococcaceae*	25 ALS patients (2 familial, 23 sporadic) and 32 controls	Fecal samples collected, extraction of nucleic acids, qRT-PCR, 16S rRNA sequencing analysis	https://pubmed.ncbi.nlm.nih.gov/29065369/
Gut inflammation and dysbiosis	A low *Firmicutes/Bacteroidetes* ratio and low *Ruminococcus* spp. in ALS patients 1,3, and 5, low *Clostridium* spp. and *Roseburia* spp. in patient 1, high *Bacteroides-Prevotella*, *Odoribacter* spp. *Barnesiella* spp., and *Bacteroides vulgatus* in patient 5, and high *Bacteroides vulgatus* in patient 3	5 ALS patients and 96 healthy individuals	Collected stool samples, commensal bacteria PCR, bacterial and mycological cultures, mass spectrometry, enzyme immunoassay	https://pubmed.ncbi.nlm.nih.gov/28947596/
A prospective longitudinal study on the microbiota composition in ALS	longitudinal study addressing the microbiota composition in ALS patients and the role of a probiotic supplementation on the gut microbiota and disease progression. Lower *Clostridium* and higher *E. coli and* Enterobacteriaceae were detected in ALS patients. Members of the Cyanobacteria phylum were significantly higher in ALS patients. The control group showed a higher relative abundance of Veillonellaceae, Promicromonosporaceae, and Peptostreptococcaceae.	50 ALS patients and 50 matched controls	DNA extraction from fecal samples, quantitative PCR, PCR-DGGE	(Di Gioia et al., 2020) https://pubmed.ncbi.nlm.nih.gov/32546239/
Assessment of bidirectional relationships between 98 genera of the human gut microbiota and ALS	*OTU10032 unclassified Enterobacteriaceae* was associated with a higher risk of ALS. *Unclassified Acidaminococcaceae* was associated with a higher risk of ALS. Gamma-glutamylphenylalanine showed a significantly increased risk of ALS. Two metabolites, 1-arachidonoyl-GPI and 3-methyl-2-oxobutyrate, were associated with a higher risk of ALS. A genetically predicted increase in the levels of 4-acetamidobutanoate may lower the risk of ALS. Genetically predicted ALS was associated with an increase in the relative abundance of *OTU4607_Sutterella* and *Lactobacillales_order*.	98 genera of gut microbiota, GWAS of ALS involving 20,806 patients and 59,804 controls of European ancestry	Genome wide association study, Mendelian randomization analysis, identification of independently significant SNPs	https://pubmed.ncbi.nlm.nih.gov/34979977/
The human gut microbiota in people with ALS	There was a significantly lower abundance of both *Roseburia intestinalis* and *Eubacterium rectale* in patients in their first year of diagnosis compared to healthy controls. These results were similar to patients who were not taking Riluzole at the time of sample collection, and patients not using a gastrostomy tube. The relative abundance of the family *Lachnospiraceae* was lower in ALS patients compared to healthy controls, but the results were not statistically significant. The total relative abundance of the eight dominant butyrate producers was significantly lower in ALS patients compared to controls. A higher abundance of butyrate-producing bacteria was associated with a significantly lower risk of ALS.	66 patients with ALS, 61 healthy controls, 12 neurodegenerative controls	Clinical information was collected, stool sample self-collected, DNA and RNA extraction, metagenomic shotgun sequencing and profiling, ribosomal sequencing and profiling	https://pubmed.ncbi.nlm.nih.gov/33135936/
The Gut Microbiota-Immunity Axis in ALS: A role in deciphering disease heterogeneity	ALS patients displayed lower amounts of cytokines IL-15, IL-8, MCP-1 and VEGF-A compared to healthy controls ALS patients with a classical phenotype showed a lower microbial diversity compared to others	19 ALS patients and 9 healthy family caregivers matched for sex and closely aligned age	Fecal samples collected, tested 30 cytokines using Luminex MAGPIX detection system, qualitative and quantitative evaluation of SCFAs, genomic DNA extraction and sequencing	https://pubmed.ncbi.nlm.nih.gov/34209688/
Progression and Survival of patients	There were no significant differences in the distribution of bacteria, Proteobacteria, and F/B between ALS patients and	64 patients with ALS and 74 controls	Measurements of whole-body composition and resting energy expenditure, fecal	https://pubmed.

(Continued)

TABLE 4 | Continued

Study	Key Bacterial changes	Sample size	Methods	Reference
with motor neuron disease relative to their fecal microbiota	controls. ALS patients with greater richness and evenness of their fecal microbiome had worse survival from the onset of their symptoms than ALS patients who had a decreased richness and evenness.	(spouses, friends, or family members of ALS patients)	sample collection, DNA extraction, and 16s rRNA amplicon sequencing	ncbi.nlm.nih.gov/32643435/
Bacterial and archaeal communities, butyrate concentration analyses in ALS patients	The richness and evenness of bacterial and archaeal communities of healthy controls was "healthier" than those found in ALS patients. The average relative abundance of phylum *Firmicutes* in ALS patients was 4.7% higher than the relative abundance in controls. The relative abundance of *Negativicutes* and *Bacili* on the class levels were decreased in ALS patients compared to controls. The relative abundance of phylum Euryarchaeota, class Methanobacteria and genus *Methanobrevibacter* was significantly increased in ALS patients. There was no significant difference in fecal butyrate concentration between patients with ALS and healthy controls.	8 patients with ALS and 8 healthy individuals	Fecal samples collected, PCR amplification, enzyme linked immunosorbent assay (ELISA), fecal metabolites, endotoxin, short-chain fatty acids, NO2-N/NO3-N, and γ-aminobutyric acid, were evaluated by spectrophotometry	https://pubmed.ncbi.nlm.nih.gov/31306225/
Potential roles of gut microbiome and metabolite nicotinamide in modulating ALS	Confirm the dysbiosis and aberrant metabolism of nicotinamide in the ALS patients. Significant alterations in key molecules of the tryptophan–nicotinamide metabolic pathway in sera of patients with ALS—among them indoleacetate, kynurenine, serotonin and circulating nicotinamide.	37 human ALS patients and 29 age-matched controls	For human patients, collected their stool samples and sequenced their gut microbiome metagenomes, targeted serum metabolomics	https://pubmed.ncbi.nlm.nih.gov/31330533/
Gut microbiome differences between ALS patients and spouse controls	ALS patients lacked ASVs of the *Prevotella* genus compared to their spouses, particularly *P. timonensis.* There was no difference in stool and plasma inflammatory biomarkers between ALS patients and their spouses. ALS patients had a difference in beta diversity from their spouses. Butanoate metabolic pathway enzymes were missing in patients with ALS.	10 individuals with ALS and their spouses and 10 healthy couples	Rectal and blood sample collection, DNA extractions and 16s rRNA gene sequencing, measurement of inflammatory markers, enzyme linked immunosorbent assay (ELISA) bioinformatics	https://pubmed.ncbi.nlm.nih.gov/33818222/
Potential Role of Gut Microbiota in ALS Pathogenesis and Possible Novel Therapeutic Strategies	There was a lower DNA concentration in ALS patients compared to the healthy controls. There was a low abundance of *Clostridium* and yeast and a high abundance of *E. coli* and *Enterobacteria* in ALS patients compared to controls. There was a clear cluster division between the bacterial profiles of ALS patients compared to the healthy controls. There was no association between yeast profiles and the presence or absence of ALS.	50 ALS patients and 50 healthy controls, matched for sex, age, and origin	Fecal sample collection, total genomic DNA extraction, PCR-denaturing gradient gel electrophoresis analysis, quantitative pCR, double-blind treatment of ALS patients	https://pubmed.ncbi.nlm.nih.gov/29782468/

and Koh, 2020). Dysregulation of the peripheral immune system and reduced levels of regulatory T cells are associated with disease progression. Monocytes are activated in ALS and dendritic cells play a role in pathogenesis. In the brain and spinal cord of patients with ALS, microglia respond to injury in a beneficial way, but chronic microglial activation is thought to contribute to the pathogenesis of the neurodegenerative disease. There is activation of microglia ('neuroinflammation') in ALS. Microglia can have opposite roles at different times. However, the local inflammation in other organs, e.g GI, was less explored in the ALS research.

Intestinal Paneth cells in the small intestine produce most of the antimicrobial peptides (AMPs) and play an important role in regulating the innate immunity and inflammation (Deretic et al., 2008; Lu et al., 2021). The Paneth cell is a unique that can sense commensals and secrete AMPs, thereby playing critical roles in the maintenance of homeostasis at the intestinal-microbial interface. There was a decreased number of normal Paneth cells in the small intestine of $SOD1^{G93A}$ mice, whereas abnormal Paneth cells were significantly increased in $SOD1^{G93A}$ mice. There was a decreased amount of the AMP defensin 5 alpha in the intestine of the 3-month-old $SOD1^{G93A}$ mice with disease symptoms. There was a significant reduction in lysozyme 1 in the G93A intestine, but lysozyme 2 was not altered. There were no pathological changes in the small intestine of G93A mice. There were increased serum IL-17 levels in the young G93A mice at 2 months of age. IL 17 was increased in the intestine of G93A mice, suggesting a preinflammatory state in ALS mice before disease onset. There were higher FITC readings that indicated higher permeability of the intestine. Our study has demonstrated Paneth cell dysfunction and inflammation in the intestine of G93A mice. The intestinal inflammation may occur earlier than the systemic inflammation.

A recent study (Figueroa-Romero et al., 2019) in the $SOD1^{G93A}$ model showed that dysbiosis was observed in the 37 day-old mice. Moderate differences in the peripheral system beginning at 90 days, with elevated CD8T cells in the spleen, and then at 120 days with CD4T cell counts in peripheral blood. The CNS inflammation occurs during late-stage disease in the $SOD1^{G93A}$ mice. This study provides a roadmap to the chronological changes that occur in the intestinal microbiome and immune system relative to disease onset and progression in

the SOD1^{G93A} mouse model. It further supports the critical role of microbiome in the early stage of ALS pathogenesis (Zhang et al., 2021). Limitations of this study include that there may have been some variation introduced by a separate cohort of 120-day-old SOD1 G93A mice. Additionally, the number of males versus females was not always equal during the course of the study.

A previous study (Zhang et al., 2009) reported that the level of plasma LPS showed a significant increase, correlating with blood monocyte/macrophage activation in sALS groups, especially those with advanced sALS disease (Zhang et al., 2009). A report (Tortelli et al., 2020) studied a panel of five cytokines (IL-2, IL-6, IL-10, IFN-gamma, and TNF-alpha) in plasma in ALS (79 patients with ALS and 79 age- and sex-matched healthy controls). All the five cytokines were significantly increased in plasma samples of patients compared with controls, with IL-6 having the highest median concentration (10.11 pg/ml) in the ALS group. Furthermore, IL-6 was the plasma cytokine with the highest discrimination ability between patients and controls according to the receiver operating characteristic analysis. The elevated inflammatory cytokines in ALS support a possible role of inflammation in disease progression. Recent animal experimental studies (Butovsky et al., 2012) have tried to modulate of inflammatory monocytes with microRNAs in ALS mice.

In the animal model of C9orf72 study (Burberry et al., 2020), transplantation of pro-survival intestinal microbiome in the C9orf72^{Harvard} resulted in a significant decrease of inflammatory and autoimmune phenotypes, compared with transplantation of pro-inflammatory microbiome. These results suggested that signals from gut microbiota can help maintain the suppression of certain inflammatory and autoimmune phenotypes. *Helicobacter* spp. was found in both pro-inflammatory environments, but not in pro-survival environments. The abundance of 62 of 301 bacterial species were significantly changed when comparing the two pro-survival environments to the two-pro-inflammatory environments.

Gut microbes can influence the innate and adaptive immune systems, including immune cells in the gut and the systemic immunity.

Neuroinflammation contributes to the pathogenesis of ALS through various mechanisms including enhanced reactive oxygen species (ROS), activation of innate immune systems, increased proinflammatory cytokines, and infiltration of immune cells. Dysbiosis leads to an inflammatory state and altered gut microbiota could influence the immune system in ALS. Moreover, the nutrition has a direct impact on gut microbiome, which shapes local intestinal immune responses and in turn affects even autoimmune responses. Conjugated linoleic acid (CLA), a naturally occurring fatty acid in meat and dairy products of ruminants. A recent study (Fleck et al., 2021) identified CLA as a potent modulator of the gut–CNS axis by targeting myeloid cells in the intestine, which in turn controlled encephalitogenic T-cell responses in mouse model of multiple sclerosis and a pilot human study. However, microbiome, nutrients, immune cells, and cytokines in early disease pathology of human ALS are still unclear. The precise role of inflammation in various organs, include GI, in ALS needs to be further investigated on larger samples and with more mechanistic studies.

TARGETING MICROBIOME AND METABOLITES IN ALS TREATMENT

Significant alterations in the levels of various metabolites were identified in the sera of ALS patients. Bacterial product butyrate is beneficial and slows down the progression of ALS, based on our studies in the SOD1^{G93A} mice (Zhang et al., 2017; Zhang et al., 2021). There is a trial (Paganoni et al., 2020; Turnbull, 2020) using sodium phenylbutyrate-taurursodiol in ALS patients. It reported Sodium phenylbutyrate-taurursodiol resulted in slower functional decline than placebo as measured by the ALSFRS-R score over a period of 24 weeks.

There were alterations in the levels of key components of the tryptophan-nicotinamide (NAM) pathway in some ALS patients. Specifically, the levels of the metabolite NAM in the CSF of 14 patients with ALS were significantly lower than the levels in 17 healthy controls. These results suggested that ALS does interact in some manner with the intestinal microbiota of patients and creates a cycle in which the increase in pathogens and decrease in probiotic organisms in the intestines of ALS patients can upregulate or downregulate the production of NO, GABA, and SCFAs, increasing the pathogenesis of ALS which furthers the imbalance of the intestinal microbiota. The gut microbiota produces metabolites of tryptophan that play a role in regulating astrocyte and microglial activation. Combined, these findings suggest a possible role for microbial metabolites in the control of neuroinflammation during ALS disease progression. Targeting the gut-brain axis could modify the neuronal function (Huang et al., 2019; Kang et al., 2019) and possibly contribute to some of the non-motor cognitive and behavioral symptoms in ALS, but this would require further study.

Riluzole is FDA proved disease-modifying treatment for ALS and has the potential to extend life by only a few months (Miller et al., 2012). Interestingly, Riluzole was significantly metabolized by 40 of the bacteria, based on a 2019 study of on drug metabolism by microbiome (Zimmermann et al., 2019). Many of these bacteria are known to vary in prevalence in the human population. However, the role of the microbiome has not been linked to the drug metabolism and management of ALS treatment.

The use of proton pump inhibitor (PPI) drugs has been associated with a decrease in gastric pH, leading to gastrointestinal dysbiosis (Minalyan et al., 2017). It has also been suggested that PPI use may be related to an increased risk of neurodegenerative diseases (Erber et al., 2020). A retrospective cohort study conducted in Austria reported that 97.5% of ALS patients use other drugs together with riluzole, including PPI and centrally acting muscle relaxants (CAMR). A significant trend in PPI in reducing the survival of ALS was noted; however, after statistical corrections were applied, this effect was no longer

observed. On the other hand, the use of CAMR in association with riluzole showed a beneficial effect on the survival of these patients (Cetin et al., 2015). In a case-control study conducted with 2,484 ALS patients in Sweden, no correlation between PPI use and risk of ALS was observed in a lag window of 1-3 years (Cetin et al., 2020). Thus, it is still lacking evidence to support the detrimental effect of PPI in ALS; nevertheless, 50% of ALS patients report using PPI, raising the importance of further investigations to better understand this correlation.

The increase in the abundance of *Rikenellaceae* was significant in ALS patients who underwent a 6-month probiotic treatment administered daily (Di Gioia et al., 2020). The probiotic formulation is a mixture of five lactic acid bacteria: *Streptococcus thermophilus* ST10–DSM 25246, *Lactobacillus fermentum* LF10–DSM 19187, and *Lactobacillus delbrueckii* subsp. *delbrueckii* LDD01–DSM 22106, *Lactobacillus plantarum* LP01–LMG P-21021, and *Lactobacillus salivarius* LS03–DSM 22776. No adverse events attributed to probiotic supplementation. The significant increase in *Cyanobacteria* at the phylum, family, and genus level in ALS patients compared to controls, suggesting that cyanobacteria play a critical role in the pathogenesis of neurodegenerative diseases. The abundance of cyanobacteria decreased overtime in both the probiotic and placebo groups, although the difference was not significant.

Fecal microbiota transplantation (FMT) could improve gastrointestinal and behavioral symptoms in neurological diseases (Kang et al., 2017; Kang et al., 2019). Parkinson's disease patients after one-week treatment of FMT improved constipation and motor symptoms such as leg tremors. The tremors recurred 2 months after FMT, whereas constipation was relieved even after 3 months (Huang et al., 2019). Intestinal bacteria as an external trigger could explain the rare cases of ALS in spouses or in some clusters (Sabel et al., 2003). To reconstruct the intestinal microbiome, FMT has been used to transfer the gut microbiota from healthy individuals to ALS patients (Xu et al., 2021). ClinicalTrials.gov (https://clinicaltrials.gov/ct2/show/NCT03766321) has an ongoing FMT trial for a year with 42 ALS patients, at an early stage (28 FMT-treated patients *vs.* 14 controls). This will promote a further understanding of microbiota restoration and GI function.

DIET, MICROBIOME, AND ALS

Many factors are involved in the gut microbiota composition and function, including genetics, gender, age, geographic location, lifestyle, and diet (Ballan et al., 2020; de Vos et al., 2022). Also, the gut microbiota is responsible for metabolization of compounds from the diet producing several metabolites (Ballan et al., 2020; de Vos et al., 2022). As a consequence, different gut microbiota compositions will result in different metabolite profiles, that will interact with cell receptors, having a direct impact on different signaling pathways and on the host health (de Vos et al., 2022).

Diet is associated with the ALS risk, onset, and progression, providing potential modifiers of disease (Pape and Grose, 2020). Two mutant SOD1(G86R and G93A) mice exhibited an altered metabolic status (Dupuis et al., 2004). This study provided evidence that transgenic ALS mice suffer from a dramatic defect in energy homeostasis, likely linked to a hypermetabolism mainly of muscular origin. High-energy-diet improved the altered metabolic phenotype of G86R mice and delays disease onset (Dupuis et al., 2004). Several epidemiological studies have identified diets that positively affect ALS patients, including various high-calorie fat or sugar-based diet, summarized in a recent review (Pape and Grose, 2020).

Gluten-induced autoimmunity was speculated to trigger ALS. However, the data supporting this link are weak (Group, 2016). Leptin is an adipokine involved in food intake regulation and energy balance. Lower level of circulating leptin is associated with fat mass loss, and thus, body weight loss in ALS. Moreover, it has been suggested that fat mass loss occurs prior to muscle atrophy. Thus, leptin could be a potential biomarker of adipose tissue wasting in the early stages of ALS. The appetite-stimulating hormone ghrelin also seems to be downregulated in an ALS mice model, compared with their WT controls, contributing to lower food intake and body weight loss (Ferrer-Donato et al., 2021).

Earlier studies suggested that polyphenols (e.g., resveratrol, curcumin, epigallocatechin gallate, quercetin, and phenolic acids), which can be found in fruits, vegetables, coffee, tea, and whole grains, may have a promising neuroprotective effect in ALS. It was observed, *in vivo* and *in vitro*, that these bioactive compounds may have the potential to regulate mitochondrial biogenesis, improve energy metabolism, reduce toxic protein aggregation, reduce microglia and astrocytes inflammation, and improve motor functions and survival (Solanki et al., 2015; Novak et al., 2021).

Resveratrol, an antioxidant compound found in grapes, has been widely studied due to its neuroprotective properties. Resveratrol may reduce the *in vitro* neurotoxicity of cerebrospinal fluid (CSF) from ALS patients, preventing neuronal loss and improving Ca^{2+} homeostasis, which seems to be related to the antioxidant capacity of resveratrol. Curiously, co-incubation with riluzole inhibited this protective effect (Yanez et al., 2011). In fact, Ca^{2+} dyshomeostasis is related to impaired autophagy mechanisms and toxic protein aggregation in neurodegenerative disorders, including ALS (Tedeschi et al., 2019). Therapeutic interventions aiming to modulate autophagy pathways seemed to be an interesting approach to reduce protein aggregates, mainly in the early stages of ALS (Cipolat Mis et al., 2016).

A prospective study reported that probiotics may promote beneficial effects in ALS patients. The administration of probiotics to ALS patients increased the relative abundance of groups related to propionate and butyrate production in gut microbiota, which may improve energy provision. Also, it has been speculated a possible metabolization of neurotoxic compounds (Di Gioia et al., 2020).

In our previous work, we observed the administration of butyrate, a beneficial microbial metabolite, delayed the disease progress and significantly extended the survival time of the SOD1 ^{G93A} mice and prolonged the life span by 38 days on average (Zhang et al., 2017). Butyrate treatment improved the gut microbiome and restored the Paneth cells and the signaling

of lysozyme 1 and anti-microbial peptide defensin 5 alpha in the SOD1 ^{G93A} mice. Meanwhile, the intestinal protein aggregation of SOD1^{G93A} was significantly decreased by butyrate treatment (Zhang et al., 2017). Furthermore, butyrate treatment was able to enhance healthy metabolites by longitudinal untargeted metabolomic analysis in ALS (Destiny Ogbu and Claud, 2022).

Nicotinamide showed slightly delay of the disease progression in the ALS SOD1^{G93A} mice (Blacher et al., 2019). The survival curve showed only several days difference between the SOD1^{G93A} mice with or without nicotinamide treatment. Aberrant metabolism of nicotinamide was observed in the sera of patients with ALS. There was a trial of nicotinamide/pterostilbene supplement in ALS (https://clinicaltrials.gov/ct2/show/NCT04562831). The results have not been reported yet.

Emerging evidence support the roles of gut-neuron-microbiome in various human diseases (Ogbu et al., 2020; Fang et al., 2020). For examples, in a human pilot study in patients with multiple sclerosis, dietary conjugated linoleic acid-supplementation for 6 months significantly enhanced the anti-inflammatory profiles and functional signatures of circulating myeloid cells (Fleck et al., 2021). To move forward, we hypothesize that dietary interventions with functional foods (e.g., prebiotics, probiotics, polyphenols) or the administration of probiotic metabolites (e.g., butyrate) aiming the gut microbiota modulation may enhance intestinal barrier functions and autophagy regulation, restore healthy host-microbial interactions, and reduce toxic protein aggregation and inflammation, thus, delaying the progression of ALS.

CONCLUSION AND FUTURE DIRECTIONS

ALS is a progressive neurodegenerative disorder involving brain and spinal cord motor neuron death resulting in weakness and wasting of musculature and leaning inexorably to death. There are GI abnormalities involved liver, intestine, and pancreas in ALS. Subclinical gastrointestinal motor dysfunction, delayed colonic transit time, delayed gastric emptying an increased prevalence of constipation and anal sphincter abnormalities have been noted, but the mechanism of these issues is unclear. In various mouse models of ALS, the altered microbiome and GI issues are also observed. However, factors such as changes of diet, GI issues, inflammation, and infection were not well considered for the ALS diagnosis.

Emerging evidence has demonstrated the novel roles of microbiome and metabolites in the early stage of disease development and progression, suggesting the potential biomarkers (Chatterjee et al., 2020; Donatti et al., 2020). SALS and fALS forms of ALS manifest similar pathological and clinical phenotypes, suggesting that different initiating causes lead to a mechanistically similar neurodegenerative pathway. ALS patients have abnormal GI systems, elevated intestinal inflammation and dysbiosis. The ENS and smooth muscle automatism are unable to modulate the motor functions of the digestive tract, which provide an anatomical explanation for these clinical manifestations. Studies on microbiome changes with pro-inflammatory serum cytokines and LPS in patients will help with the early diagnosis of the disease. To treat gut microbiota dysbiosis through microbiota restoration would have the potential to interfere and slow ALS progression (Mandrioli et al., 2019). The therapeutic methods to target microbiome and intestinal functions, e.g, FMT, prebiotics, and probiotics, will help both SALS and FALS patients. ALS studies should be performed by considering diet, microbiome, lifestyle, and gender difference. Studies that examine the microbiome together with intestinal pathogenesis will help to determine when, where, and whether microbiome and metabolites are critical to disease progression of ALS. Understanding the pathogenesis of ALS GI will provide innovative strategies for accurate diagnosis and better treatment for this challenging disease.

AUTHOR CONTRIBUTIONS

SM and JS performed literature search and detailed analyses and summary of related literature, prepared the draft text. CB contributed to literature search of ALS mouse models, nutrition, and the draft text. JS designed the study/project, obtained funds, summarize literature, and directed the project. All authors contributed to the article and approved the submitted version.

FUNDING

We would like to acknowledge the VA Merit Award 1 I01BX004824-01, the NIDDK/National Institutes of Health grant R01 DK105118, and R01DK114126 to JS. The study sponsors play no role in the study design, data collection, analysis, and interpretation of data. The contents do not represent the views of the United States Department of Veterans Affairs or the United States Government.

REFERENCES

Ballan, R., Battistini, C., Xavier-Santos, D., and Saad, S. M. I. (2020). Interactions of Probiotics and Prebiotics With the Gut Microbiota. *Prog. Mol. Biol. Transl. Sci.* 171, 265–300. doi: 10.1016/bs.pmbts.2020.03.008

Banack, S. A., and Cox, P. A. (2003). Biomagnification of Cycad Neurotoxins in Flying Foxes: Implications for ALS-PDC in Guam. *Neurology* 61, 387–389. doi: 10.1212/01.WNL.0000078320.18564.9F

Baquero, F., and Nombela, C. (2012). The Microbiome as a Human Organ. *Clin. Microbiol. Infect.* 18, 2–4. doi: 10.1111/j.1469-0691.2012.03916.x

Blacher, E., Bashiardes, S., Shapiro, H., Rothschild, D., Mor, U., Dori-Bachash, M., et al. (2019). Potential Roles of Gut Microbiome and Metabolites in Modulating ALS in Mice. *Nature* 572, 474–480. doi: 10.1038/s41586-019-1443-5

Brenner, D., Hiergeist, A., Adis, C., Mayer, B., Gessner, A., Ludolph, A. C., et al. (2018). The Fecal Microbiome of ALS Patients. *Neurobiol. Aging* 61, 132–137. doi: 10.1016/j.neurobiolaging.2017.09.023

Brown, K. J., Jewells, V., Herfarth, H., and Castillo, M. (2010). White Matter Lesions Suggestive of Amyotrophic Lateral Sclerosis Attributed to Celiac Disease. *AJNR Am. J. Neuroradiol.* 31, 880–881. doi: 10.3174/ajnr.A1826

Burberry, A., Wells, M. F., Limone, F., Couto, A., Smith, K. S., Keaney, J., et al. (2020). C9orf72 Suppresses Systemic and Neural Inflammation Induced by Gut Bacteria. *Nature* 582, 89–94. doi: 10.1038/s41586-020-2288-7

Butovsky, O., Siddiqui, S., Gabriely, G., Lanser, A. J., Dake, B., Murugaiyan, G., et al. (2012). Modulating Inflammatory Monocytes With a Unique microRNA Gene Signature Ameliorates Murine ALS. *J. Clin. Invest.* 122, 3063–3087. doi: 10.1172/JCI62636

Cetin, H., Klickovic, U., Rath, J., Zulehner, G., Fuzi, J., Reichardt, B., et al. (2015). Associations Between Co-Medications and Survival in ALS-A Cohort Study From Austria. *J. Neurol.* 262, 1698–1705. doi: 10.1007/s00415-015-7767-7

Cetin, H., Sun, J., Almqvist, C., Reichardt, B., Tomschik, M., Zimprich, F., et al. (2020). No Association Between Proton Pump Inhibitor Use and ALS Risk: A Nationwide Nested Case-Control Study. *Sci. Rep.* 10, 13371. doi: 10.1038/s41598-020-70373-8

Charchaflie, R. J., Bustos Fernandez, L., Perec, C. J., Gonzalez, E., and Marzi, A. (1974). Functional Studies of the Parotid and Pancreas Glands in Amyotrophic Lateral Sclerosis. *J. Neurol. Neurosurg. Psychiatry* 37, 863–867. doi: 10.1136/jnnp.37.7.863

Chatterjee, I., Lu, R., Zhang, Y., Zhang, J., Dai, Y., Xia, Y., et al. (2020). Vitamin D Receptor Promotes Healthy Microbial Metabolites and Microbiome. *Sci. Rep.* 10, 7340. doi: 10.1038/s41598-020-64226-7

Chio, A., Logroscino, G., Traynor, B. J., Collins, J., Simeone, J. C., Goldstein, L. A., et al. (2013). Global Epidemiology of Amyotrophic Lateral Sclerosis: A Systematic Review of the Published Literature. *Neuroepidemiology* 41, 118–130. doi: 10.1159/000351153

Cipolat Mis, M. S., Brajkovic, S., Frattini, E., Di Fonzo, A., and Corti, S. (2016). Autophagy in Motor Neuron Disease: Key Pathogenetic Mechanisms and Therapeutic Targets. *Mol. Cell Neurosci.* 72, 84–90. doi: 10.1016/j.mcn.2016.01.012

Deretic, V., Master, S., and Singh, S. (2008). Autophagy Gives a Nod and a Wink to the Inflammasome and Paneth Cells in Crohn's Disease. *Dev. Cell* 15, 641–642. doi: 10.1016/j.devcel.2008.10.009

Destiny Ogbu, Y. Z., and Claud, K. (2022). Yinglin Xia, Un Sun, Dietary Butyrate Treatment Enhances Healthy Metabolites by Longitudinal Untargeted Metabolomic Analysis in Amyotrophic Lateral Sclerosis Mice. *Biorxiv*. doi: 10.1101/2022.01.15.476456

de Vos, W. M., Tilg, H., Van Hul, M., and Cani, P. D. (2022). Gut Microbiome and Health: Mechanistic Insights. *Gut*. doi: 10.1136gutjnl-2021-326789

Di Gioia, D., Bozzi Cionci, N., Baffoni, L., Amoruso, A., Pane, M., Mogna, L., et al. (2020). A Prospective Longitudinal Study on the Microbiota Composition in Amyotrophic Lateral Sclerosis. *BMC Med.* 18, 153. doi: 10.1186/s12916-020-01607-9

Donatti, A., Canto, A. M., Godoi, A. B., da Rosa, D. C., and Lopes-Cendes, I. (2020). Circulating Metabolites as Potential Biomarkers for Neurological Disorders-Metabolites in Neurological Disorders. *Metabolites* 10 (10), 389. doi: 10.3390/metabo10100389

Dupuis, L., Oudart, H., Rene, F., Gonzalez de Aguilar, J. L., and Loeffler, J. P. (2004). Evidence for Defective Energy Homeostasis in Amyotrophic Lateral Sclerosis: Benefit of a High-Energy Diet in a Transgenic Mouse Model. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11159–11164. doi: 10.1073/pnas.0402026101

Erber, A. C., Cetin, H., Berry, D., and Schernhammer, E. S. (2020). The Role of Gut Microbiota, Butyrate and Proton Pump Inhibitors in Amyotrophic Lateral Sclerosis: A Systematic Review. *Int. J. Neurosci.* 130, 727–735. doi: 10.1080/00207454.2019.1702549

Esmaeili, M. A., Panahi, M., Yadav, S., Hennings, L., and Kiaei, M. (2013). Premature Death of TDP-43 (A315T) Transgenic Mice Due to Gastrointestinal Complications Prior to Development of Full Neurological Symptoms of Amyotrophic Lateral Sclerosis. *Int. J. Exp. Pathol.* 94, 56–64. doi: 10.1111/iep.12006

Fang, X. (2016). Potential Role of Gut Microbiota and Tissue Barriers in Parkinson's Disease and Amyotrophic Lateral Sclerosis. *Int. J. Neurosci.* 126, 771–776. doi: 10.3109/00207454.2015.1096271

Fang, P., Kazmi, S. A., Jameson, K. G., and Hsiao, E. Y. (2020). The Microbiome as a Modifier of Neurodegenerative Disease Risk. *Cell Host Microbe* 28, 201–222. doi: 10.1016/j.chom.2020.06.008

Fang, X., Wang, X., Yang, S., Meng, F., Wang, X., Wei, H., et al. (2016). Evaluation of the Microbial Diversity in Amyotrophic Lateral Sclerosis Using High-Throughput Sequencing. *Front. Microbiol.* 7, 1479. doi: 10.3389/fmicb.2016.01479

Ferrer-Donato, A., Contreras, A., Frago, L. M., Chowen, J. A., and Fernandez-Martos, C. M. (2021). Alterations in Leptin Signaling in Amyotrophic Lateral Sclerosis (ALS). *Int. J. Mol. Sci.* 22 (19), 10305. doi: 10.3390/ijms221910305

Figueroa-Romero, C., Guo, K., Murdock, B. J., Paez-Colasante, X., Bassis, C. M., Mikhail, K. A., et al. (2019). Temporal Evolution of the Microbiome, Immune System and Epigenome With Disease Progression in ALS Mice. *Dis. Model Mech.* 13 (2), dmm041947. doi: 10.1242/dmm.041947

Fleck, A. K., Hucke, S., Teipel, F., Eschborn, M., Janoschka, C., Liebmann, M., et al. (2021). Dietary Conjugated Linoleic Acid Links Reduced Intestinal Inflammation to Amelioration of CNS Autoimmunity. *Brain* 144 (4), 1152–1166. doi: 10.1093/brain/awab040

Gadoth, A., Nefussy, B., Bleiberg, M., Klein, T., Artman, I., and Drory, V. E. (2015). Transglutaminase 6 Antibodies in the Serum of Patients With Amyotrophic Lateral Sclerosis. *JAMA Neurol.* 72, 676–681. doi: 10.1001/jamaneurol.2015.48

Group, A. L. S. U. (2016). ALSUntangled No. 32: Gluten-Free Diet. *Amyotroph. Lateral Scler. Frontotemporal Degener.* 17, 307–309. doi: 10.3109/21678421.2015.1096496

Guo, Y., Wang, Q., Zhang, K., An, T., Shi, P., Li, Z., et al. (2012). HO-1 Induction in Motor Cortex and Intestinal Dysfunction in TDP-43 A315T Transgenic Mice. *Brain Res.* 1460, 88–95. doi: 10.1016/j.brainres.2012.04.003

Ham, H., Lee, B. I., Oh, H. J., Park, S. H., Kim, J. S., Park, J. M., et al. (2017). A Case of Celiac Disease With Neurologic Manifestations Misdiagnosed as Amyotrophic Lateral Sclerosis. *Intest. Res.* 15, 540–542. doi: 10.5217/ir.2017.15.4.540

Hatzipetros, T., Bogdanik, L. P., Tassinari, V. R., Kidd, J. D., Moreno, A. J., Davis, C., et al. (2014). C57BL/6J Congenic Prp-TDP43A315T Mice Develop Progressive Neurodegeneration in the Myenteric Plexus of the Colon Without Exhibiting Key Features of ALS. *Brain Res.* 1584, 59–72. doi: 10.1016/j.brainres.2013.10.013

Herdewyn, S., Cirillo, C., Van Den Bosch, L., Robberecht, W., Vanden Berghe, P., and Van Damme, P. (2014). Prevention of Intestinal Obstruction Reveals Progressive Neurodegeneration in Mutant TDP-43 (A315T) Mice. *Mol. Neurodegener.* 9, 24. doi: 10.1186/1750-1326-9-24

Hertzberg, V. S., Singh, H., Fournier, C. N., Moustafa, A., Polak, M., Kuelbs, C. A., et al. (2022). Gut Microbiome Differences Between Amyotrophic Lateral Sclerosis Patients and Spouse Controls. *Amyotroph Lateral Scler Frontotemporal Degener* 23, 91–99. doi: 10.1080/21678421.2021.1904994

Huang, H., Xu, H., Luo, Q., He, J., Li, M., Chen, H., et al. (2019). Fecal Microbiota Transplantation to Treat Parkinson's Disease With Constipation: A Case Report. *Med. (Baltimore)* 98, e16163. doi: 10.1097/MD.0000000000016163

Kang, D. W., Adams, J. B., Coleman, D. M., Pollard, E. L., Maldonado, J., McDonough-Means, S., et al. (2019). Long-Term Benefit of Microbiota Transfer Therapy on Autism Symptoms and Gut Microbiota. *Sci. Rep.* 9, 5821. doi: 10.1038/s41598-019-42183-0

Kang, D. W., Adams, J. B., Gregory, A. C., Borody, T., Chittick, L., Fasano, A., et al. (2017). Microbiota Transfer Therapy Alters Gut Ecosystem and Improves Gastrointestinal and Autism Symptoms: An Open-Label Study. *Microbiome* 5, 10. doi: 10.1186/s40168-016-0225-7

Kwon, H. S., and Koh, S. H. (2020). Neuroinflammation in Neurodegenerative Disorders: The Roles of Microglia and Astrocytes. *Transl. Neurodegener.* 9, 42. doi: 10.1186/s40035-020-00221-2

Labarre, A., Guitard, E., and Parker, J. A. (2017). Variation of Gut Microbiome Rescues Paralysis and Neurodegeneration Profiles in C. Elegans ALS Models. *Amyotrophic Lateral Scler Frontotemporal Degener*, 245–246.

Lee, S. H., and Yang, E. J. (2018). Relationship Between Liver Pathology and Disease Progression in a Murine Model of Amyotrophic Lateral Sclerosis. *Neurodegener. Dis.* 18, 200–207. doi: 10.1159/000491392

Luesma, M. J., Cantarero, I., Álvarez-Dotu, J. M., Santander, S., and Junquera, C. (2014). New Insights Into C-Ret Signalling Pathway in the Enteric Nervous System and its Relationship With ALS. *BioMed. Res. Int.* 2014, 328348. doi: 10.1155/2014/328348

Lu, R., Zhang, Y. G., Xia, Y., Zhang, J., Kaser, A., Blumberg, R., et al. (2021). Paneth Cell Alertness to Pathogens Maintained by Vitamin D Receptors. *Gastroenterology* 160, 1269–1283. doi: 10.1053/j.gastro.2020.11.015

Mandrioli, J., Amedei, A., Cammarota, G., Niccolai, E., Zucchi, E., D'Amico, R., et al. (2019). FETR-ALS Study Protocol: A Randomized Clinical Trial of Fecal Microbiota Transplantation in Amyotrophic Lateral Sclerosis. *Front. Neurol.* 10, 1021. doi: 10.3389/fneur.2019.01021

Mazzini, L., Mogna, L., De Marchi, F., Amoruso, A., Pane, M., Aloisio, I., et al. (2018). Potential Role of Gut Microbiota in ALS Pathogenesis and Possible Novel Therapeutic Strategies. *J. Clin. Gastroenterol.* 52, S68–S70. Proceedings from the 9th Probiotics, Prebiotics and New Foods, Nutraceuticals and Botanicals for Nutrition & Human and Microbiota Health Meeting, held in Rome, Italy from September 10 to 12, 2017. doi: 10.1097/MCG.0000000000001042

McCombe, P. A., and Henderson, R. D. (2011). The Role of Immune and Inflammatory Mechanisms in ALS. *Curr. Mol. Med.* 11, 246–254. doi: 10.2174/156652411795243450

Miller, R. G., Mitchell, J. D., and Moore, D. H. (2012). Riluzole for Amyotrophic Lateral Sclerosis (ALS)/motor Neuron Disease (MND). *Cochrane Database Syst. Rev.*, CD001447. doi: 10.1002/14651858.CD001447.pub3

Minalyan, A., Gabrielyan, L., Scott, D., Jacobs, J., and Pisegna, J. R. (2017). The Gastric and Intestinal Microbiome: Role of Proton Pump Inhibitors. *Curr. Gastroenterol. Rep.* 19, 42. doi: 10.1007/s11894-017-0577-6

Murch, S. J., Cox, P. A., Banack, S. A., Steele, J. C., and Sacks, O. W. (2004). Occurrence of Beta-Methylamino-L-Alanine (BMAA) in ALS/PDC Patients From Guam. *Acta Neurol Scand* 110, 267–269. doi: 10.1111/j.1600-0404.2004.00320.x

Nakano, Y., Hirayama, K., and Terao, K. (1987). Hepatic Ultrastructural Changes and Liver Dysfunction in Amyotrophic Lateral Sclerosis. *Arch. Neurol.* 44, 103–106. doi: 10.1001/archneur.1987.00520130079022

Ngo, S. T., Restuadi, R., McCrae, A. F., Van Eijk, R. P., Garton, F., Henderson, R. D., et al. (2020). Progression and Survival of Patients With Motor Neuron Disease Relative to Their Fecal Microbiota. *Amyotroph. Lateral Scler. Frontotemporal Degener.* 21, 549–562. doi: 10.1080/21678421.2020.1772825

Niccolai, E., Di Pilato, V., Nannini, G., Baldi, S., Russo, E., Zucchi, E., et al. (2021). The Gut Microbiota-Immunity Axis in ALS: A Role in Deciphering Disease Heterogeneity? *Biomedicines* 9, 1–17. doi: 10.3390/biomedicines9070753

Nicolas, A., Kenna, K. P., Renton, A. E., Ticozzi, N., Faghri, F., Chia, R., et al. (2018). Genome-Wide Analyses Identify KIF5A as a Novel ALS Gene. *Neuron* 97, 1268–126+. doi: 10.1016/j.neuron.2018.02.027

Niesler, B., Kuerten, S., Demir, I. E., and Schafer, K. H. (2021). Disorders of the Enteric Nervous System - a Holistic View. *Nat. Rev. Gastroenterol. Hepatol.* 18, 393–410. doi: 10.1038/s41575-020-00385-2

Novak, V., Rogelj, B., and Zupunski, V. (2021). Therapeutic Potential of Polyphenols in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. *Antioxidants (Basel)* 10 (8), 1328. doi: 10.3390/antiox10081328

Nubling, G. S., Mie, E., Bauer, R. M., Hensler, M., Lorenzl, S., Hapfelmeier, A., et al. (2014). Increased Prevalence of Bladder and Intestinal Dysfunction in Amyotrophic Lateral Sclerosis. *Amyotroph. Lateral Scler. Frontotemporal. Degener.* 15, 174–179. doi: 10.3109/21678421.2013.868001

Ogbu, D., Xia, E., and Sun, J. (2020). Gut Instincts: Vitamin D/vitamin D Receptor and Microbiome in Neurodevelopment Disorders. *Open Biol.* 10, 200063. doi: 10.1098/rsob.200063

Paganoni, S., Macklin, E. A., Hendrix, S., Berry, J. D., Elliott, M. A., Maiser, S., et al. (2020). Trial of Sodium Phenylbutyrate-Taursodiol for Amyotrophic Lateral Sclerosis. *N Engl. J. Med.* 383, 919–930. doi: 10.1056/NEJMoa1916945

Pape, J. A., and Grose, J. H. (2020). The Effects of Diet and Sex in Amyotrophic Lateral Sclerosis. *Rev. Neurol. (Paris)* 176, 301–315. doi: 10.1016/j.neurol.2019.09.008

Philips, T., and Rothstein, J. D. (2015). Rodent Models of Amyotrophic Lateral Sclerosis. *Curr. Protoc. Pharmacol.* 69, 5 67 1–5 67 21. doi: 10.1002/0471141755.ph0567s69

Quick, D. T., and Greer, M. (1967). Pancreatic Dysfunction in Patients With Amyotrophic Lateral Sclerosis. *Neurology* 17, 112–116. doi: 10.1212/WNL.17.2.112

Rao, M., and Gershon, M. D. (2016). The Bowel and Beyond: The Enteric Nervous System in Neurological Disorders. *Nat. Rev. Gastroenterol. Hepatol.* 13, 517–528. doi: 10.1038/nrgastro.2016.107

Roberts, A. L., Johnson, N. J., Cudkowicz, M. E., Eum, K. D., and Weisskopf, M. G. (2016). Job-Related Formaldehyde Exposure and ALS Mortality in the USA. *J. Neurol. Neurosurg Psychiatry* 87, 786–788. doi: 10.1136/jnnp-2015-310750

Rowin, J., Xia, Y., Jung, B., and Sun, J. (2017). Gut Inflammation and Dysbiosis in Human Motor Neuron Disease. *Physiol. Rep.* 5, 1–6. doi: 10.14814/phy2.13443

Sabel, C. E., Boyle, P. J., Loytonen, M., Gatrell, A. C., Jokelainen, M., Flowerdew, R., et al. (2003). Spatial Clustering of Amyotrophic Lateral Sclerosis in Finland at Place of Birth and Place of Death. *Am. J. Epidemiol.* 157, 898–905. doi: 10.1093/aje/kwg090

Solanki, I., Parihar, P., Mansuri, M. L., and Parihar, M. S. (2015). Flavonoid-Based Therapies in the Early Management of Neurodegenerative Diseases. *Adv. Nutr.* 6, 64–72. doi: 10.3945/an.114.007500

Sun, J., Zhan, Y., Mariosa, D., Larsson, H., Almqvist, C., Ingre, C., et al. (2019). Antibiotics Use and Risk of Amyotrophic Lateral Sclerosis in Sweden. *Eur. J. Neurol.* 26, 1355–1361. doi: 10.1111/ene.13986

Tedeschi, V., Petrozziello, T., and Secondo, A. (2019). Calcium Dyshomeostasis and Lysosomal Ca(2+) Dysfunction in Amyotrophic Lateral Sclerosis. *Cells* 8 (10), 1216. doi: 10.3390/cells8101216

Tortelli, R., Zecca, C., Piccininni, M., Benmahamed, S., Dell'Abate, M. T., Barulli, M. R., et al. (2020). Plasma Inflammatory Cytokines Are Elevated in ALS. *Front. Neurol.* 11, 552295. doi: 10.3389/fneur.2020.552295

Turnbull, J. (2020). Sodium Phenylbutyrate-Taursodiol for ALS. *N. Engl. J. Med.* 383, 2293–2294. doi: 10.1056/NEJMc2030710

Turner, M. R., Al-Chalabi, A., Chio, A., Hardiman, O., Kiernan, M. C., Rohrer, J. D., et al. (2017). Genetic Screening in Sporadic ALS and FTD. *J. Neurol Neurosurg Psychiatry* 88, 1042–1044. doi: 10.1136/jnnp-2017-315995

Turner, M. R., Chohan, G., Quaghebeur, G., Greenhall, R. C., Hadjivassiliou, M., and Talbot, K. (2007). A Case of Celiac Disease Mimicking Amyotrophic Lateral Sclerosis. *Nat. Clin. Pract. Neurol.* 3, 581–584. doi: 10.1038/ncpneuro0631

Utterback, R. A., Cummins, A. J., Cape, C. A., and Goldenberg, J. (1970). Pancreatic Function in Amyotrophic Lateral Sclerosis. *J. Neurol. Neurosurg Psychiatry* 33, 544–547. doi: 10.1136/jnnp.33.4.544

Volk, A. E., Weishaupt, J. H., Andersen, P. M., Ludolph, A. C., and Kubisch, C. (2018). Current Knowledge and Recent Insights Into the Genetic Basis of Amyotrophic Lateral Sclerosis. *Med. Genet-Berlin* 30, 252–258. doi: 10.1007/s11825-018-0185-3

Wang, M. D., Little, J., Gomes, J., Cashman, N. R., and Krewski, D. (2017). Identification of Risk Factors Associated With Onset and Progression of Amyotrophic Lateral Sclerosis Using Systematic Review and Meta-Analysis. *Neurotoxicology* 61, 101–130. doi: 10.1016/j.neuro.2016.06.015

Weisskopf, M. G., Morozova, N., O'Reilly, E. J., McCullough, M. L., Calle, E. E., Thun, M. J., et al. (2009). Prospective Study of Chemical Exposures and Amyotrophic Lateral Sclerosis. *J. Neurol. Neurosurg. Psychiatry* 80, 558–561. doi: 10.1136/jnnp.2008.156976

Whipps, J., Lewis, K., and Cooke, R. (1988). "Mycoparasitism and Plant Disease Control," in *Fungi in Biological Control Systems*. Ed. B. M. (Manchester: Manchester University Press), 161–187.

Wingate, D. (1999). Gastrointestinal Dysfunction in ALS. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 1, 2.

Wu, S., Yi, J., Zhang, Y. G., Zhou, J., and Sun, J. (2015). Leaky Intestine and Impaired Microbiome in an Amyotrophic Lateral Sclerosis Mouse Model. *Physiol. Rep.* 3, 1–10. doi: 10.14814/phy2.12356

Xu, H. M., Huang, H. L., Zhou, Y. L., Zhao, H. L., Xu, J., Shou, D. W., et al. (2021). Fecal Microbiota Transplantation: A New Therapeutic Attempt From the Gut to the Brain. *Gastroenterol. Res. Pract.* 2021, 6699268. doi: 10.1155/2021/6699268

Yanez, M., Galan, L., Matias-Guiu, J., Vela, A., Guerrero, A., and Garcia, A. G. (2011). CSF From Amyotrophic Lateral Sclerosis Patients Produces Glutamate Independent Death of Rat Motor Brain Cortical Neurons: Protection by Resveratrol But Not Riluzole. *Brain Res.* 1423, 77–86. doi: 10.1016/j.brainres.2011.09.025

Zeng, Q., Shen, J., Chen, K., Zhou, J., Liao, Q., Lu, K., et al. (2020). The Alteration of Gut Microbiome and Metabolism in Amyotrophic Lateral Sclerosis Patients. *Sci. Rep.* 10, 12998. doi: 10.1038/s41598-020-69845-8

Zhai, C. D., Zheng, J. J., An, B. C., Huang, H. F., and Tan, Z. C. (2019). Intestinal Microbiota Composition in Patients With Amyotrophic Lateral Sclerosis:

Establishment of Bacterial and Archaeal Communities Analyses. *Chin. Med. J. (Engl)* 132, 1815–1822. doi: 10.1097/CM9.0000000000000351

Zhang, Y. G., Lu, R., Xia, Y., Zhou, D., Petrof, E., Claud, E. C., et al. (2019). Lack of Vitamin D Receptor Leads to Hyperfunction of Claudin-2 in Intestinal Inflammatory Responses. *Inflamm. Bowel Dis.* 25, 97–110. doi: 10.1093/ibd/izy292

Zhang, R., Miller, R. G., Gascon, R., Champion, S., Katz, J., Lancero, M., et al. (2009). Circulating Endotoxin and Systemic Immune Activation in Sporadic Amyotrophic Lateral Sclerosis (sALS). *J. Neuroimmunol.* 206, 121–124. doi: 10.1016/j.jneuroim.2008.09.017

Zhang, Y. G., Ogbu, D., Garrett, S., Xia, Y., and Sun, J. (2021). Aberrant Enteric Neuromuscular System and Dysbiosis in Amyotrophic Lateral Sclerosis. *Gut Microbes* 13, 1996848. doi: 10.1080/19490976.2021.1996848

Zhang, Y. G., Wu, S., and Sun, J. (2013). Vitamin D, Vitamin D Receptor, and Tissue Barriers. *Tissue Barriers* 1 (1). doi: 10.4161/tisb.23118

Zhang, Y. G., Wu, S., Yi, J., Xia, Y., Jin, D., Zhou, J., et al. (2017). Target Intestinal Microbiota to Alleviate Disease Progression in Amyotrophic Lateral Sclerosis. *Clin. Ther.* 39, 322–336. doi: 10.1016/j.clinthera.2016.12.014

Zhang, L., Zhuang, Z., Zhang, G., Huang, T., and Fan, D. (2022). Assessment of Bidirectional Relationships Between 98 Genera of the Human Gut Microbiota and Amyotrophic Lateral Sclerosis: A 2-Sample Mendelian Randomization Study. *BMC Neurol.* 22, 8. doi: 10.1186/s12883-021-02522-z

Zimmermann, M., Zimmermann-Kogadeeva, M., Wegmann, R., and Goodman, A. L. (2019). Mapping Human Microbiome Drug Metabolism by Gut Bacteria and Their Genes. *Nature* 570, 462–467. doi: 10.1038/s41586-019-1291-3

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/fcimb.2022.854122

The Infant Gut Commensal *Bacteroides dorei* Presents a Generalized Transcriptional Response to Various Human Milk Oligosaccharides

Sivan Kijner[1], *Avital Cher*[1] *and Moran Yassour*[1,2]*

[1] Microbiology & Molecular Genetics Department, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel, [2] The Rachel and Selim Benin School of Computer Science and Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel

OPEN ACCESS

Edited by:
Gislane Lelis Vilela de Oliveira, São Paulo State University, Brazil

Reviewed by:
Daniel A. Medina, San Sebastián University, Chile
Christopher James Stewart, Newcastle University, United Kingdom
Sabrina Neves Casarotti, Federal University of Mato Grosso, Brazil

***Correspondence:**
Moran Yassour
moranya@mail.huji.ac.il

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *13 January 2022*
Accepted: *22 February 2022*
Published: *18 March 2022*

Citation:
Kijner S, Cher A and Yassour M (2022) The Infant Gut Commensal Bacteroides dorei Presents a Generalized Transcriptional Response to Various Human Milk Oligosaccharides. Front. Cell. Infect. Microbiol. 12:854122. doi: 10.3389/fcimb.2022.854122

Human milk oligosaccharides (HMOs) are a family of glycans found in breastmilk with over 200 identified structures. Despite being the third-largest solid component in breastmilk, HMOs are indigestible by infants, and they serve as food for the infant gut bacteria. Most research thus far has focused on *Bifidobacterium* species that harbor many glycoside hydrolases (GHs) tailored to break the carbon bonds in HMO molecules. However, there are additional microbes in the infant gut, such as *Bacteroides* species, with increasing evidence that they, too, are able to break-down HMOs. To study the unbiased impact of breastfeeding on the infant gut microbiome, we need to investigate the underlying mechanisms of HMO utilization by all members of the infant gut. Here, we developed an optimized system for isolating *Bacteroides* strains from infant stool samples. We then examined the HMO utilization capacity of multiple *Bacteroides* isolates by performing growth curves on six common HMOs (2'-FL, DFL, 3'-SL, 6'-SL, LNT, LNnT). Isolates often displayed similar growth characteristics on similarly-structured HMOs, like sialylated or fucosylated sugars. We identified variation in HMO utilization across multiple strains of the same species, and chose to focus here on a *Bacteroides dorei* isolate that was able to utilize the test HMOs. We performed RNA sequencing on *B. dorei* cultures, comparing the transcriptional profile in minimal media supplemented with glucose or HMOs. We showed that *B. dorei* employs an extensive metabolic response to HMOs. Surprisingly, there was no clear up-regulation for most GH families previously known to break-down HMOs, possibly because they were almost exclusively described in *Bifidobacterium* species. Instead, *B. dorei* exhibits a generalized response to HMOs, markedly up-regulating several shared GH families across all conditions. Within each GH family, *B. dorei* displays a consistent pattern of up-regulation of some genes with down-regulation of

the others. This response pattern to HMOs has yet to be described in other commensals of the infant gut. Our work highlights the importance of expanding the HMO-microbiome studies beyond *Bifidobacterium* species, sheds light on the differences across *Bacteroides* strains in terms of HMO utilization, and paves the way to understanding the mechanisms enabling *Bacteroides* HMO utilization.

Keywords: infant microbiome, human milk oligosaccharides (HMO), *Bacteroides*, microbial RNA seq, breastmilk

INTRODUCTION

The infant gut microbiome is very dynamic (Yassour et al., 2016; Ferretti et al., 2018), and stabilizes to an adult-like state around the age of three (Yatsunenko et al., 2012; Yassour et al., 2016). The initial colonization of the infant gut is a complex process (La Rosa et al., 2014; Lax et al., 2014), mainly influenced by delivery mode (Dominguez-Bello et al., 2010; Azad et al., 2013; Madan et al., 2016; Yassour et al., 2016) and infant feeding (formula vs. breast milk) (Azad et al., 2013; Bäckhed et al., 2015; Madan et al., 2016; Stewart et al., 2018). Human Milk Oligosaccharides (HMOs) are a family of glycans found in breast milk, and despite being the third-largest solid component in human milk (Vandenplas et al., 2018), infants themselves are unable to digest these sugars (Engfer et al., 2000; Gnoth et al., 2000), and HMOs are exclusively digested by bacteria in the infant gut. HMOs take part in many important processes, such as infant immune system maturation (Plaza-Díaz et al., 2018) and brain development (Jacobi et al., 2016; Hegar et al., 2019), but one of their most-studied roles is the promotion of a healthy infant gut microbial community, by supporting the colonization of beneficial gut bacteria that can break down the HMO chemical structure (Walsh et al., 2020). Mounting evidence show that early establishment of a "correct" microbial community in the infant gut is crucial for future health, in microbiota-mediated colonization resistance against intestinal pathogens (Buffie and Pamer, 2013; McLaren and Callahan, 2020), even under antibiotic disturbance (Letten et al., 2021a), and the ability of the microbiome to modulate persistence of antibiotic resistant strains (Letten et al., 2021b). The importance of HMOs in shaping the unique infant microbiome has been a subject of avid research in the past two decades, however most of it focused on *Bifidobacterium* species (Dethlefsen et al., 2007; Sela et al., 2008).

Bifidobacterium species abundance is strongly associated with HMO consumption, as demonstrated by multiple studies comparing breastfed to non-breastfed infants (Davis et al., 2016; Smith-Brown et al., 2016; Bai et al., 2018; Lawson et al., 2020; Laursen et al., 2021; Liu et al., 2021). The first HMO-specific utilization cluster (H1) was discovered in 2008 (Sela et al., 2008). It is 43kb long and is conserved in all *B. longum subsp. infantis* genomes sequenced thus far. Since 2008, five additional HMO-utilizing gene clusters (H2, H3, H4, H5, urease) have been identified (LoCascio et al., 2010), albeit they are less conserved. Recently, additional genes capable of HMO utilization were discovered, which do not necessarily belong to the known clusters identified thus far, and revealed variation in differential expression of genes across *Bifidobacterium longum subsp. infantis* bacteria (Zabel et al., 2020). Importantly, none of these well-studied gene clusters were found in any non-*Bifidobacterium* species (Sela and Mills, 2010; Marcobal and Sonnenburg, 2012).

Despite the well-established connection between *Bifidobacterium* and HMOs, recent empirical evidence support the HMO utilization by other, non-*Bifidobacterium* species. First, some breastfeeding infants do not harbor any *Bifidobacterium* species throughout their breastfeeding period (Yassour et al., 2016), and findings exhibit inter-study heterogeneity (Harmsen et al., 2000; Favier et al., 2002; Palmer et al., 2007). Importantly, it was recently reported that the best known HMO-utilizer strain, *Bifidobacterium longum subsp. infantis*, considered to be predominant in the infant gut (Chichlowski et al., 2020), is not evident in 90% of infants in the US (Casaburi et al., 2021) and Finland (Vatanen et al., 2019). Second, there are significant correlations between specific HMO levels and the relative abundance of other genera, such as *Lactobacillus* (Bai et al., 2018), *Bacteroides* and *Parabacteroides* (Wang et al., 2015; Borewicz et al., 2019; Borewicz et al., 2020). Specifically, the immediate next candidates for HMO utilization are *Bacteroides* species, which are known degraders of other complex versatile glycans (Hooper et al., 2002; Bjursell et al., 2006; Martens et al., 2008), like mucin coating the colon epithelium (Marcobal et al., 2011; Pruss et al., 2021). When examining the genomes of common infant gut microbes, we can computationally annotate some genes as potential HMO-utilizers (Xu et al., 2003; Yassour et al., 2016; Vatanen et al., 2019), mostly from the *Bacteroides* genus. Lastly, there is experimental evidence that various *Bacteroides* strains can utilize HMOs as their sole carbon source (Salli et al., 2021), and some of the mechanisms have been further characterized (Marcobal et al., 2011). *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* utilized both host mucus and HMOs, using a similar set of upregulated genes (Marcobal et al., 2011). The variety of findings concerning non-*Bifidobacterium* HMO-degraders highlight the importance of expanding our knowledge of HMO utilization among other gut commensals.

We therefore place *Bacteroides* at the heart of this research. First, we developed an optimized system for isolation of *Bacteroides* strains from infant stool samples, utilizing *Bacteroides*-specific qPCR and *Bacteroides*-selective media. Second, we examined the HMO utilization capacity of our *Bacteroides* isolates by analyzing growth curves on six common HMO molecules: 2-Fucosyllactose (2'-FL), Difucosyllactose (DFL), 3-Sialyllactose (3'-SL), 6-Sialyllactose (6'-SL), Lacto-N-

Tetraose (LNT) and Lacto-N-neotetraose (LNnT). Isolates often displayed similar growth characteristics on similarly-structured HMOs, like sialylated or fucosylated sugars. We identified variation in HMO utilization across multiple strains of the same species, and chose to focus here on a *Bacteroides dorei* isolate that was able to utilize the test HMOs. We performed RNA sequencing on *B. dorei* cultures, comparing the transcriptional profile in minimal media supplemented with glucose or HMO. We showed that *B. dorei* employs an extensive metabolic response to HMOs: 17 GH families were upregulated when grown on 2'-FL, 21 on DFL, 19 on 3'-SL, 23 on 6'-SL, 15 on LNT, and 18 on LNnT. In addition, *B. dorei* exhibits a generalized response to HMOs, markedly up-regulating several shared GH families across all conditions, in contrast to *Bifidobacterium* species, previously shown to employ specific GH families under different growth conditions. Within each GH family, *B. dorei* displays a consistent pattern of up-regulation of some genes with down-regulation of the others.

MATERIALS AND METHODS

Bacteroides Isolation From Stool Samples

Ten stool samples were collected from seven exclusively breastfed infants less than six month old (S4-5, S5-6, S9-10 were collected twice from the same infants with an average of one week interval). Infants were vaginally-born, and did not receive any antibiotic treatment from birth to sample collection time. All seven mothers have agreed to participate in our study, which was approved by the Hebrew University's Institutional Review Board (IRB), and signed our consent forms. Samples were collected from diapers using a small plastic spoon and tube, stored at 4°C, then transferred to -80°C within 8 hours. The samples were frozen for a maximal time of two months, and thawed twice: once for DNA extraction, and once for plating and *Bacteroides* isolation.

DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN) and used as a template for quantitative PCR (qPCR) reactions, with both general 16S primers (Ivanov et al., 2009; Yassour et al., 2018) (16S_F 5′ GGTGAATACGTTCCCGG 3′, 16S_R 5′ TACGGCTACCTTGTTACGACTT 3′) and *Bacteroides*-specific primers (Matsuki et al., 2002) (g-Bfra-F 5′ ATAGCCTTTCGAAAGRAAGAT 3′, g-Bfra-R 5′ CCAGTA TCAACTGCAATTTTA 3′). qPCR reaction volumes were 1 µL 2.5 ng/µL DNA, 0.25 µL 10µM forward primer, 0.25 µL 10µM reverse primer, 3.5 µL Nuclease-free water, 5 µL SYBR Green PCR Master Mix. The amplification program for the *Bacteroides*-specific qPCR consisted of (1) 94°C for 5 min, (2) 94°C for 20s (3) 50°C for 20 s (4) 72°C for 50 s (5) repeat 2 - 4 for 39 times (6) 94°C for 15 s. The amplification program for the general 16S qPCR consisted of (1) 95°C for 10 min, (2) 95°C for 15 s, (3) 60°C for 30 s, and (4) repeat 2 and 3 39 times. The fluorescent products were detected at the last step of each cycle, and reactions were conducted on a BioRad CFX96 Real-Time System.

The cycle threshold (Ct) value was recorded for each sample, and it is inversely proportional to the amount of *Bacteroides* DNA in the sample. The negative control samples (NC1, NC2), that did not contain *Bacteroides* according to previously obtained in-lab 16S metagenomic sequencing, had a high Ct in *Bacteroides-specific* qPCR experiments. In contrast, DNA extracted from a pure *Bacteroides fragilis* (ATCC 25285) culture, serving as a positive control, had a low Ct value.

Samples that passed the initial qPCR screening were plated on blood agar plates (hylabs, PD005) and incubated at 37°C for 48 hours in an anaerobic chamber (COY). Then, colonies were re-streaked onto Bile Esculin Agar (BEA) plates and incubated in the same conditions. PCR reaction for the 16S region was performed using the universal primers 27F AGAGTTTGATCMTGGCTCAG, 1492R GGTTACCTTGTTACGACTT (Lane, 1991). Reaction volumes were 1 µL DNA, 1.25 µL 10µM forward primer, 1.25 µL 10µM reverse primer, 9 µL Nuclease-free water, 12.5 µL SYBR NEB Q5 High-Fidelity MasterMix. The program consisted of (1) 98°C for 30 s, (2) 98°C for 10s (3) 55°C for 15 s (4) 72°C for 30 s (5) repeat 2 - 4 for 29 times (6) 72°C for 2 min. AMPure XP (Beckman Coulter, A63881) bead cleaning of PCR products was performed according to manufacturer's instructions. Then, Sanger sequencing of the 16S region was performed with the primer 515F GTGCCAGCMGCCGCGGTAA (Caporaso et al., 2011), at Hylabs (Rehovot, Israel). Bacterial identity identification was achieved using BLAST (Altschul et al., 1990) alignment with default settings.

16S Ribosomal Gene Sequencing of Stool Samples

Samples that were found to contain *Bacteroides* according to qPCR screening were chosen for 16S rRNA sequencing. 16S rRNA gene sequencing was performed as previously described (Caporaso et al., 2012), and the 16S gene data set consists of sequences targeting the V4 variable region. Sequencing was performed on the Illumina MiSeq platform according to the manufacturer's specifications, with addition of 5% PhiX, generating single-end reads of 250 bp in length. Human reads were excluded from further processing with Bowtie2 (Langmead and Salzberg, 2012), in addition to quality-based filtering of reads performed using Fastqmcf (Aronesty, 2013). Taxonomic classification was assigned using BURST (Al-Ghalith and Knights, 2020).

Growth Curves

For each growth curve experiment, an isolate from infant stool samples was plated on a brain heart infusion (BHI) agar plate (Sigma 70138) supplemented with 50 ml/L fetal bovine serum (FBS; Sigma F2442), 10 ml/L trace vitamins (ATCC® MD-VS™), 10 ml/L trace minerals (ATCC® MD-TMS™), 10 ml/L vitamin K1 and hemin (BBL, 212354), 1 g/L D-(+)-Cellobiose (Alfa Aesar, 528507), 1 g/L D-(+)-Maltose (Caisson, 6363537), 1 g/L D-(+)-Fructose (Sigma Aldrich, 1286504), and 0.5 g/L L-Cysteine (Acros Organics, 52904). The individual HMOs were received as a donation from the DSM Nutritional Products Ltd (previously known as Glycom).

The plates were incubated in an anaerobic chamber (COY) for 48 hours, at 37°C. Two single colonies (biological replicates) were transferred to liquid BHI media (Sigma 53286),

supplemented as described above, for overnight incubation. These cultures were then diluted 1:100 in *Bacteroides*-specific minimal-media (MM) with 0.5% (w/v) glucose (Martens et al., 2008). After another overnight incubation, the culture was diluted 1:100 into the same *Bacteroides*-specific minimal media, this time with individual HMOs, glucose or lactose as carbon sources (0.5% w/v). All experiments were conducted with two biological replicates and three technical replicates per carbon source, under anaerobic conditions. Growth (OD600) was monitored every 30 minutes for 60h, at 37°C, using the Epoch2 plate reader (Agilent).

Optical density data over time was analyzed using an in-house custom script, the pheatmap (RRID : SCR_016418) and the growthcurver (Sprouffske and Wagner, 2016) packages under R statistical language [R Core Team (2021)].

RNA Sequencing of *B. dorei* Cultures

Pure cultures of *B. dorei* growing on various carbon sources (in replicates) were harvested at log phase (OD ~ 0.7) using the Direct-zol™ RNA Miniprep Plus kit (Zymo research, R2071). Agilent 2100 Bioanalyzer (Agilent Technologies) was employed for RNA quality control. Samples were processed according to a previously described protocol (Shishkin et al., 2015) and sequenced in two separate pools: HMOs (2'-FL, DFL, 3'-SL, 6'-SL, LNT, LNnT) and glucose. Single-end 75bp sequencing was performed on a NextSeq device.

The raw sequencing data were further filtered by trim_galore (https://github.com/FelixKrueger/TrimGalore), and classification of the *B. dorei* isolate to the strain level was achieved by alignment of reads using BLAST (Altschul et al., 1990). Principal Component Analysis (PCA) of transcriptional profiles on the various carbon sources was performed on the VST (variance stabilizing transformation)-transformed data (Love et al., 2014). Genome mapping to *B. dorei DSM 17855* genome (GenBank: CP046176.1) was performed by Bowtie2 (Langmead and Salzberg, 2012), and differential expression analysis between HMOs and glucose was carried out using featureCounts (Liao et al., 2014) and DEseq2 (Love et al., 2014). Significantly up-regulated genes were selected based on the padj < 0.05 and log2 fold-change > 1 parameters. Gene annotation of glycoside hydrolases (GHs) was downloaded from the CAZy (Carbohydrate Active enZYmes) (Lombard et al., 2014) database. The statistical enrichment of GH genes from multiple families among the up-regulated gene group, compared to all *B. dorei*'s genes, was calculated with hypergeometric test, using the *phyper* function (*stats* package, R version 4.1.1).

RESULTS

An Optimized System for Isolation of *Bacteroides* Strains From Infant Stool

To experimentally test the ability of infant *Bacteroides* strains to utilize HMOs as a carbon source, we first need to be able to isolate these infant strains from infant stool samples. In general, the ease of strain isolation differs depending on the species, however, infant stool samples may add an additional layer of complexity due to the low bacterial biomass (compared to adult stool) (Mitchell et al., 2020). Since the isolation process is laborious and time consuming, we aimed to apply it on a selective subset of samples for which we are confident that they harbor *Bacteroides* strains. The earliest possible step to identify the samples containing *Bacteroides* strains is following DNA extraction, for example, by using qPCR. We found that the previously published g-Bfra-F/R (Matsuki et al., 2002) primers enabled a clear distinction between *Bacteroides*-containing and non-*Bacteroides*-containing samples (**Figure 1A**). To control for differences in general bacterial DNA content, we compared the *Bacteroides*-specific Ct value to the universal bacterial 16S Ct value (**Figure 1A**).

Next, we turned to optimize a system for isolation of *Bacteroides* species from samples with probable *Bacteroides* strains. We found that plating stool samples on a rich medium (such as blood agar plates) prior to plating on a more "harsh" selective medium, resulted in enhanced growth of *Bacteroides* and higher isolation success rates, perhaps due to the challenging nature of these *Bacteroides* strains (Medeiros, 1972; Pricop et al., 2020). Thus, we first plated the samples on blood agar plates, then we re-streaked multiple colonies on Bile Esculin Agar (BEA) *Bacteroides*-specific plates (Livingston et al., 1978). BEA plates contain both gentamicin, for which *Bacteroides* are inherently resistant to, and esculin, which *Bacteroides* are able to hydrolyze, producing a black pigment. Then, we picked single black colonies, and performed a second qPCR with *Bacteroides*-specific primers to exclude any non-*Bacteroides* gentamicin-resistant esculin hydrolyzing bacteria that might be present in the stool sample. Finally, Sanger sequencing of the 16S region was performed in order to receive an identification of the bacteria isolated at the genus level.

Indeed, we were able to successfully isolate 11 *Bacteroides* strains from infant samples that passed our qPCR filtering step (S4-S10, **Figure 1A**), whereas no *Bacteroides* were detected after plating and 16S sequencing of the samples that failed the qPCR step (S1-S3, **Figure 1A**).In addition, to test that the isolated strains match the overall *Bacteroides* composition of each sample, we also performed 16S sequencing the samples S4-S10 (**Figure 1B** and **Supplementary Table 1**). All isolated strains belong to species that were identified in the 16S sequencing of each sample. We were able to isolate strains at least from the most abundant species in each sample, and sometimes even from less abundant species, indicating the efficiency of our isolation method (**Figure 1B**).

Variation in HMO Utilization Across *Bacteroides* Isolates

Once we have established a strain collection composed of all successfully isolated *Bacteroides* strains from infant stool, we next wanted to examine the growth of the unique strains on different complex carbon sources. While there are many types of HMOs, in our study we use synthetic versions of HMOs from all

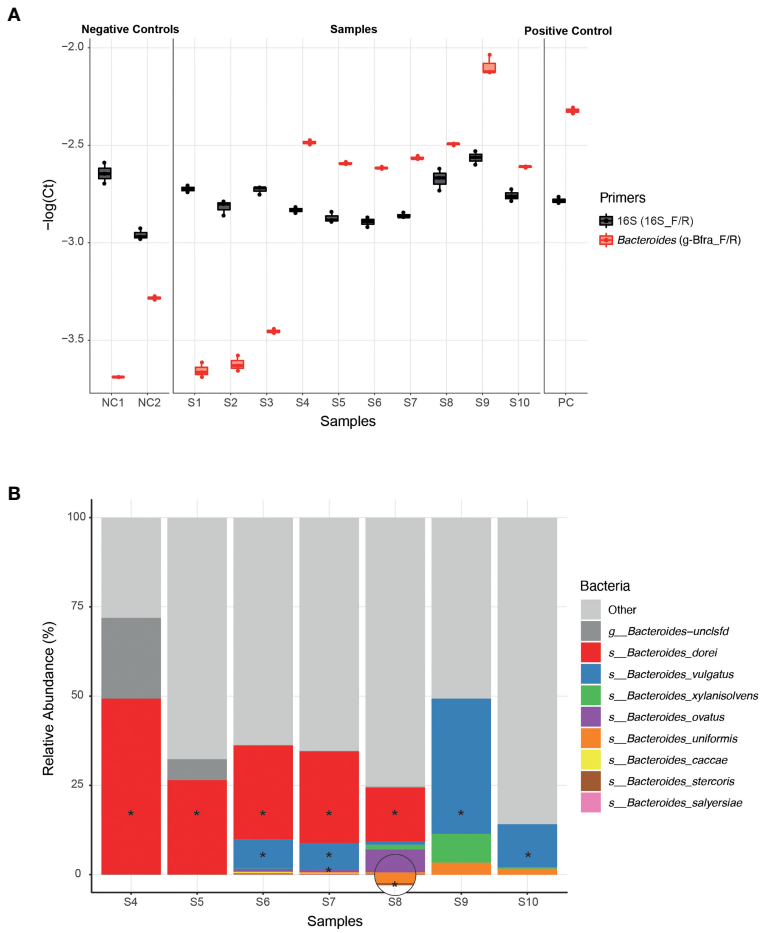


FIGURE 1 | Isolation of *Bacteroides* strains from infant stool samples. **(A)** A comparison of qPCR Ct (Cycle threshold; **Methods**) values of infant stool samples DNA, using *Bacteroides*-specific primers (g-Bfra-F, g-Bfra-R) and universal 16S primers (16_F, 16_R). Each qPCR reaction was performed in triplicates, and each replicate is indicated by a dot. **(B)** Relative abundance of *Bacteroides* species, as identified by 16S rRNA gene sequencing of stool samples found to contain *Bacteroides* according to qPCR screening. *Bacteroides* species that were successfully isolated from stool samples are indicated with asterisks.

three sub-types, including neutral, fucosylated and sialylated sugars: Lacto-N-Tetraose (LNT), Lacto-N-neotetraose (LNnT), 2′-fucosyllactose (2′-FL), difucosyllactose (DFL), 3-Sialyllactose (3'-SL), and 6-Sialyllactose (6'SL). Growth curve assays on some of the isolated strains were performed with *Bacteroides*-specific minimal media (MM) mixed with the chosen carbon source to a final concentration of 0.5% (weight/volume). Our results indicate that our isolated *Bacteroides* strains differ in their HMO utilization capability and dynamics (**Figures 2A, B** and **Supplementary Figure 1**). Some strains do not grow on any tested HMO as a single carbon source (*B. stercoris*), whereas others exhibit growth on various sugars (*B. doeri 2, B. vulgatus*; **Figures 2A, B** and **Supplementary Figure 1**). Furthermore, we also observed intra-species variation, in both *B. vulgatus* and *B. dorei* strains, where two strains of the same species exhibit distinct utilization patterns. As one might expect, although it has not been reported yet, strains tended to display similar growth characteristics on similarly structured HMOs, for example, the growth pattern of *B. dorei 2* on 3'SL is similar to its growth on 6'SL, and these sugars are both sialylated (**Figure 2B**).

To make sure that the strains cannot grow on any of the minimal media (MM) components on its own, the negative control in our growth experiments was MM without an added carbon source. The positive control was supplemented BHI media, which is a rich nonselective media that supports the growth of many bacteria. Some strains are inherently more difficult to grow in the lab, and thus we used the positive control to evaluate the ease of growth for each strain. In addition, we also measure the strains' ability to grow on glucose and lactose (the main carbohydrate in breast-milk) (Ballard and Morrow, 2013). Growth experiments can be evaluated by the kinetics of growth (using the OD600 over time), and by emphasizing the quantitative ability of a strain to utilize a carbon source (using the area under the OD600 curve; AUC; **Figure 2A**). The AUC statistic was calculated for all experiments, varied across carbon sources, yet showed consistency across biological replicates (**Figure 2A**).

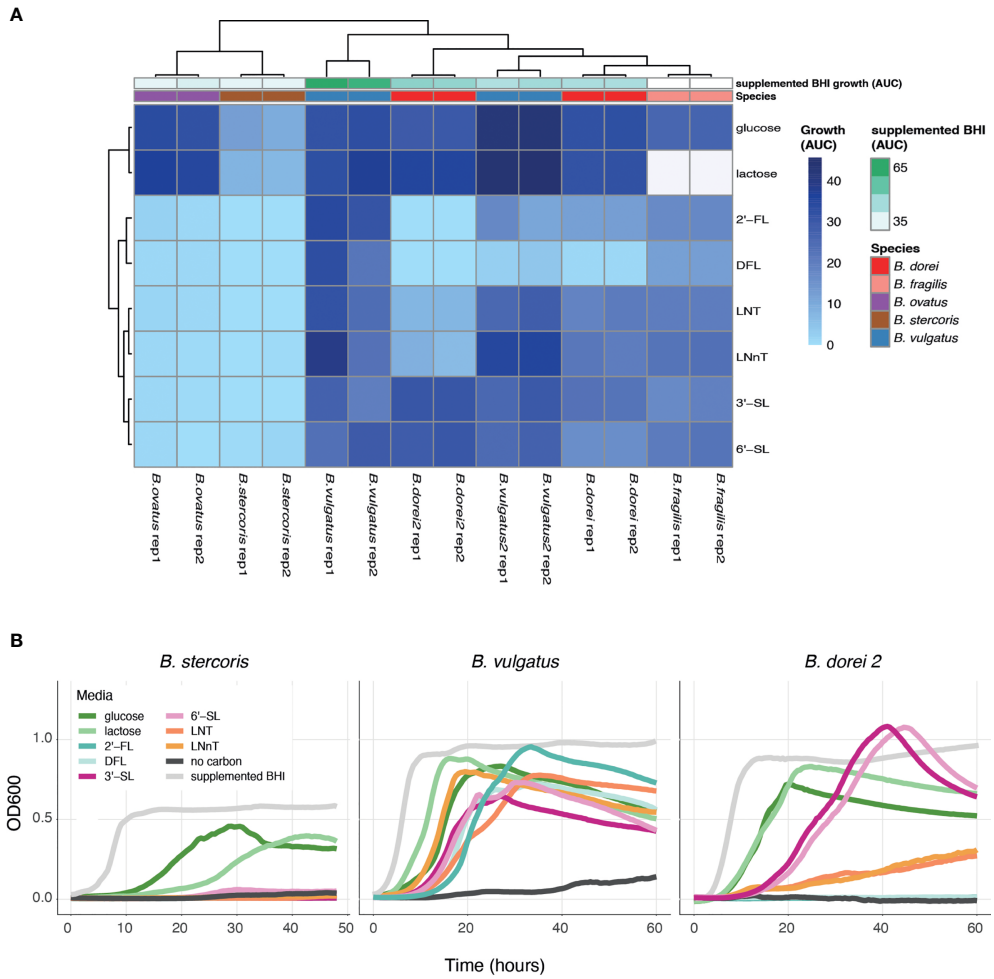


FIGURE 2 | HMO utilization ability varies across *Bacteroides* isolates. Growth curves were measured for all chosen strains and all chosen sources (0.5% weight/volume): glucose, lactose, 2-Fucosyllactose (2'-FL), Difucosyllactose (DFL), 3-Sialyllactose (3'-SL), 6-Sialyllactose (6'-SL), Lacto-N-Tetraose (LNT) and Lacto-N-neotetraose (LNnT), supplemented BHI media (positive control), no carbon media (negative control). **(A)** Area under the curve (AUC) of the growth curve plots, shown in two biological replicates for each strain. Missing data is represented by white cells, in both heat map and annotations. **(B)** Growth curve plots of *B. stercoris*, *B. vulgatus* and *B. dorei 2* on all selected carbon sources.

Searching for *B. dorei's* Differentially Expressed Genes Across Multiple HMO Conditions

Differential expression of genes under various conditions can shed light on the enzymes, mechanisms and pathways enabling the utilization of the specific carbon sources. We next chose the isolate of *Bacteroides dorei* that was able to utilize all HMOs tested to some extent, *B. dorei 1* (**Supplementary Figure 1**), to search the genes that enable this utilization. We want to compare the transcribed genes in each condition to identify the upregulated genes that can account for the HMO utilization capability. We grew *B. dorei* on the full set of HMOs described above and harvested the cultures during the mid-log phase to perform transcriptional profiling using RNA sequencing (RNA-Seq).

To identify the genes up regulated by *B. dorei* during HMO consumption, whole-genome transcriptional profiling of *B. dorei* on MM supplemented with individual HMOs was compared to growth on MM-glucose (**Supplementary Table 2** and **Supplementary Figure 2**). The two biological replicates of each condition were similar to one another, and more similar within a condition than across conditions (**Supplementary Figure 3**). Fucosylated sugars (2'-FL, DFL) clustered separately from sialylated (3'-SL, 6'-SL), and HMO core structures (LNT, LNnT) clustered in-between (**Figure 3A**). A possible explanation could be that LNT and LNnT lack unique sialyl or fucosyl residues distinguishing them from the other sugars. Overall, the examined conditions are relatively similar, as the tested sugars differ from one another by small modifications only. Thus, we expected the overall gene-expression profiles to be rather similar, with small, yet distinct differences, across all conditions (**Supplementary Figure 3**).

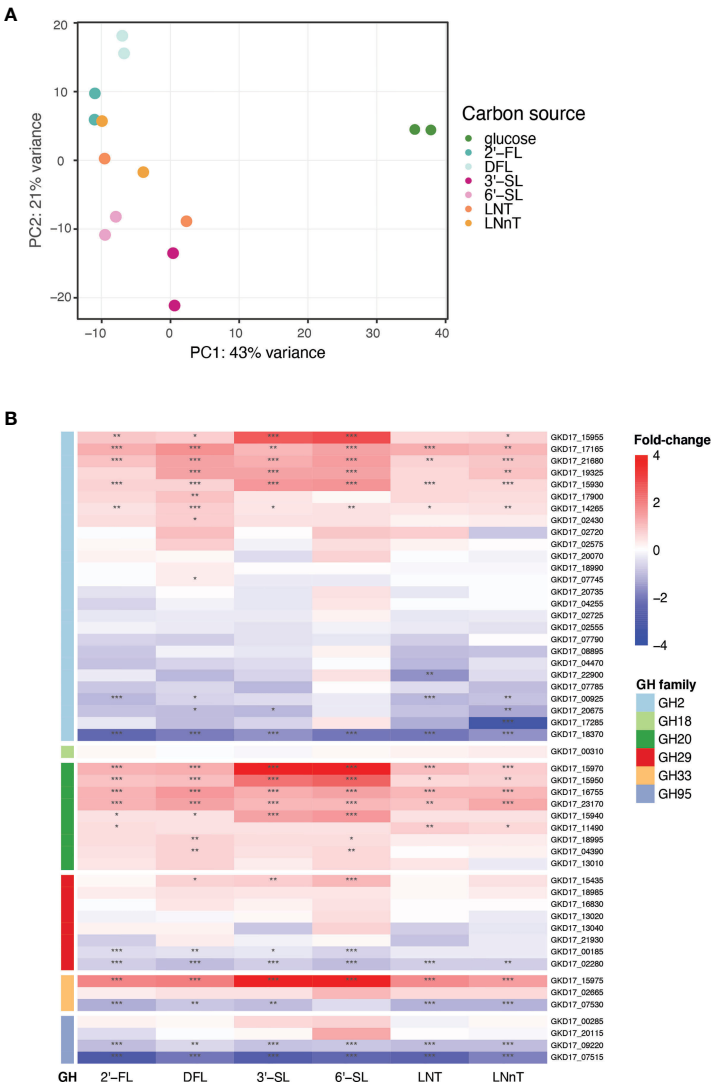


FIGURE 3 | *B. dorei's* transcriptional response to HMOs is similar across conditions. **(A)** Principal Component Analysis (PCA) of transcriptional profiles of pure cultures of *B. dorei* on various carbon sources, in two biological replicates, using RNA-Sequencing (Methods). **(B)** Heat map of log2 fold-change of genes (rows) belonging to glycoside hydrolase (GH) families previously known to break down HMOs. Gene expression data on HMOs (2'-FL, DFL, 3'-SL, 6'-SL, LNT, LNnT; columns) was compared to gene expression on glucose. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

B. dorei possesses a repertoire of predicted glycoside hydrolases (GHs), carbohydrate active enzymes (CAZy) capable of breaking the carbon bond found in all human milk oligosaccharides. *B. dorei's* 213 glycoside hydrolases comprise 59 unique GH families (Lombard et al., 2014). Differential expression analysis of the RNA-Seq data revealed that *B. dorei* exhibits an expansive glycoside hydrolase response during consumption of HMOs *in vitro*. Many unique GH families were upregulated (log2foldChange > 1, padj < 0.05) when growing on HMOs compared to glucose: 17 GH families were upregulated when grown on 2'-FL, 21 on DFL, 19 on 3'-SL, 23 on 6'-SL, 15 on LNT, and 18 on LNnT.

First, we examined the differential expression of GH families previously annotated as capable of breaking down the HMO carbon bond (Marcobal et al., 2011): GH2, GH18, GH20, GH29, GH33, and GH95 (**Figure 3B**). Two additional GH families have been reported in the literature (GH85, GH112) (Ioannou et al., 2021), however they are not encoded in the *B. dorei* genome. Most research regarding HMO utilization by infant gut microbes has focused on *Bifidobacterium* species, hence the knowledge on *Bacteroides* cellular response to HMOs is rather limited. Indeed, some GH families that are well annotated in *Bifidobacterium* species did not exhibit any clear differential expression here, like GH18 and GH95. Surprisingly, some of the GH95 and GH29 *B. dorei* genes, which break the α1-2 and α1-3/4 fucosyl bonds, accordingly, were up-regulated in non-fucosylated HMOs. This lack of specificity is unexpected as we would expect GH95 and GH29 to be upregulated only on fucosylated HMOs. Similarly,

GH33 which is responsible for sialic acid release from sialylated HMOs, was up-regulated across all HMO conditions (sialylated and non-sialylated HMOs), although the up-regulation in sialylated oligosaccharides is the most prominent. Lastly, in GH2, the largest GH family in *B. dorei*'s genome (Lombard et al., 2014), some genes were up-regulated while others were down regulated, which might be confusing, and is further discussed below.

Multiple Glycoside Hydrolase Families Are Upregulated During *B. dorei's* Growth on HMOs

We next performed an unbiased search for all up-regulated GH families in our data, regardless of their annotations. To identify the GH families that could be responsible for *B. dorei*'s HMO utilization, we searched for all GH families that were upregulated in similarly-structured HMO-pairs: 2'-FL and DFL, 3'-SL and 6'-SL, LNT and LNnT (**Figure 4A**). A GH family that is upregulated in two structurally-similar HMOs is more likely to play a role in breaking down these common carbon bonds.

First, we focused on GH families that had a minimum of two upregulated genes for at least one HMO-pair, and found five such GH families: GH2, G20, GH43, GH92, and GH97 (**Figure 4B**). We then expanded our analysis to all genes that belong to these families to compare their regulation across the various HMO structures (**Figure 4C**). We did not find a specific GH family response for a specific HMO-pair, rather, it seems that *B. dorei*'s response to growth on various HMOs is quite generalized, regardless of the specific HMO molecule. Furthermore, even though the response is not specific towards a single HMO, it is specific in the transcriptional changes. Namely, in most cases, only a small subset of the genes in each GH family were up-regulated, and the others were often even down-regulated, suggesting that a specific set of genes, rather than all genes in a certain GH family, responded to the change in carbon source. For example, we found that among *B. dorei*'s 27 GH2 genes, two genes only (GKD17_17165, GKD17_15930) were consistently, significantly upregulated for all conditions. Likewise, for GH43 and GH97, GKD17_02610 and GKD17_19390 were the only consistently upregulated genes, respectively (**Figure 4C**).

Second, we were interested to inspect if the pattern of simultaneous up- and down-regulation of different genes from the same GH family is evident for additional GH families. We looked at GH families for which only a single gene was upregulated in at least one HMO-pair (**Figure 4D**), as a single up-regulated gene can be sufficient for HMO utilization. Similarly to the analysis in the first step (**Figure 4C**), oftentimes, for each GH family, only one or two specific genes were consistently up-regulated, while other members of the family were down-regulated compared to MM-glucose. This observation of specificity in upregulated genes was also evident in smaller GH families, such as GH3 (GKD17_22625), GH33 (GKD17_15975), GH36 (GKD17_20055), GH51 (GKD17_18785). Lastly, GH140 with only two genes presented an interesting pattern, where one was strongly upregulated (GKD17_02615) while the other was strongly down-regulated (GKD17_07525), which may merit further study of this particular GH family. Interestingly, when looking at all GH28 genes encoded in *B. dorei*'s genome, our results suggest that all conditions invoke a strong down-regulation response for many of the GH genes. This consistent broad down-regulation might indicate the up-regulation of other, yet to be annotated, GH genes.

Additionally, enrichment analysis of the shared upregulated GH families revealed that some GH families were enriched in the upregulated gene group compared to the total amount of genes in *B. dorei*'s genome: GH20, GH43 and GH92 were significantly ($p<0.05$) enriched for DFL, 3'-SL and 6'-SL, GH3 was significantly ($p<0.05$) enriched for LNT, LNnT, 3'-SL and 6'-SL, GH2 was significantly ($p<0.05$) enriched for 3'-SL and 6'-SL, and GH36 was significantly ($p<0.05$) enriched for 6'-SL.

DISCUSSION

In our quest to investigate HMO utilization by *Bacteroides* infant strains, we have established a system for screening, culturing, measuring growth curves and performing RNA sequencing of *Bacteroides* isolates. We constructed growth curves of *Bacteroides* isolates from infant stool and demonstrated variation in HMO-utilization patterns across *Bacteroides* species and within strains of the same species. Some *Bacteroides* isolates do not grow on HMOs at all, whereas others can utilize all tested HMOs to some extent. This is consistent with previous studies, showing that *Bacteroides* are metabolically diverse and are capable of utilizing many versatile carbon sources (Salyers et al., 1977; Sonnenburg et al., 2005; Bjursell et al., 2006; Marcobal et al., 2011; Wexler and Goodman, 2017; Carrow et al., 2020; García-Bayona and Comstock, 2020; Fultz et al., 2021). Pioneering work in *Bacteroides* demonstrated *B. thetaiotomicron*'s ability to expand and adapt its metabolism, from mainly utilizing glucose and lactose, to also being able to break down more complex plant polysaccharides (Bjursell et al., 2006). Thus, species isolated from infant stool at different time points might alter their metabolic repertoire as the infant ages. A strain's ability to utilize a wide variety of HMOs may confer a selective advantage over strains that do not utilize these sugars, and could select for its survival in the infant gut.

Our growth curve results demonstrate similar growth patterns on similarly structured HMOs, which may suggest unique carbohydrate utilization pathways being activated for HMOs with similar residues. *Bacteroides* are known to have a wide range of polysaccharide utilization loci (PUL) that encode common classes of proteins involved in polysaccharide utilization, and a large percentage of their genome is dedicated to the sensing, import, and hydrolysis of diverse glycans (Bjursell et al., 2006; Martens et al., 2008). However, RNA-Seq experiments do not show unique genes upregulated under specific conditions. When examining differential expression of glycoside hydrolase (GH) genes of various families, a generalized HMO response was observed, with several GH families upregulated across all six

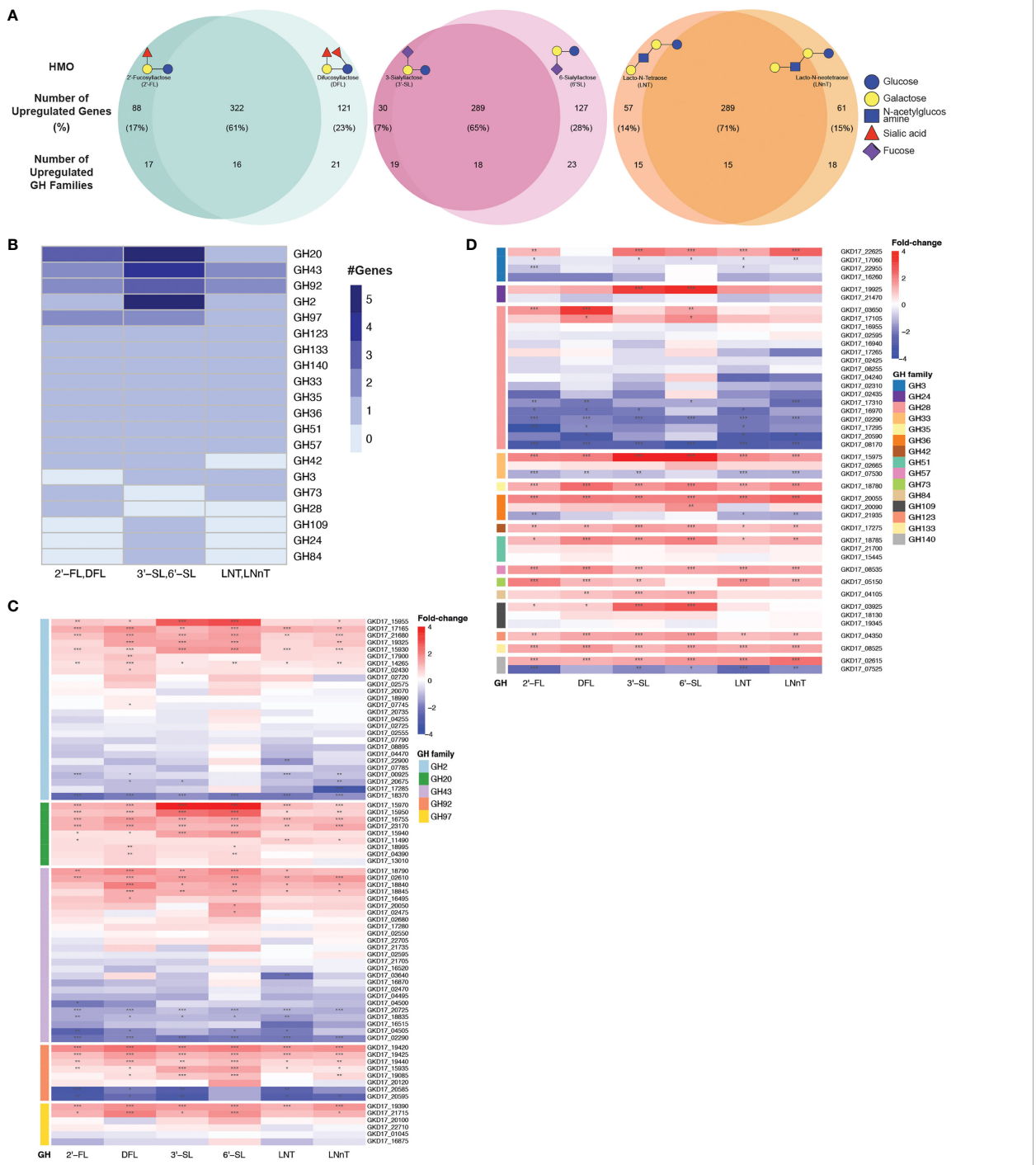


FIGURE 4 | *B. dorei* shares up-regulated genes upon growth on HMOs compared to glucose. **(A)** Venn diagrams of *B. dorei*'s up-regulated genes and glycoside hydrolase (GH) families across HMO types, highlighting the similar transcriptional response to HMOs, regardless of specific conditions. HMOs were divided into pairs based on structural similarity. Percentage values refer to the fraction of up-regulated genes from the total up-regulated genes in both members of an HMO pair. **(B)** A heat map showing the number of up-regulated genes in each up-regulated GH family in at least one HMO pair. **(C, D)** Heat maps of log2 fold-change of genes (rows) belonging to GH families with **(C)** at least two; or **(D)** a single up-regulated gene(s). Gene expression data on HMOs (2'-FL, DFL, 3'-SL, 6'-SL, LNT, LNnT; columns) was compared to gene expression on glucose. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

HMOs tested: 2'-FL, DFL, 3'-SL, 6'-SL, LNT, and LNnT. Hence, our findings suggest a unique HMO-utilization strategy among some *Bacteroides* strains, diverging from the extensively studied *Bifidobacterium* HMO utilization mechanisms, where distinct enzymes were recognized for specific carbon bonds in HMO molecules. A possible explanation for the similar response of *B. dorei* to all tested HMOs could be that overall, the conditions tested are rather close, with identical minimal media composition for all samples and small changes in HMO modifications.

Bifidobacterium species are considered to be well-adapted to HMO utilization, and they have evolved two distinct strategies for this purpose. One is transporter-dependent (intracellular digestion strategy), and the other is extracellular glycosidase-dependent (extracellular digestion strategy), utilizing the same enzymes, only in a membrane-bound form (Sela et al., 2008; Turroni et al., 2014; Bunesova et al., 2016). Both strategies employ specific enzymes (Ioannou et al., 2021), some of them clustered in specific genomic regions (Sela et al., 2008; LoCascio et al., 2010) whereas others are spread across additional genomic locations (Zabel et al., 2020). In contrast, very little is known about *Bacteroides* HMO utilization, and only a few papers have discussed this issue in terms of phenotypic growth (Salli et al., 2021) or transcriptional profiling (Marcobal et al., 2011). Due to the high-prevalence of *Bacteroides* in the infant gut and a seeming deviation from the traditional *Bifidobacterium*-dominated infant gut microbiome (Vatanen et al., 2019; Casaburi et al., 2021), it is increasingly important to decipher the HMO utilization strategies employed by *Bacteroides* species as well.

B. dorei's differential expression pattern in response to HMOs does not appear to be HMO-specific, however, we show that for the upregulated GH families in our data, a particular set of genes is up-regulated among all members of the GH family encoded in the genome. Moreover, the remaining members of the GH families explored are down-regulated, perhaps meaning that *B. dorei*'s response to HMOs is tailored to exploit some GH genes over others. The novel pattern of HMO utilization presented here can indicate that for a given *Bacteroides* strain, we can discuss specific genes responsible for HMO break-down, in addition to discussing the results on the broader, less-selective, GH family level.

The pioneering work of exploring HMO utilization mechanisms by the type strains of *B. thetaiotaomicron* and *B. fragilis* (Marcobal et al., 2011) found that certain genes are heavily upregulated upon growth on HMOs. These genes belong to several GH families: GH2, GH18, GH20, GH29, GH33, and GH95. There are a few notable differences between this early study and our results. First, the researchers used an HMO mixture, while we used individual HMO molecules. Second, to our knowledge, no study has looked at the transcriptional response of *Bacteroides* strains that were isolated from infant sool to HMOs, which could be different from the type strain response, due to large variation across *Bacteroides* strains of the same species. Lastly, in the 2011 manuscript, the transcriptional response was examined on a pre-selected set of genes (using microarrays designed to capture these specific RNA molecules), whereas we used an unbiased approach of total RNA sequencing. These differences make it difficult to compare the results of both studies, highlighting the need for additional research in additional species, preferably in an unbiased manner.

Notably, the *Bacteroides* genus is characterized by great variations in carbon-utilization abilities, both across species and within strains of the same species (Lange et al., 2016; Husain et al., 2017; Pasolli et al., 2019). Therefore, our findings, exploring one isolate only, do represent a general HMO utilization pattern, and every *Bacteroides* strain or isolate of interest should be individually examined. Second, we compared gene expression profiles of *B. dorei* cultures grown with various HMOs as a single carbon source, in comparison to MM-glucose only. While glucose is the most common reference in microbiology literature, follow up studies should also include galactose and lactose as a reference. Lastly, we should keep in mind that bacteria in the infant gut are simultaneously exposed to multiple types of HMOs, in the context of a complex microbial ecosystem. Experiments such as the ones presented test the utilization capacity of individual strains with a specific HMO molecule. These studies are critical stepping stones towards constructing a comprehensive map of how the infant gut microbes can utilize glycans found in breastmilk, thus shedding light on how breastfeeding, and breastmilk composition, impact the nursing infant gut microbiome.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) repository, accession number PRJNA804725.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hebrew University's Institutional Review Board (IRB) (approval number 20042021). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SK - Established the experimental system, performed experiments and analysis, and wrote the manuscript. AC-Assisted in establishing the experimental system. MY- Guided the work and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded in part by the Azrieli Foundation grant for faculty fellows. SK, AC, and MY were supported by the Azrieli Foundation and MY was also supported by the Israel Science Foundation grant 2660/18.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.854122/full#supplementary-material

Supplementary Figure 1 | (A) Growth curves of *B. dorei*, *B. vulgatus 2*, *B. ovatus*, and *B. fragilis* on various HMOs (2'-FL, DFL, 3'-SL, 6'-SL, LNT and LNnT), glucose, lactose, supplemented BHI media (positive control), and no carbon media (negative control). **(B)** Principal Component Analysis (PCA) of *Bacteroides* isolates based on the ability to grow on all media types, as measured by the area under the curve (AUC) of each growth curve.

Supplementary Figure 2 | MA plots of HMO transcriptional profiles compared to glucose, shown as M (log ratio) on the y-axis and A (mean average) on the x-axis. Here, the log ratio is calculated as the log2 of the change in expression values of the HMOs vs. glucose, and the mean average is of the glucose read counts. Genes from GH families are labeled with larger dots and display the GH family they belong to. The plot highlights up-regulated (red) and down-regulated (blue) genes, using a threshold of 1 for the absolute log2 fold change, and a threshold of 4 for the log2 glucose mean read count.

Supplementary Figure 3 | *B. dorei*'s transcriptional profile, as characterized by RNA-Seq experiments, is similar across conditions. The scatterplots display the normalized read count value for all annotated genes in *B. dorei*'s genome (dots), compared between replicates of the same carbon source (diagonal plots) and between different carbon sources (bottom triangle). Pearson coefficient was calculated for each comparison.

Supplementary Table 1 | Bacterial relative abundances (%) as detected by 16S ribosomal RNA gene sequencing, at the species level.

Supplementary Table 2 | *B. dorei*'s transcriptional response upon growth in minimal media (MM) supplemented with various HMOs, compared to MM-glucose. Each sheet represents a different HMO compared to glucose, and the last sheet contains explanations of all columns in previous sheets.

REFERENCES

Al-Ghalith, G., and Knights, D. (2020). BURST Enables Mathematically Optimal Short-Read Alignment for Big Data. *Zenodo*. doi: 10.1101/2020.09.08.287128. 2020.09.08.287128.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic Local Alignment Search Tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Aronesty, E. (2013). Comparison of Sequencing Utility Programs. *Open Bioinforma. J.* 7, 1–8. doi: 10.2174/1875036201307010001

Azad, M. B., Konya, T., Maughan, H., Guttman, D. S., Field, C. J., Chari, R. S., et al. (2013). Gut Microbiota of Healthy Canadian Infants: Profiles by Mode of Delivery and Infant Diet at 4 Months. *CMAJ* 185, 385–394. doi: 10.1503/cmaj.121189

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and Stabilization of the Human Gut Microbiome During the First Year of Life. *Cell Host Microbe* 17, 690–703. doi: 10.1016/j.chom.2015.04.004

Bai, Y., Tao, J., Zhou, J., Fan, Q., Liu, M., Hu, Y., et al. (2018). Fucosylated Human Milk Oligosaccharides and N-Glycans in the Milk of Chinese Mothers Regulate the Gut Microbiome of Their Breast-Fed Infants During Different Lactation Stages. *mSystems* 3, e00206–18. doi: 10.1128/mSystems.00206-18

Ballard, O., and Morrow, A. L. (2013). Human Milk Composition: Nutrients and Bioactive Factors. *Pediatr. Clin. North Am.* 60, 49–74. doi: 10.1016/j.pcl.2012.10.002

Bjursell, M. K., Martens, E. C., and Gordon, J. I. (2006). Functional Genomic and Metabolic Studies of the Adaptations of a Prominent Adult Human Gut Symbiont, Bacteroides Thetaiotaomicron, to the Suckling Period. *J. Biol. Chem.* 281, 36269–36279. doi: 10.1074/jbc.M606509200

Borewicz, K., Gu, F., Saccenti, E., and Arts, I. C. W. (2019). Correlating Infant Fecal Microbiota Composition and Human Milk Oligosaccharide Consumption by Microbiota of 1-Month-Old Breastfed Infants. *Mol. Nutr. Food Res.* 63 (13), e1801214. doi: 10.1002/mnfr.201801214

Borewicz, K., Gu, F., Saccenti, E., Hechler, C., Beijers, R., de Weerth, C., et al. (2020). The Association Between Breastmilk Oligosaccharides and Faecal Microbiota in Healthy Breastfed Infants at Two, Six, and Twelve Weeks of Age. *Sci. Rep.* 10, 4270. doi: 10.1038/s41598-020-61024-z

Buffie, C. G., and Pamer, E. G. (2013). Microbiota-Mediated Colonization Resistance Against Intestinal Pathogens. *Nat. Rev. Immunol.* 13, 790–801. doi: 10.1038/nri3535

Bunesova, V., Lacroix, C., and Schwab, C. (2016). Fucosyllactose and L-Fucose Utilization of Infant Bifidobacterium Longum and Bifidobacterium Kashiwanohense. *BMC Microbiol.* 16, 248. doi: 10.1186/s12866-016-0867-4

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global Patterns of 16S rRNA Diversity at a Depth of Millions of Sequences Per Sample. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl 1, 4516–4522. doi: 10.1073/pnas.1000080107

Carrow, H. C., Batachari, L. E., and Chu, H. (2020). Strain Diversity in the Microbiome: Lessons From Bacteroides Fragilis. *PloS Pathog.* 16, e1009056. doi: 10.1371/journal.ppat.1009056

Casaburi, G., Duar, R. M., Brown, H., Mitchell, R. D., Kazi, S., Chew, S., et al. (2021). Metagenomic Insights of the Infant Microbiome Community Structure and Function Across Multiple Sites in the United States. *Sci. Rep.* 11, 1472. doi: 10.1038/s41598-020-80583-9

Chichlowski, M., Shah, N., Wampler, J. L., Wu, S. S., and Vanderhoof, J. A. (2020). Bifidobacterium Longum Subspecies Infantis (B. Infantis) in Pediatric Nutrition: Current State of Knowledge. *Nutrients* 12, 1581–1599. doi: 10.3390/nu12061581

Davis, J. C. C., Totten, S. M., Huang, J. O., Nagshbandi, S., Kirmiz, N., Garrido, D. A., et al. (2016). Identification of Oligosaccharides in Feces of Breast-Fed Infants and Their Correlation With the Gut Microbial Community. *Mol. Cell. Proteomics* 15, 2987–3002. doi: 10.1074/mcp.M116.060665

Dethlefsen, L., McFall-Ngai, M., and Relman, D. A. (2007). An Ecological and Evolutionary Perspective on Human-Microbe Mutualism and Disease. *Nature* 449, 811–818. doi: 10.1038/nature06245

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al. (2010). Delivery Mode Shapes the Acquisition and Structure of the Initial Microbiota Across Multiple Body Habitats in Newborns. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11971–11975. doi: 10.1073/pnas.1002601107

Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G., and Daniel, H. (2000). Human Milk Oligosaccharides are Resistant to Enzymatic Hydrolysis in the Upper Gastrointestinal Tract. *Am. J. Clin. Nutr.* 71, 1589–1596. doi: 10.1093/ajcn/71.6.1589

Favier, C. F., Vaughan, E. E., De Vos, W. M., and Akkermans, A. D. L. (2002). Molecular Monitoring of Succession of Bacterial Communities in Human Neonates. *Appl. Environ. Microbiol.* 68, 219–226. doi: 10.1128/AEM.68.1.219-226.2002

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., et al. (2018). Mother-To-Infant Microbial Transmission From Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe* 24, 133–145.e5. doi: 10.1016/j.chom.2018.06.005

Fultz, R., Ticer, T., Ihekweazu, F. D., Horvath, T. D., Haidacher, S. J., Hoch, K. M., et al. (2021). Unraveling the Metabolic Requirements of the Gut Commensal Bacteroides Ovatus. *Front. Microbiol.* 12, 745469. doi: 10.3389/fmicb.2021.745469

García-Bayona, L., and Comstock, L. E. (2020). Utilizing Ribose Compounds: How Bacteroides PUL It Off. *Cell Host Microbe* 27, 6–8. doi: 10.1016/j.chom.2019.12.010

Gnoth, M. J., Kunz, C., Kinne-Saffran, E., and Rudloff, S. (2000). Human Milk Oligosaccharides are Minimally Digested *In Vitro*. *J. Nutr.* 130, 3014–3020. doi: 10.1093/jn/130.12.3014

Harmsen, H. J., Wildeboer-Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., et al. (2000). Analysis of Intestinal Flora Development in Breast-Fed and Formula-Fed Infants by Using Molecular Identification and Detection Methods. *J. Pediatr. Gastroenterol. Nutr.* 30, 61–67. doi: 10.1097/00005176-200001000-00019

Hegar, B., Wibowo, Y., Basrowi, R. W., Ranuh, R. G., Sudarmo, S. M., Munasir, Z., et al. (2019). The Role of Two Human Milk Oligosaccharides, 2′-Fucosyllactose and Lacto-N-Neotetraose, in Infant Nutrition. *Pediatr. Gastroenterol. Hepatol. Nutr.* 22, 330–340. doi: 10.5223/pghn.2019.22.4.330

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How Host-Microbial Interactions Shape the Nutrient Environment of the Mammalian Intestine. *Annu. Rev. Nutr.* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259

Husain, F., Tang, K., Veeranagouda, Y., Boente, R., Patrick, S., Blakely, G., et al. (2017). Novel Large-Scale Chromosomal Transfer in Bacteroides Fragilis Contributes to its Pan-Genome and Rapid Environmental Adaptation. *Microb. Genom.* 3, e000136. doi: 10.1099/mgen.0.000136

Ioannou, A., Knol, J., and Belzer, C. (2021). Microbial Glycoside Hydrolases in the First Year of Life: An Analysis Review on Their Presence and Importance in Infant Gut. *Front. Microbiol.* 12, 631282. doi: 10.3389/fmicb.2021.631282

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* 139, 485–498. doi: 10.1016/j.cell.2009.09.033

Jacobi, S. K., Yatsunenko, T., Li, D., Dasgupta, S., Yu, R. K., Berg, B. M., et al. (2016). Dietary Isomers of Sialyllactose Increase Ganglioside Sialic Acid Concentrations in the Corpus Callosum and Cerebellum and Modulate the Colonic Microbiota of Formula-Fed Piglets. *J. Nutr.* 146, 200–208. doi: 10.3945/jn.115.220152

Lane, D. J. (1991). 16S/23S rRNA Sequencing. In: Stackebrandt, E., and Goodfellow, M., Eds., *Nucleic Acid Techniques in Bacterial Systematic*. (New York: John Wiley and Sons), 115–175. doi: 10.1177/016344391013001010

Lange, A., Beier, S., Steimle, A., Autenrieth, I. B., Huson, D. H., and Frick, J.-S. (2016). Extensive Mobilome-Driven Genome Diversification in Mouse Gut-Associated Bacteroides Vulgatus Mpk. *Genome Biol. Evol.* 8, 1197–1207. doi: 10.1093/gbe/evw070

Langmead, B., and Salzberg, S. L. (2012). Fast Gapped-Read Alignment With Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

La Rosa, P. S., Warner, B. B., Zhou, Y., Weinstock, G. M., Sodergren, E., Hall-Moore, C. M., et al. (2014). Patterned Progression of Bacterial Populations in the Premature Infant Gut. *Proc. Natl. Acad. Sci. U. S. A.* 111, 12522–12527. doi: 10.1073/pnas.1409497111

Laursen, M. F., Pekmez, C. T., Larsson, M. W., Lind, M. V., Yonemitsu, C., Larnkjær, A., et al. (2021). Maternal Milk Microbiota and Oligosaccharides Contribute to the Infant Gut Microbiota Assembly. *ISME Commun.* 1, 1–13. doi: 10.1038/s43705-021-00021-3

Lawson, M. A. E., O'Neill, I. J., Kujawska, M., Gowrinadh Javvadi, S., Wijeyesekera, A., Flegg, Z., et al. (2020). Breast Milk-Derived Human Milk Oligosaccharides Promote Bifidobacterium Interactions Within a Single Ecosystem. *ISME J.* 14, 635–648. doi: 10.1038/s41396-019-0553-2

Lax, S., Smith, D. P., Hampton-Marcell, J., Owens, S. M., Handley, K. M., Scott, N. M., et al. (2014). Longitudinal Analysis of Microbial Interaction Between Humans and the Indoor Environment. *Science*. 345, 1048–1052. doi: 10.1126/science.1254529

Letten, A. D., Baumgartner, M., Pfrunder-Cardozo, K. R., Levine, J. M., and Hall, A. R. (2021a). Human-Associated Microbiota Suppress Invading Bacteria Even Under Disruption by Antibiotics. *ISME J.* 15, 2809–2812. doi: 10.1038/s41396-021-00929-7

Letten, A. D., Hall, A. R., and Levine, J. M. (2021b). Using Ecological Coexistence Theory to Understand Antibiotic Resistance and Microbial Competition. *Nat. Ecol. Evol.* 5, 431–441. doi: 10.1038/s41559-020-01385-w

Liao, Y., Smyth, G. K., and Shi, W. (2014). Featurecounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics* 30, 923–930. doi: 10.1093/bioinformatics/btt656

Liu, F., Yan, J., Wang, X., Wang, C., Chen, L., Li, Y., et al. (2021). Maternal Fucosyltransferase 2 Status Associates With the Profiles of Human Milk Oligosaccharides and the Fecal Microbiota Composition of Breastfed Infants. *J. Agric. Food Chem.* 69, 3032–3043. doi: 10.1021/acs.jafc.0c04575

Livingston, S. J., Kominos, S. D., and Yee, R. B. (1978). New Medium for Selection and Presumptive Identification of the Bacteroides Fragilis Group. *J. Clin. Microbiol.* 7, 448–453. doi: 10.1128/jcm.7.5.448-453.1978

LoCascio, R. G., Desai, P., Sela, D. A., Weimer, B., and Mills, D. A. (2010). Broad Conservation of Milk Utilization Genes in Bifidobacterium Longum Subsp. Infantis as Revealed by Comparative Genomic Hybridization. *Appl. Environ. Microbiol.* 76, 7373–7381. doi: 10.1128/AEM.00675-10

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B. (2014). The Carbohydrate-Active Enzymes Database (CAZy) in 2013. *Nucleic Acids Res.* 42, D490–D495. doi: 10.1093/nar/gkt1178

Love, M. I., Huber, W., and Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With Deseq2. *Genome Biol.* 15, 550. doi: 10.1186/s13059-014-0550-8

Madan, J. C., Hoen, A. G., Lundgren, S. N., Farzan, S. F., Cottingham, K. L., Morrison, H. G., et al. (2016). Association of Cesarean Delivery and Formula Supplementation With the Intestinal Microbiome of 6-Week-Old Infants. *JAMA Pediatr.* 170, 212–219. doi: 10.1001/jamapediatrics.2015.3732

Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., et al. (2011). Bacteroides in the Infant Gut Consume Milk Oligosaccharides *via* Mucus-Utilization Pathways. *Cell Host Microbe* 10, 507–514. doi: 10.1016/j.chom.2011.10.007

Marcobal, A., and Sonnenburg, J. L. (2012). Human Milk Oligosaccharide Consumption by Intestinal Microbiota. *Clin. Microbiol. Infect.* 18 (Suppl 4), 12–15. doi: 10.1111/j.1469-0691.2012.03863.x

Martens, E. C., Chiang, H. C., and Gordon, J. I. (2008). Mucosal Glycan Foraging Enhances Fitness and Transmission of a Saccharolytic Human Gut Bacterial Symbiont. *Cell Host Microbe* 4, 447–457. doi: 10.1016/j.chom.2008.09.007

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., et al. (2002). Development of 16S rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of Predominant Bacteria in Human Feces. *Appl. Environ. Microbiol.* 68, 5445–5451. doi: 10.1128/AEM.68.11.5445-5451.2002

McLaren, M. R., and Callahan, B. J. (2020). Pathogen Resistance may be the Principal Evolutionary Advantage Provided by the Microbiome. *Philos. Trans. R. Soc Lond. B Biol. Sci.* 375, 20190592. doi: 10.1098/rstb.2019.0592

Medeiros, A. A. (1972). Bacteroides Bacillemia. Sepsis for Surgeons. *Arch. Surg.* 105, 819–820. doi: 10.1001/archsurg.1972.04180120006001

Mitchell, C. M., Mazzoni, C., Hogstrom, L., Bryant, A., Bergerat, A., Cher, A., et al. (2020). Delivery Mode Affects Stability of Early Infant Gut Microbiota. *Cell Rep. Med.* 1, 100156. doi: 10.1016/j.xcrm.2020.100156

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., and Brown, P. O. (2007). Development of the Human Infant Intestinal Microbiota. *PloS Biol.* 5, e177. doi: 10.1371/journal.pbio.0050177

Pasolli, E., Asnicar, F., Manara, S., Zolfo, M., Karcher, N., Armanini, F., et al. (2019). Extensive Unexplored Human Microbiome Diversity Revealed by Over 150,000 Genomes From Metagenomes Spanning Age, Geography, and Lifestyle. *Cell* 176, 649–662.e20. doi: 10.1016/j.cell.2019.01.001

Plaza-Díaz, J., Fontana, L., and Gil, A. (2018). Human Milk Oligosaccharides and Immune System Development. *Nutrients* 10, 1038–1055. doi: 10.3390/nu10081038

Pricop, G. R., Gheorghe, I., Pircalabioru, G. G., Cristea, V., Popa, M., Marutescu, L., et al. (2020). Resistance and Virulence Features of Bacteroides Spp. Isolated From Abdominal Infections in Romanian Patients. *Pathogens* 9, 940–950. doi: 10.3390/pathogens9110940

Pruss, K. M., Marcobal, A., Southwick, A. M., Dahan, D., Smits, S. A., Ferreyra, J. A., et al. (2021). Mucin-Derived O-Glycans Supplemented to Diet Mitigate Diverse Microbiota Perturbations. *ISME J.* 15, 577–591. doi: 10.1038/s41396-020-00798-6

R Core Team (2021). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing* (Vienna, Austria). Available at: https://www.R-project.org/.

Salli, K., Hirvonen, J., Siitonen, J., Ahonen, I., Anglenius, H., and Maukonen, J. (2021). Selective Utilization of the Human Milk Oligosaccharides 2'-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose by Various Probiotic and Pathogenic Bacteria. *J. Agric. Food Chem.* 69, 170–182. doi: 10.1021/acs.jafc.0c06041

Salyers, A. A., Vercellotti, J. R., West, S. E., and Wilkins, T. D. (1977). Fermentation of Mucin and Plant Polysaccharides by Strains of Bacteroides From the Human Colon. *Appl. Environ. Microbiol.* 33, 319–322. doi: 10.1128/aem.33.2.319-322.1977

Sela, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., et al. (2008). The Genome Sequence of Bifidobacterium Longum Subsp. Infantis Reveals Adaptations for Milk Utilization Within the Infant Microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 105, 18964–18969. doi: 10.1073/pnas.0809584105

Sela, D. A., and Mills, D. A. (2010). Nursing Our Microbiota: Molecular Linkages Between Bifidobacteria and Milk Oligosaccharides. *Trends Microbiol.* 18, 298–307. doi: 10.1016/j.tim.2010.03.008

Shishkin, A. A., Giannoukos, G., Kucukural, A., Ciulla, D., Busby, M., Surka, C., et al. (2015). Simultaneous Generation of Many RNA-Seq Libraries in a Single Reaction. *Nat. Methods* 12, 323–325. doi: 10.1038/nmeth.3313

Smith-Brown, P., Morrison, M., Krause, L., and Davies, P. S. W. (2016). Mothers Secretor Status Affects Development of Childrens Microbiota Composition and Function: A Pilot Study. *PloS One* 11, e0161211. doi: 10.1371/journal.pone.0161211

Sonnenburg, J. L., Xu, J., Leip, D. D., Chen, C.-H., Westover, B. P., Weatherford, J., et al. (2005). Glycan Foraging *In Vivo* by an Intestine-Adapted Bacterial Symbiont. *Science* 307, 1955–1959. doi: 10.1126/science.1109051

Sprouffske, K., and Wagner, A. (2016). Growthcurver: An R Package for Obtaining Interpretable Metrics From Microbial Growth Curves. *BMC Bioinf.* 17, 172. doi: 10.1186/s12859-016-1016-7

Stewart, C. J., Ajami, N. J., O'Brien, J. L., Hutchinson, D. S., Smith, D. P., Wong, M. C., et al. (2018). Temporal Development of the Gut Microbiome in Early Childhood From the TEDDY Study. *Nature* 562, 583–588. doi: 10.1038/s41586-018-0617-x

Turroni, F., Duranti, S., Bottacini, F., Guglielmetti, S., Van Sinderen, D., and Ventura, M. (2014). Bifidobacterium Bifidum as an Example of a Specialized Human Gut Commensal. *Front. Microbiol.* 5, 437. doi: 10.3389/fmicb.2014.00437

Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagström, H., Sanchez Luna, M., et al. (2018). Human Milk Oligosaccharides: 2'-Fucosyllactose (2'-FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. *Nutrients* 10, 1161–1173. doi: 10.3390/nu10091161

Vatanen, T., Plichta, D. R., Somani, J., Münch, P. C., Arthur, T. D., Hall, A. B., et al. (2019). Genomic Variation and Strain-Specific Functional Adaptation in the Human Gut Microbiome During Early Life. *Nat. Microbiol.* 4, 470–479. doi: 10.1038/s41564-018-0321-5

Walsh, C., Lane, J. A., van Sinderen, D., and Hickey, R. M. (2020). Human Milk Oligosaccharides: Shaping the Infant Gut Microbiota and Supporting Health. *J. Funct. Foods* 72, 104074. doi: 10.1016/j.jff.2020.104074

Wang, M., Li, M., Wu, S., Lebrilla, C. B., Chapkin, R. S., Ivanov, I., et al. (2015). Fecal Microbiota Composition of Breast-Fed Infants is Correlated With Human Milk Oligosaccharides Consumed. *J. Pediatr. Gastroenterol. Nutr.* 60, 825–833. doi: 10.1097/MPG.0000000000000752

Wexler, A. G., and Goodman, A. L. (2017). An Insider's Perspective: Bacteroides as a Window Into the Microbiome. *Nat. Microbiol.* 2, 17026. doi: 10.1038/nmicrobiol.2017.26

Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., et al. (2003). A Genomic View of the Human-Bacteroides Thetaiotaomicron Symbiosis. *Science* 299, 2074–2076. doi: 10.1126/science.1080029

Yassour, M., Jason, E., Hogstrom, L. J., Arthur, T. D., Tripathi, S., Siljander, H., et al. (2018). Strain-Level Analysis of Mother-To-Child Bacterial Transmission During the First Few Months of Life. *Cell Host Microbe* 24, 146–154.e4. doi: 10.1016/j.chom.2018.06.007

Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A.-M., Härkönen, T., Ryhänen, S. J., et al. (2016). Natural History of the Infant Gut Microbiome and Impact of Antibiotic Treatment on Bacterial Strain Diversity and Stability. *Sci. Transl. Med.* 8, 343ra81. doi: 10.1126/scitranslmed.aad0917

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human Gut Microbiome Viewed Across Age and Geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Zabel, B. E., Gerdes, S., Evans, K. C., Nedveck, D., Singles, S. K., Volk, B., et al. (2020). Strain-Specific Strategies of 2′-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose Assimilation by Bifidobacterium Longum Subsp. Infantis Bi-26 and ATCC 15697. *Sci. Rep.* 10, 15919–15937. doi: 10.1038/s41598-020-72792-z

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 11 May 2022
doi: 10.3389/fcimb.2022.873683

Subgingival Microbiome in Pregnancy and a Potential Relationship to Early Term Birth

Irene Yang[1], Henry Claussen[2], Robert Adam Arthur[2], Vicki Stover Hertzberg[1], Nicolaas Geurs[3], Elizabeth J. Corwin[4] and Anne L. Dunlop[5]*

[1] Nell Hodgson Woodruff School of Nursing, Emory University, Atlanta, GA, United States, [2] Emory Integrated Computational Core, Emory University, Atlanta, GA, United States, [3] Department of Periodontology, School of Dentistry, University of Alabama at Birmingham, Birmingham, AL, United States, [4] School of Nursing, Columbia University, New York, NY, United States, [5] Department of Gynecology and Obstetrics, School of Medicine, Emory University, Atlanta, GA, United States

Background: Periodontal disease in pregnancy is considered a risk factor for adverse birth outcomes. Periodontal disease has a microbial etiology, however, the current state of knowledge about the subgingival microbiome in pregnancy is not well understood.

Objective: To characterize the structure and diversity of the subgingival microbiome in early and late pregnancy and explore relationships between the subgingival microbiome and preterm birth among pregnant Black women.

Methods: This longitudinal descriptive study used 16S rRNA sequencing to profile the subgingival microbiome of 59 Black women and describe microbial ecology using alpha and beta diversity metrics. We also compared microbiome features across early (8-14 weeks) and late (24-30 weeks) gestation overall and according to gestational age at birth outcomes (spontaneous preterm, spontaneous early term, full term).

Results: In this sample of Black pregnant women, the top twenty bacterial taxa represented in the subgingival microbiome included a spectrum representative of various stages of biofilm progression leading to periodontal disease, including known periopathogens *Porphyromonas gingivalis* and *Tannerella forsythia.* Other organisms associated with periodontal disease reflected in the subgingival microbiome included several *Prevotella* spp., and *Campylobacter* spp. Measures of alpha or beta diversity did not distinguish the subgingival microbiome of women according to early/late gestation or full term/spontaneous preterm birth; however, alpha diversity differences in late pregnancy between women who spontaneously delivered early term and women who delivered full term were identified. Several taxa were also identified as being differentially abundant according to early/late gestation, and full term/spontaneous early term births.

Conclusions: Although the composition of the subgingival microbiome is shifted toward complexes associated with periodontal disease, the diversity of the microbiome remains stable throughout pregnancy. Several taxa were identified as being associated with spontaneous early term birth. Two, in particular, are promising targets of further investigation. Depletion of the oral commensal *Lautropia mirabilis* in early pregnancy

OPEN ACCESS

Edited by:
Maria De Lourdes Pereira, University of Aveiro, Portugal

Reviewed by:
Ana Sofia Duarte, Portuguese Catholic University, Portugal
Maria Correia, Centre for Interdisciplinary Research in Health, Portugal

****Correspondence:***
Irene Yang
irene.yang@emory.edu

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *11 February 2022*
Accepted: *13 April 2022*
Published: *11 May 2022*

Citation:
Yang I, Claussen H, Arthur RA, Hertzberg VS, Geurs N, Corwin EJ and Dunlop AL (2022) Subgingival Microbiome in Pregnancy and a Potential Relationship to Early Term Birth. Front. Cell. Infect. Microbiol. 12:873683. doi: 10.3389/fcimb.2022.873683

and elevated levels of *Prevotella melaninogenica* in late pregnancy were both associated with spontaneous early term birth.

Keywords: pregnancy, oral microbiome, periodontal disease, preterm birth, subgingival microbiome

1 INTRODUCTION

Pregnant women are susceptible to periodontal disease (Raju and Berens, 2021) due to hormonally driven hyper-reactivity of the gingiva to bacteria in the subgingival biofilm (Usin et al., 2013). Other factors that increase risk for poor oral health during pregnancy include changes in dietary habits (frequent snacking or increased consumption of carbohydrate rich or decay-promoting foods), stomach acids from nausea and vomiting that contribute to the breakdown of tooth enamel, and decreased likelihood of seeking dental care during pregnancy (Detman et al., 2010).

Periodontal disease can be understood as two sub-conditions. Gingivitis, the milder form of periodontal disease, occurs in 50-70% of all pregnancies (Anil et al., 2015). Gingivitis is an inflammation of the gingiva in which the connective tissue attachment to the tooth remains intact. The inflammation is limited to the soft-tissue compartment of the epithelium and connective tissue (Beck and Arbes, 2006). Symptoms typically emerge during the first trimester (Giglio et al., 2009) and present as red, swollen gingival margins and bleeding that occurs with the slightest provocation (Barak et al., 2003). The severity of gingivitis increases as pregnancy progresses (Cohen et al., 1969). Progressive gingivitis can lead to periodontitis, a more severe and chronic form of periodontal disease involving the irreversible destruction of supportive soft tissue and bone, ultimately leading to tooth loss (Darby et al., 2000). Diagnosis of periodontitis is based on clinical measurements of subgingival pocket depth, bleeding on probing, a plaque index, clinical attachment level, and radiographic examination (Papapanou et al., 2018). Symptoms of periodontal disease typically become more overt during the second trimester (American Pregnancy Association, 2014) in approximately 5-20% of pregnant women (Jared and Boggess, 2008), however, oral assessment, a basic component of any physical exam, is frequently overlooked in prenatal care. Dental care utilization in pregnancy ranges from 25% to 75% and is highly associated with demographic, socioeconomic, and perceived need factors (Rocha et al., 2018).

Decades of epidemiological research suggest that periodontal disease is an independent risk factor for various adverse birth outcomes, including preterm birth (Komine-Aizawa et al., 2019). Since 1996 when Offenbacher et al. (1996) reported that women with periodontal disease had a seven-fold increase in the risk of preterm birth, results from several clinical studies have confirmed this association. For example, after controlling for other known risk factors of preterm birth such as systemic disease, smoking, or complications during previous pregnancies, moderate to strong associations were found between periodontal disease and/or inflammation and spontaneous preterm birth (Khader et al., 2009; Guimarães et al., 2010; Macedo et al., 2014; Stadelmann et al., 2014). These associations, however, must be considered in light of a high degree of variability in study populations, and clinical assessment (Agueda et al., 2008; Ide and Papapanou, 2013) informing our choice to focus our investigation on a within-race sample of African American women, who are at greater risk for both preterm birth and periodontal disease (Henshaw et al., 2018; Martin et al., 2018).

Periodontal disease is initiated by microbial dysbiosis. The human oral cavity has a characteristic microbiome, with over 700 bacterial species (Aas et al., 2005), representing the second most complex ecosystem in the body, after the colon (Huttenhower et al., 2012). The key to oral health is an ecologically balanced and diverse oral microbiome that is in a state of commensalism within itself and mutualism with its host (Zaura et al., 2009; Zarco et al., 2012). This balanced biodiversity is mutually beneficial for both host and microbial community. Acute disruptions like pregnancy can challenge a stable ecosystem, increasing the risk of infection by opportunistic pathogens (Wade, 2013). Persistent and prolonged disruptions can permanently shift the composition of a microbial community. Overgrowth of opportunistic pathogens may lead to a cascade of interactions between host and community, which over time, lead to the development of a new community. This new community is equally as stable, but reflective of a diseased state (Socransky and Haffajee, 2005). Local oral pathogenesis is now accepted as an ecological phenomenon. Dysbiosis within an ecological niche plays a role in all major oral disease including periodontal disease (Dewhirst et al., 2010) shifting biodiversity to initiate an infectious/inflammatory state (Zarco et al., 2012). The microbial etiology of periodontal disease suggests that a thorough understanding of the microbiome is essential for any investigation of the association between periodontal disease and preterm birth.

The prenatal oral microbiome, however, is not well understood. Early attempts to characterize the ecological shift that occurs in pregnancy were limited to the identification of specific pathogenic organisms like *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia* (Holt and Ebersole, 2005). Results were inconsistent (Jarjoura et al., 2005; Lin et al., 2007; Novak et al., 2008; Vettore et al., 2008; Adriaens et al., 2009; Carrillo-de-Albornoz et al., 2010) due to the methodologies employed in studies which only targeted known pathogens. Emerging periodontal disease research utilizing 16S rRNA next-generation sequencing in the non-pregnant population implicate many more associated taxa and uncultivated species in the subgingival biofilm (Griffen et al., 2012; Liu et al., 2012) that have not been investigated in the prenatal population. These include genera more abundant in periodontal disease like *Spirochaetes*, *Synergistetes, Bacteriodetes,*

Clostridia, Negativicutes, and *Erysipelotrichia* (Griffen et al., 2012), *Selenomonas, Prevotella, Treponema, Tannerella, Haemophilus*, and *Catonella* (Liu et al., 2012), whereas, *Bacilli, Proteobacteria* (Griffen et al., 2012), *Streptococcus, Actinomyces*, and *Granulicatella* (Liu et al., 2012) were associated with healthy gingiva. Furthermore, this shift in the relative abundance of microbial organisms associated with oral health and disease appears to be present even in early and mild cases, i.e., gingivitis, where frank periodontitis is not yet evident (Liu et al., 2012). This ecological shift, however, is not yet fully understood for periodontal disease in general, and for pregnant women, in particular.

The purpose of this study, therefore, was to characterize the structure and diversity of the subgingival microbiome of African American women in early and late pregnancy and explore relationships between the microbiome and preterm birth.

2 MATERIALS AND METHODS

2.1 Design

This study utilized a longitudinal design with a sample of 59 participants drawn from among a larger group of participants taking part in the Emory University African American Vaginal, Oral, and Gut microbiome in Pregnancy cohort study. Institutional review board approval was obtained for this study the primary purpose of which was to characterize the subgingival microbiome at two points during pregnancy: early gestation (8-14 weeks) and late gestation (24-30 weeks).

2.2 Setting and Sample

Participants for this study were recruited from an ongoing investigation of the association between a woman's microbiome and preterm birth (Corwin et al., 2017). The parent study prospectively enrolled a socioeconomically diverse cohort of pregnant women between 8-14 weeks gestation who were US born Black or African American and followed them through delivery. Participants who were enrolled in the parent study were approached at the initial data collection period within the 8-14 week gestation period to ascertain their interest in and eligibility for this study. Additional eligibility criteria for this study included having: 1) minimum of 20 natural teeth and 2) no dental cleaning in the past three months. These requirements are routinely used in oral health studies (Eick and Pfister, 2002; Griffen et al., 2012; Matthews et al., 2013) to ensure adequate oral sampling sites and an undisturbed subgingival environment. Use of antibiotics was collected to address potential confounding effects on the microbiome, however, none of the participants whose samples were included in our microbiome analyses reported recent use of antibiotics.

2.3 Procedures

2.3.1 Oral Microbiome Samples

Subgingival plaque samples were collected for microbiome analysis using protocols based on the Human Microbiome Project (McInnes and Cutting, 2010). Participants were asked about oral sex within 48 hours of sample collection (McInnes and Cutting, 2010), oral hygiene (tooth brushing, flossing, and mouth washes) within 12 hours of sample collection (Kumar et al., 2014), and food, drink or gum within 30 minutes of sample collection (Kumar et al., 2014) as potential confounders of microbiome analysis. Samples were collected in early pregnancy (8-14 weeks) and again in late pregnancy (24-30 weeks).

Subgingival plaque was collected by inserting a scaler into the gingival sulcus of three tooth sites exhibiting visible signs of inflammation (McInnes and Cutting, 2010). If no visible signs of inflammation were present, three tooth sites were chosen randomly. After collection, each scaler tip was immediately swirled and placed in 750uL of MoBio buffer contained in sterile MoBio bead tubes (Mobio laboratories, Inc., Carlsbad, CA). These tubes were then placed on ice in a biohazards transport bag and transported for storage at -80°C until ready for DNA extraction.

2.3.2 DNA Isolation and 16S rRNA Library Preparation and Sequencing

Specimens were sent to the Emory Integrated Genomics Core (Atlanta, GA). DNA was isolated using the Qiagen DNeasy Powersoil Kit (Qiagen; 12888). Libraries were made using a modification of the Illumina 16S Meta-genomic Sequencing Library Preparation workflow (Illumina Inc., 2020). Briefly, the highly conserved 16S rRNA gene, which is widely used to characterize taxonomic diversity in microbial communities, was amplified targeting the third and fourth hypervariable region (v3-v4). Final 16S libraries were approximately 630 base pairs (bp) in length and were pooled in equal amounts based on fluorescence quantification. Final library pools were quantitated *via* quantitative Polymerase Chain Reaction (qPCR) (Kapa Biosystems; KK4824). The pooled library was sequenced on an Illumina MiSeq using MiSeq v3 600 cycle chemistry (Illumina; MS-102-3003) at a loading density of 6–8 pM with 20% PhiX, generating roughly 20 million, 300 bp paired-end reads.

2.3.3 Quality Control and Amplicon Bioinformatics

All samples were collected using standard sterile technique. Consistent reagents were used throughout DNA extraction with positive control (*Escherichia coli* bacterial pellet) to ensure appropriate extraction and negative control (sterile water) to confirm no contamination in extraction kit reagent. Additional controls (positive: mock community with known microbiome diversity; negative: sterile water) were used in the PCR amplification process.

Amplicon sequence reads in compressed fastq.gz format were produced by the above extraction and subsequent sequencing protocols and were then checked for quality control with FastQC and MultiQC packages as seen in Yang et al., 2021 (Andrews, 2010; Ewels et al., 2016). Following, reads were analyzed *via* Quantitative Insights Into Microbial Ecology (QIIME2) 2020.2 (Bolyen et al., 2019). Data denoising and dereplication was implemented by use of the DADA2 module in QIIME2 (Callahan et al., 2016), and the amplicon sequencing variant (ASV) feature table was built. DADA2 read trimming and truncation parameters of trim-left-f and trim-left-r 30 and

trunc-len-f and trunc-len-r 240 were used. Taxonomic assignment using the v3-v4 hypervariable regions of the 16S gene for primers was conducted, and data were compared to GreenGenes (v13_8, 99% clustered OTUs) (DeSantis et al., 2006), Silva v132 (Quast et al., 2012), and the Human Oral Microbiome Database (HOMD) v15.2 (Chen et al., 2010) through QIIME2 taxonomy modules (Bolyen et al., 2019).

In further detail, reference reads were pulled *via* QIIME2 feature-classifier extract-reads, trimming as above to match the raw reads using v3/v4 primers, then fitted to a naïve-Bayesian classifier using QIIME2 feature-classifier fit-classifier-naïve-bayes, and applied to the ASV feature table using QIIME2 feature-classifier sklearn (Bolyen et al., 2019). The HOMD database best resolved our Zymo microbial mock community controls and positive controls, thus HOMD taxonomic assignment was used downstream. Data were then exported into.BIOM format for downstream analyses.

2.3.4 Clinical and Questionnaire Data

Demographic and health behavior variables were collected from parent study survey data and birth outcome data were collected from parent study medical record abstraction reports. Gestational age at birth outcomes were adjudicated to include the full range of classifications as identified in the American College of Obstetrics and Gynecology (ACOG) guidelines. Preterm births, identified as birth prior to 37 weeks gestation (American College of Obstetricians and Gynecologists, 2013), were grouped according to whether they were spontaneous or induced. Births after 37 weeks were categorized as "early term" from 37 0/7 weeks through 38 6/7 weeks, and full term from 37 0/7 weeks through 38 6/7 weeks (American College of Obstetricians and Gynecologists, 2013). Spontaneous abortions (loss of pregnancy at less than 20 weeks gestation) (American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Gynecology, 2018) were also included as a group. All participants received early pregnancy dating by last menstrual period (LMP) and/or early ultrasound, given enrollment criteria. Type of Labor (spontaneous, induced, none) and mode of delivery (vaginal, C-section) along with indication for induction and/or C-section were obtained and used to further phenotype birth outcomes.

2.3.5 Analysis

From a total of 106 samples (across both time points), we excluded 34 samples due to low yield, specifically, any samples with less than 20,000 reads remaining after DADA2 dereplication and filtering were removed. As a result, the final study sample consisted of 72 subgingival samples (38 early pregnancy samples and 34 late pregnancy samples) from 50 participants. Sociodemographic and self-reported oral symptom and behavior data for these 50 participants were described. Analysis comparing the subgingival microbiome at early and late gestation overall was conducted among samples, regardless of birth outcome (N [Time 1] = 38; N [Time 2] = 34). Analysis comparing the subgingival microbiome according to gestational age at birth outcomes excluded inductions and included spontaneous abortions and spontaneous births (preterm, early term, and full term). Comparative analyses were conducted with the full term group as the referent group, and were conducted both at early and late gestation.

Alpha diversity, a measure of species diversity within a particular community, was calculated using the Shannon index within the QIIME2 platform. The Shannon index provides a measure of both richness and evenness (Magurran, 2013). Communities numerically dominated by one or a few species exhibit a low Shannon score, whereas communities in which abundance is distributed equally among species will exhibit high evenness. Beta diversity, which measures similarity/dissimilarity between a pairs of communities, were calculated using the Bray-Curtis (abundance-weighted) and Jaccard (presence/absence of detected ASV) distances. The communities were visualized on a principal coordinates analysis (PCoA) plot based on these distance matrices to assess any clustering by groups of interest. The significance of the cluster differences (i.e., variation in community structure in relationship to group status) was assessed using the permutational multivariate analysis of variance (PERMANOVA).

The Linear Decomposition Model (LDM) (Hu and Satten, 2020) was used to determine differences at the individual ASV level with permutation-based p-values, in terms of relative abundance, controlling for false discovery rate at a nominal level of 5%. Because this is an exploratory study, significant p-values, even though differences did not persist with correction, were reported as potential signals for future targeted investigation. Sample groups were classified by spontaneous pre-term, spontaneous early term, or full-term gestation. Taxa, by relative abundance, were tested for differences using LDM's global test of the microbiome effect. To account for the difference in sample size, random sampling with replacement was implemented to match sample group sizes and the global test repeated for 1,000 permutations.

3 RESULTS

3.1 Sociodemographic Characteristics and Birth Outcomes

The mean age of the participants was 26.04 ± 5.37 years. Other sociodemographic characteristics and birth outcomes may be found in **Table 1**.

3.2 Self-reported Oral Healthy Symptom and Behavior

Only one participant self-reported gingivitis at the second time point. Other oral health symptoms and behaviors are in **Table 2**.

3.3 Characterization of the Oral Microbiome Across Early and Late Pregnancy

There was no difference in measures of alpha or beta diversity for samples from early and late gestation, controlling for participants. The top twenty taxa (**Figure 1**) represented in the subgingival microbiome of participants across gestation include

TABLE 1 | Sociodemographic characteristics of participants according to birth outcomes (*N* = 50).

Sociodemographic Characteristics	Preterm (*n* = 5)	Early Term (*n* = 16)	Full Term (*n* = 24)	Spontaneous Abortions (*n* = 3)
Education				
≤ High School	4 (80.0)	9 (56.25)	13 (54.2)	2 (0.67)
> High School	1 (20.0)	3 (18.75)	9 (37.5)	1 (0.33)
Missing	0	4 (2.5)	2 (8.3)	0
Income				
< 100% Federal Poverty Level	2 (40.0)	9 (56.25)	9 (37.5)	3 (100)
≥ 100% Federal Poverty Level	3 (60.0)	2 (12.5)	8 (33.3)	0
Missing	0	5 (31.25)	7 (29.2)	0
Medical Insurance				
Medicaid	4 (80.0)	11 (68.75)	20 (83.4)	3 (100)
Private Insurance	1 (20.0)	1 (6.25)	2 (8.3)	0
Missing	0	4 (25.0)	2 (8.3)	0

Twin birth (n = 1); Missing sociodemographic data (n = 1).

a spectrum of bacteria representative of various stages of biofilm progression leading to periodontal disease. These include *Porphyromonas gingivalis* and *Tannerella forsythia.* Other bacteria associated with periodontal disease that were reflected in the microbiome profile of our participants included several *Prevotella* spp., and *Campylobacter* spp.

The relative abundance of six taxa differentiated the subgingival microbiome of our cohort between early and late pregnancy. Five organisms (*Porphyromonas* sp.*_HMT_284, Catonella* sp.*_HMT_164, Peptostreptococcaceae_[XI][G-5] [XI][G-5]_saphenum, Johnsonella ignava, Actinomyces massiliensis* were more abundant in early pregnancy, while one, *Cryptobacterium curtum*, was more abundant in late pregnancy (**Table 3**).

3.4 Characterization of the Oral Microbiome According to Birth Outcomes

No association between microbiome features and spontaneous abortion or spontaneous preterm birth at either time point were identified. We did, however, find that in late pregnancy both alpha and beta diversity distinguished the subgingival microbiome of women who spontaneously delivered early term from women who delivered full term (**Figures 2**, **3**). Several taxa also differentiated women who spontaneously delivered early

TABLE 2 | Self-reported oral health symptoms and behaviors in early and late gestation.

	Early Gestation (*n* = 38) Frequency, *n* (%)	Late Gestation (*n* = 34) Frequency, *n* (%)
In the past month		
Self-reported gingivitis		
Yes	0	1 (2.9)
No	38 (100)	33 (97.1)
Missing	0	1 (2.9)
Used mouthwash		
Yes	24 (63.2)	19 (55.9)
No	13 (34.2)	14 (41.2)
Missing	1 (2.6)	1 (2.9)
Flossed		
Yes	23 (60.5)	12 (35.3)
No	14 (36.8)	21 (61.8)
Missing	1 (2.6)	1 (2.9)
Visited dentist		
Yes	2 (5.3)	3 (8.8)
No	35 (92.1)	31 (91.2)
Missing	1 (2.6)	0
Current		
Red and swollen gums		
Yes	1 (2.6)	1 (2.9)
No	37 (97.4)	33 (97.1)
Bleeding gums		
Yes	11 (28.9)	8 (23.5)
No	26 (68.4)	25 (73.5)
Missing	1 (2.6)	1 (2.9)
Brushed teeth in the last 2 days		
Yes	37 (97.4)	32 (94.1)
No	0	1 (2.9)
Missing	1 (2.6)	1 (2.9)

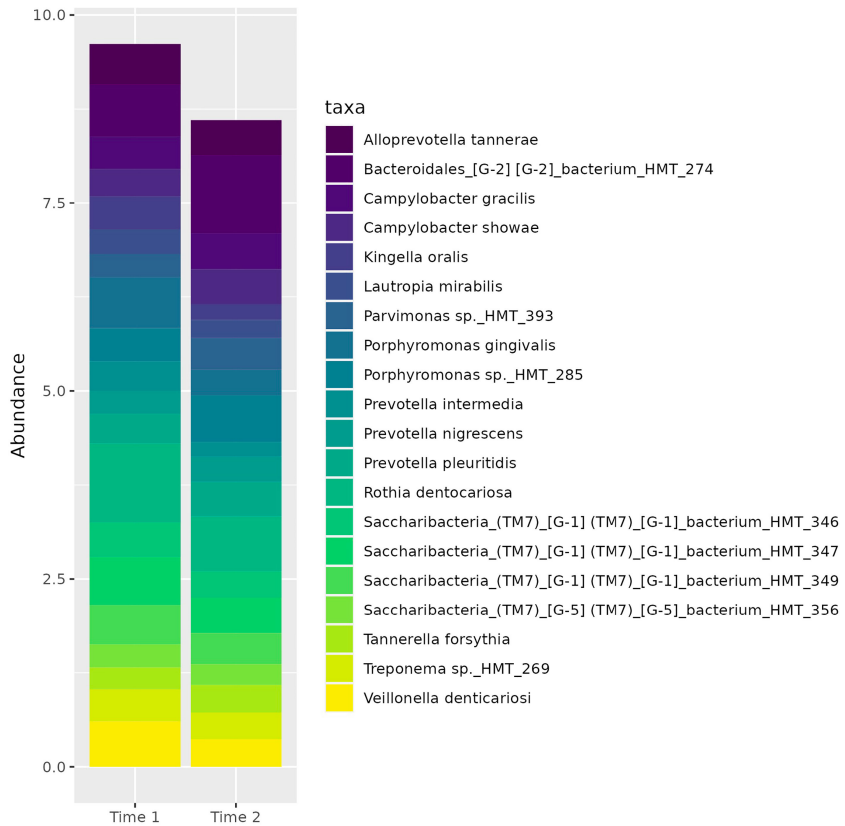


FIGURE 1 | Top 20 species (according to relative abundance) across Time 1 and Time 2.

term vs full term. In early pregnancy, eight taxa including *Actinomyces israelii, Cardiobacterium hominis, Treponema putidum*, and *Lautropia mirabilis* were more abundant among women who delivered full term compared to early term (**Table 4**). In late pregnancy, four taxa (*Campylobacter gracilis, Prevotella melaninogenica*, and two unnamed species belonging to the genera *Tannerella* and *Catonella*) were differentially abundant between women who spontaneously delivered early term and women who delivered full term. Of these, *P. melaninogenica* was the only organism that was more abundant among women who spontaneously delivered early term (**Table 5**).

4 DISCUSSION

4.1 Sociodemographic and Behavioral Characteristics

This study describes the subgingival microbiome of a group of pregnant Black women and explores associations between the microbiome and preterm birth. Although the parent study, from which this study leveraged its participants, aimed to enroll a socioeconomically diverse cohort of pregnant women, the majority of our convenience sub-sample were socioeconomically vulnerable with less than high school education, and incomes at less than the federal poverty level. Most of the participants were on

TABLE 3 | Six taxa identified to have significantly different relative abundances between early and late pregnancy.

Taxa	Early Pregnancy Mean Relative Abundance	Late Pregnancy Mean Relative Abundance	*p*.value	fdr.*q*.value
Actinomyces massiliensis	0.0311	0.01313	0.0317	0.996
Catonella sp._*HMT_164*	0.0185	0.01083	0.0078	0.7271
Cryptobacterium curtum	0.00227	0.01102	0.0127	0.7271
Johnsonella ignava	0.00851	0.00316	0.0196	0.8977
Peptostreptococcaceae_[XI][G-5][XI][G-5]_saphenum	0.00314	0.00225	0.0108	0.7271
Porphyromonas sp._*HMT_284*	0.00514	0.0051	0.0034	0.7271

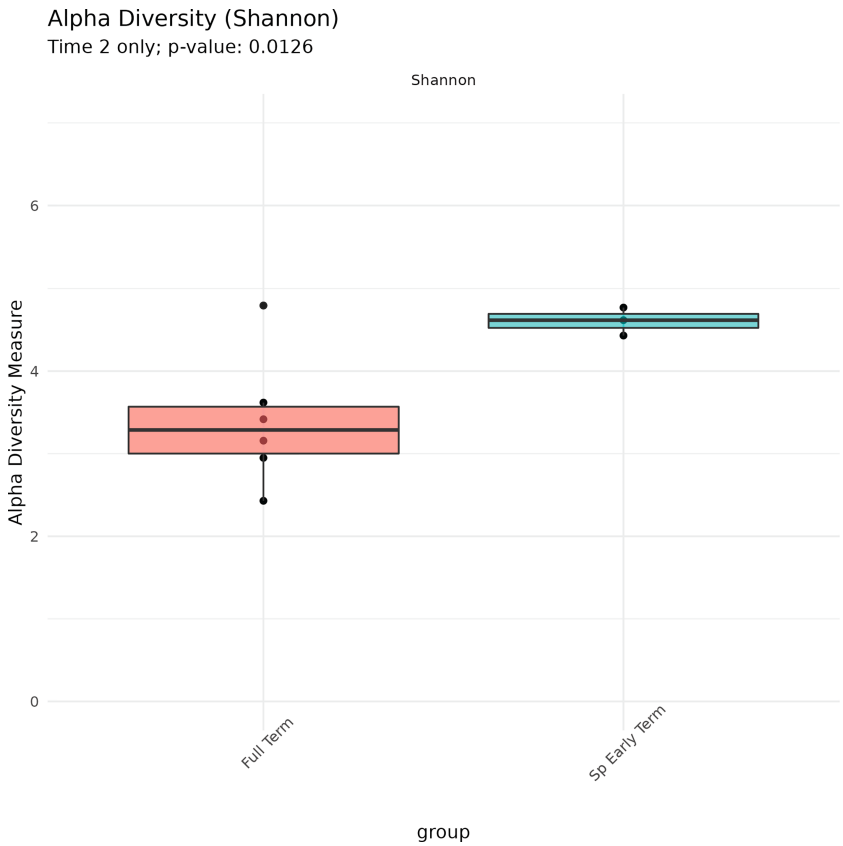


FIGURE 2 | Alpha diversity of subgingival microbiome at Time 2 according to delivery time status.

Medicaid insurance. Evidence suggests that socioeconomic inequalities are strongly associated with oral health disparities, both self-reported and clinically determined (Mejia et al., 2018). This holds true for pregnant populations as well (Azofeifa et al., 2014; Chung et al., 2014; McNeil et al., 2016). None of the women in the study self-reported gingivitis across pregnancy, although one woman reported red or swollen gums, and around a quarter of the participants reported bleeding gums across pregnancy. Both red/swollen gums and bleeding gums are symptoms of gingivitis. Self-reported gingivitis and symptomatology is less than expected within our cohort since we know that gingivitis is present in 50-70% of pregnant women (Anil et al., 2015), and tends to increase in severity across pregnancy (Cohen et al., 1969). Self-reported gingivitis and bleeding gums, while useful for public health screening, lacks validity for individual screening for gingivitis as defined by a dental professional (Kallio et al., 1994; Abbood et al., 2016). It is likely that underreporting of gingivitis and associated symptoms was present in our study.

Virtually all the women reported brushing their teeth in the last two days, however, use of other oral hygiene measures such as flossing, and mouthwash were not as ubiquitous. This can be understood in the light of previous research which indicates racial, ethnic, and economic disparities related to oral hygiene practices during pregnancy (Boggess et al., 2010). Even controlling for income, education, and insurance status, racial disparities in oral health experiences persist; an important contextual consideration for our cohort (Hwang et al., 2011).

4.2 The Subgingival Microbiome Across Pregnancy

Microbial community diversity can be described in two ways. Alpha diversity describes how many ASVs are present in a community and how evenly they are distributed; beta diversity describes the diversity between two different environments. In studies conducted among an Asian population, pregnancy itself appears to increase the alpha diversity of the subgingival microbiome (Balan et al., 2021), although both species richness and diversity remain stable throughout pregnancy (Balan et al., 2018). Our findings support the consistency of alpha diversity throughout pregnancy among African American women since we found no difference in either species richness or diversity between early and late pregnancy. More research among diverse racial and ethnic populations of pregnant women is needed to further assess the generalizability of this finding.

To contextualize our description of the most prevalent organisms identified in the subgingival space of our cohort of pregnant women, it is important to understand that periodontal disease has a polymicrobial etiology. The current scientific

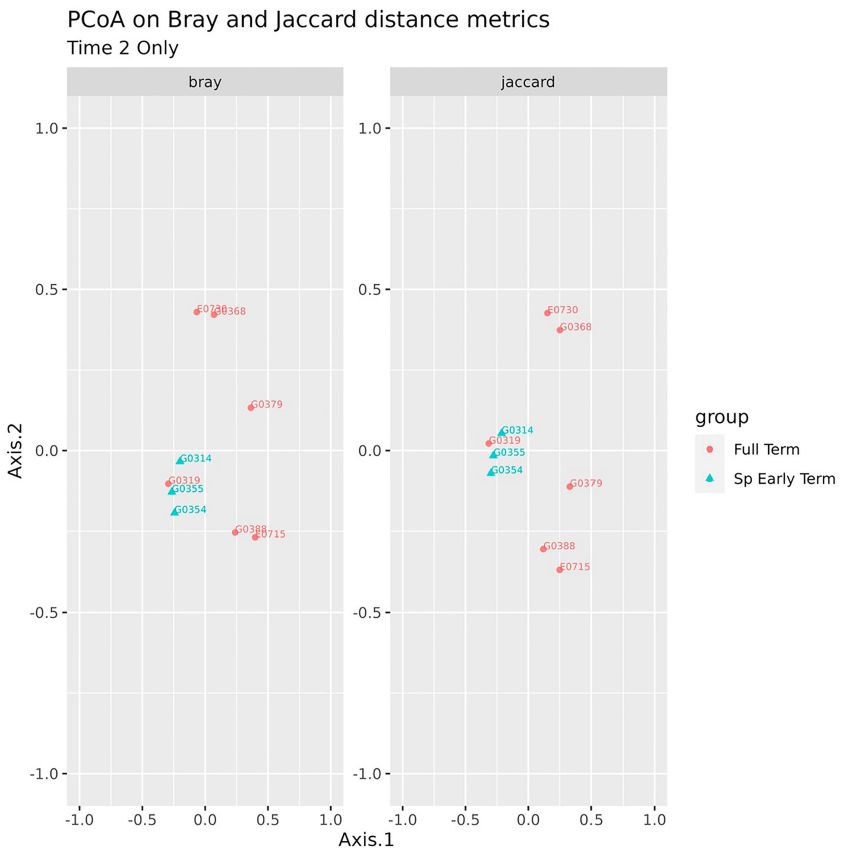


FIGURE 3 | Beta diversity of subgingival microbiome at Time 2 according to delivery time status.

understanding of periodontal disease is that of progressive dysbiosis within the biofilm, a polymicrobial ecosystem which attaches to the surface of the tooth in the subgingival pocket (Guthmiller and Novak, 2002). The dysbiotic shift results in a predominantly gram negative environment (Mohanty et al., 2019) caused by microbial succession that occurs with rapid accumulation of plaque on the teeth (Socransky and Haffajee, 2005). According to the seminal work of Socransky and Haffajee (2005), the postulated scheme of microbial succession occurs according to complexes, or co-occurring clusters of organisms. Initially, members of the yellow, green, and purple complexes dominate along with *Actinomyces* species (Socransky and Haffajee, 2005). The establishment of these three complexes contributes to the alteration of the subgingival environment such that the next two, more pathogenic, complexes become dominant (**Figure 4**) (Socransky and Haffajee, 2005). These are the "orange complex" organisms (*Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Fusobacterium nucleatum, Eubacterium nodatum, Streptococcus constellatus*, and several *Campylobacter* species). The orange complex of organisms contain bridge species and generally precedes the growth of the red complex which consists of *Treponema*

TABLE 4 | Eight taxa identified in early pregnancy to have significantly different relative abundances between spontaneous early term and full term births.

Taxa	Spontaneous Early Term Mean Relative Abundance	Full Term Mean Relative Abundance	*p*.value	fdr.*q*.value
Actinomyces israelii	0.00118	0.00132	0.003	0.2332
Leptotrichia sp._HMT_392	0.00158	0.00279	0.0047	0.2332
Cardiobacterium hominis	0.00515	0.0077	0.0076	0.2332
Capnocytophaga sp._HMT_338	0.00023	0.00216	0.0093	0.2332
Treponema putidum	0.00318	0.00765	0.0106	0.2332
Bacteroidaceae_[G-1] [G-1]_bacterium_HMT_272	0.00085	0.00289	0.0166	0.3043
Lautropia mirabilis	0.00428	0.01146	0.0419	0.6435
Prevotella loescheii	0.00024	0.00795	0.0468	0.6435

TABLE 5 | Four taxa identified in late pregnancy to have significantly different relative abundances between spontaneous early term and full term births.

Taxa	Early Term Mean Relative Abundance	Full Term Mean Relative Abundance	*p*.value	fdr.*q*.value
Campylobacter gracilis	0.00838	0.03764	0.025	0.3132
Catonella sp._*HMT_164*	0.00132	0.00364	0.0464	0.3132
Prevotella melaninogenica	0.01081	0.00366	0.0435	0.3132
Tannerella sp._*HMT_286*	0.00211	0.00512	0.0452	0.3132

denticola, Porphyromonas gingivalis, and *Tannerella forsythia* (Guthmiller and Novak, 2002). The red complex organisms are known as periopathogens and are present at sites presenting with symptoms of periodontal disease.

Notably, several of the organisms represented among the top 20 most abundant organisms in the subgingival microbiome of our cohort include members of both the red and orange complexes: *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Campylobacter gracilis*, and *Campylobacter showae*, suggesting a shift toward a pathogenic subgingival ecosystem. This finding is supported by a previous study by Balan et al. (2018) which also identified a dominance of periopathogens during pregnancy from the genera *Prevotella, Veillonella*, and *Streptococcus*, and at the species level, *P. gingivalis, P. nigrescens*, and *P. oris*.

Given that periodontal disease symptoms intensify over pregnancy (Cohen et al., 1969), we might expect to see increases in the abundance of these pathogenic organisms over pregnancy, however, the significant changes in relative abundance of organisms that we identified were not among established periodontal disease associated organisms. Instead, slight but significant differences were identified with six taxa, five of which decreased from early to late pregnancy, and one which increased in abundance across pregnancy. Of the five taxa that decreased in abundance, three were uncultivated phylotypes. Little is known about *Porphyromonas* sp._*HMT_284*, however, sister organisms within the genus are *P. gingivalis* and *P. endodontalis*, both of which have been associated with periodontal disease (Lombardo Bedran et al., 2012). The genus *Catonella* is a relatively newly identified genus (Moore and Moore, 1994) with one named species, *C. morbi*, that has been described as a putative periopathogens (Wu et al., 2017). Bacteria from the family *Peptostreptococcaceae* are a morphologically diverse group of gram positive organisms (Slobodkin, 2014). Due to the multiple genera and species within this family it is difficult to speculate on the meaning of our finding that this particular taxa decreased in abundance over pregnancy. *Johnsonella ignava* may be an opportunistic pathogen. There is modest evidence that it may be associated with chronic obstructive pulmonary disease as well as oral squamous-cell carcinoma (Wu et al., 2017). The evidence on whether it is associated with periodontal disease is mixed (Moore and Moore, 1994; Wu et al., 2017). *Actinomyces* spp. are commonly found in human oral mucosa and many novel species have been described in recent years (Renvoise et al., 2009). Although *A. massiliensis* is known to reside in the subgingival biofilm, its role within an *Actinomyces* complex associated with periodontal disease is not known (Vielkind et al., 2015). *Cryptobacterium curtum* was the

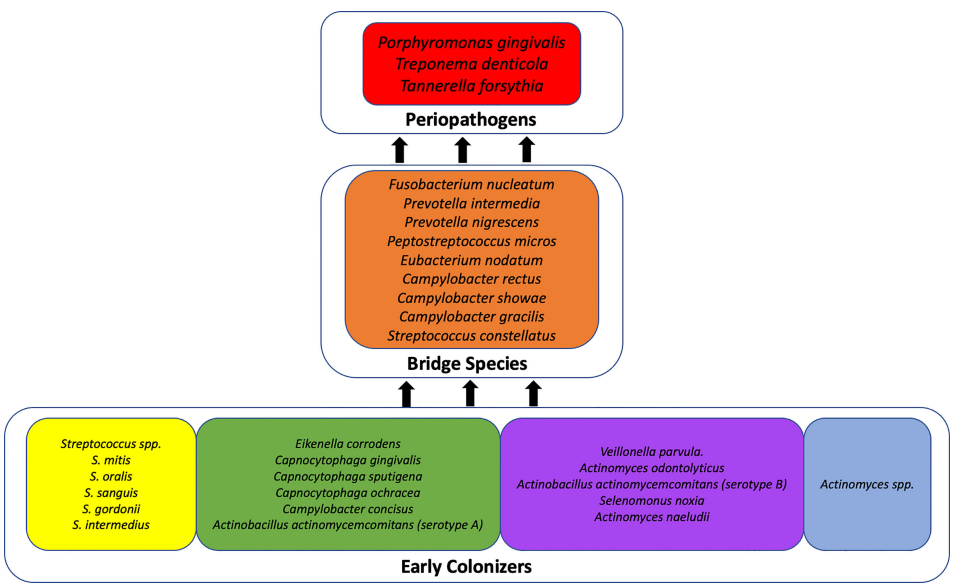


FIGURE 4 | Theoretical scheme of microbial succession in periodontal disease. This figure has been adapted from Socransky and Haffajee, 2005; Publisher: Wiley; Copyright © Blackwell Munksgaard 2005.

only taxa identified to increase from early to late pregnancy. *Cryptobacterium curtum* is an oral opportunistic pathogen that is involved in dental and oral infections (Mavrommatis et al., 2009).

Our findings suggest that despite the overall stability in alpha and beta diversity over the course of pregnancy, relative abundance shifts within individual taxa do occur over time with organisms that have some associations to periodontal disease and other opportunistic infections. Further investigation of these organisms and their clinical impact on oral disease and pregnancy outcomes are warranted.

4.3 Exploring the Association Between the Subgingival Microbiome and Preterm/ Early Term Birth

Likely due to limited power from the small number of preterm births in our cohort, we did not identify any associations between subgingival microbiome features and spontaneous preterm birth. We did, however, identify associations among microbiome features and spontaneous early term birth (37 0/7 weeks through 38 6/7 weeks). During early pregnancy, eight taxa were more abundant among women who delivered full term compared to those who delivered early term. Two were unnamed species belonging to the genera *Leptotrichia* and *Capnocytophaga*. *Leptotrichia* spp. are facultative anaerobic oral commensals that are commonly found in the oral cavity (Smid et al., 2015) and can act as opportunistic pathogens or stimulate the growth of other pathogenic organisms (Eribe and Olsen, 2017). Various pathologic conditions are associated with *Leptotrichia*, (Eribe and Olsen, 2017) including poor pregnancy outcomes. *Leptotrichia amnionii*, *L. sanguinegens*, and *L. buccalis* have been associated with outcomes such as miscarriage, chorioamnionitis, preterm labor, pregnancy loss, neonatal infection and postpartum infection (Smid et al., 2015). Several *Capnocytophaga* spp. belong to the green complex, early colonizers in the microbial succession leading to periodontal disease.

Cardiobacterium hominis was also more abundant among women who delivered full term. *C. hominis* is not commonly associated with oral disease, although one study did find it to be associated with aggressive periodontitis and suggested it as a potentially new periopathogens (Lourenço et al., 2014). *C. hominis* is better known as an oral commensal belonging to the HACEK group of five organisms which can cause infective endocarditis (Moreillon, 2010).

Oral treponemes are widely considered to play an important role in periodontal disease etiology and pathogenesis (You et al., 2013). *Treponema putidum* is a novel treponeme isolated from lesions of periodontal disease and acute necrotizing ulcerative gingivitis (Wyss et al., 2004). The genus *Prevotella* is the largest genus in the phylum *Bacteroidetes* (Dewhirst et al., 2010) and contains species such as *P. intermedia* and *P. nigrescens*, members of the orange complex. Lesser known are organisms like *P. loeschii* which is, nonetheless, associated with periodontal disease. *P. loeschii* is known to produce propionic acid, a major metabolic player in the inflammatory process of gingival tissue (Al-Lahham et al., 2010). *Actinomyces* spp. are thought to play a significant role as early colonizers in the process of plaque formation and figure prominently in the microbial succession dogma of periodontal disease. The genus contains a total of 42 species, 20 of which are relevant for human health (Vielkind et al., 2015). *A. israelii* is part of the normal human oral flora and is frequently found in dental caries and at the gingival margins of individuals with poor oral hygiene (Desouches et al., 2019). It is the most common cause of actinomycosis, and has also been associated with periodontal disease (Vielkind et al., 2015). *Lautropia mirabilis* is a Gram-negative, motile, coccus bacteria that has been isolated from the human oral cavity (Gerner-Smidt et al., 1994). While *L. mirabilis* may be associated with some disease states (Ben Dekhil et al., 1997; Rossmann et al., 1998), it is currently primarily associated with periodontal health (Colombo et al., 2009; Balan et al., 2018; Papapanou et al., 2020). Finally, *Bacteroidaceae_[G-1] [G-1]_bacterium_HMT_272* is a novel, uncultivated, unnamed, and uncharacterized taxon. Little is known about this organism, however, it has been identified as the most prevalent phylotype in apical periodontitis lesions (Amaral et al., 2020).

In late pregnancy, four taxa were identified as being differentially abundant among women who spontaneously delivered early term. Women who delivered early term had lower abundances of *Catonella* sp.*_HMT_164, Tannerella* sp.*_HMT_286*, and *Campylobacter gracilis*. Both *Catonella* sp.*_HMT_164* and *Tannerella* sp.*_HMT_286* are uncultivated phylotypes. While these strains are not yet well characterized, we do know that other species within these genera are periopathogens. *Tannerella forsythia*, for example, belongs to the red complex and is known as a keystone periopathogen. *Catonella morbi*, the only named species within the genus, has been described as a putative pathogen (Wu et al., 2017). *Campylobacter gracilis* was one of the top 20 most abundant organisms across pregnancy in our cohort. *C. gracilis* belongs to the orange complex, known to closely precede colonization by the three dominant periopathogens of the red complex.

Prevotella melaninogenica was the only taxon identified to be more abundant among women who spontaneously delivered early term. *P. melaninogenica* is an oral commensal, gram-negative anaerobe that is closely related to periodontal disease (Ibrahim et al., 2017). *P. melaninogenica* has a pro-inflammatory effect that stimulates the release of inflammatory cytokines, suggesting that it may play an important role in promoting chronic inflammation (Larsen, 2017).

Several of the taxa identified to be differentially abundant between women who delivered early term *versus* full term in both early and late pregnancy were novel, uncultivated species suggesting the importance of looking beyond known pathogens to additional microbial players that may affect oral disease and birth outcomes. Many of the taxa identified to be differentially abundant between early term and full term women have strong known or suspected associations with periodontal disease. That most were elevated in women who delivered full term challenges the hypothetical association between periodontal disease (and periodontal disease-causing organisms) and adverse birth

outcomes. Further investigation of these organisms, their role in periodontal disease and early term birth is required.

What our findings do reveal is that there are taxonomic shifts that are associated with early term birth. Two taxa are potential targets for future research. *Lautropia mirabilis* is a Gram-negative, motile, coccus bacteria that is known as an oral commensal (Gerner-Smidt et al., 1994). Studies have identified *L. mirabilis* as being associated with periodontal health (Colombo et al., 2009; Papapanou et al., 2020). In our study we identified that a depletion of this organism was associated with spontaneous early term birth suggesting that it may be a potential target of future investigation as an oral commensal associated with both oral and pregnancy health. *Prevotella melaninogenica* was the only taxa identified to be enriched in women who delivered early term. While not traditionally understood as a periopathogen, it has been identified to be highly abundant in the saliva of pregnant women (Balan et al., 2018), and enrichment of *Prevotella* spp. is associated with mucosal inflammation leading to systemic dissemination of inflammatory mediators, bacteria, and bacterial products, which in turn, may affect systemic outcomes (Larsen, 2017). Further investigation of this organism and its inflammatory effects on oral health and pregnancy are needed.

Spontaneous early term births are births that occur between 37 and 39 weeks. While infants born at this gestational age do not face the same risks as those born preterm (< 37 weeks), they do remain at increased risk for respiratory distress syndrome, transient tachypnea of the newborn, feeding difficulty, pneumonia, and hypothermia (Parikh et al., 2014). Although reasons for spontaneous early term birth are not fully understood, biological determinants may be at play including placental ischemia, diabetes, and poly- or oligohydramnios (Brown et al., 2015). To our knowledge, no studies have investigated the contribution of the oral microbiome to early term birth or tested the potential hypotheses that an oral microbiome shifted toward periodontal disease might contribute to early term labor. This study lays the groundwork and provides preliminary evidence for future targeted investigations testing this hypothetical association.

4.4 Limitations

Several limitations must be acknowledged. First, this was an exploratory study and was not fully powered to identify microbiome associations with preterm or early term birth. Second, although the Human Oral Microbiome Database offers a well-curated and up-to-date resource for 16S rRNA sequences, marker gene sequencing technology limits the resolution with which to identify taxa. Finally, the lack of periodontal disease clinical diagnosis limits our ability to interpret the clinical impact of the oral microbiome.

5 CONCLUSIONS

Despite these limitations, this exploratory study confirmed emerging findings that the diversity of the subgingival microbiome remains stable during pregnancy. While our study did not include clinical diagnosis of periodontal disease, our findings suggest that the subgingival microbiome of this group of pregnant women was shifted towards complexes associated with periodontal disease. Our exploration also identified periodontal disease associated taxa that differentiated the subgingival microbiome of women who delivered early term versus full-term, namely *Porphyromonas* sp._*HMT_284, Catonella* sp._*HMT_164, Peptostreptococcaceae_[XI][G-5] [XI][G-5]_saphenum, Johnsonella ignava, Actinomyces massiliensis* and *Cryptobacterium curtum*, suggesting a relationship between the subgingival microbiome and early term birth. Next step investigations should consider the incorporation of clinical assessment of the oral cavity; shotgun whole metagenome sequencing to allow identification of microbial taxa to the species, and even strain level; and further investigation into novel, uncultivated phylotypes which may play a role in the relationship between the subgingival microbiome in pregnancy and early term birth.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. Repository: NCBI. Accession number: PRJNA811442. The link to the data can be found below: http://www.ncbi.nlm.nih.gov/bioproject/811442.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Emory University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IY, EC, AD, NG, and VH contributed to the conception and design of the study. IY, RA, and HC contributed to the data curation. HC and IY performed the formal analysis. IY wrote the first draft of the manuscript. HC and RA wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

Support for this study came from NIH - National Institute of Nursing Research grant number R01NR014800 and NIH – National Institute of Nursing Research grant number K01NR016971.

ACKNOWLEDGMENTS

We are grateful for the support of Kristi Logue, Castalia Thorne, Cassandra Hall, and Shirleta Reid for their help in recruiting participants for this study.

REFERENCES

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005). Defining the Normal Bacterial Flora of the Oral Cavity. *J. Clin. Microbiol.* 43 (11), 5721–5732. doi: 10.1128/JCM.43.11.5721-5732.2005

Abbood, H. M., Hinz, J., Cherukara, G., and Macfarlane, T. V. (2016). Validity of Self-Reported Periodontal Disease: A Systematic Review and Meta-Analysis. *J. Periodontol.* 87 (12), 1474–1483. doi: 10.1902/jop.2016.160196

Adriaens, L. M., Alessandri, R., Spörri, S., Lang, N. P., and Persson, G. R. (2009). Does Pregnancy Have an Impact on the Subgingival Microbiota? *J. Periodontol.* 80 (1), 72–81. doi: 10.1902/jop.2009.080012

Agueda, A., Echeverría, A., and Manau, C. (2008). Association Between Periodontitis in Pregnancy and Preterm or Low Birth Weight: Review of the Literature. *Med. Oral. Patol. Oral. Cir. Bucal.* 13 (9), E609–E615.

Al-Lahham, S. H., Peppelenbosch, M. P., Roelofsen, H., Vonk, R. J., and Venema, K. (2010). Biological Effects of Propionic Acid in Humans; Metabolism, Potential Applications and Underlying Mechanisms. *Biochim. Biophys. Acta* 1801 (11), 1175–1183. doi: 10.1016/j.bbalip.2010.07.007

Amaral, R. R., Braga, T., Siqueira, J. F., Rôças, I. N., da Costa Rachid, C. T. C., Guimarães Oliveira, A. G, et al. (2020). Root Canal Microbiome Associated With Asymptomatic Apical Periodontitis as Determined by High-Throughput Sequencing. *J. Endod* 48, 487–495. doi: 10.1016/j.joen.2022.01.012

American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Gynecology. (2018). ACOG Practice Bulletin No. 200: Early Pregnancy Loss. *Obstet. Gynecol.* 132 (5), e197–e207. doi: 10.1097/AOG.0000000000002899

American College of Obstetricians and Gynecologists. (2013). ACOG Committee Opinion No 579: Definition of Term Pregnancy. *Obstet. Gynecol.* 122 (5), 1139–1140. doi: 10.1097/01.AOG.0000437385.88715.4a

American Pregnancy Association. (2014). *Swollen Gums During Pregnancy (Also Known as Pregnancy Gingivitis)*. Available at: http://americanpregnancy.org/pregnancy-health/swollen-gums-during-pregnancy/.

Andrews, S. (2010) *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Anil, S., Alrowis, R. M., Chalisserry, E. P., Chalissery, V. P., AlMoharib, H. S., and Al-Sulaimani, A. F. (2015). "Oral Health and Adverse Pregnancy Outcomes," in *Emerging Trends in Oral Health Sciences and Dentistry*. Ed. M. Virdi (London, UK: IntechOpen). doi: 10.5772/58660

Azofeifa, A., Yeung, L. F., Alverson, C. J., and Beltrán-Aguilar, E. (2014). Oral Health Conditions and Dental Visits Among Pregnant and Nonpregnant Women of Childbearing Age in the United States, National Health and Nutrition Examination Surve-2004. *Prev. Chronic. Dis.* 11, E163. doi: 10.5888/pcd11.140212

Balan, P., Brandt, B. W., Chong, Y. S., Crielaard, W., Wong, M. L., Lopez, V., et al. (2021). Subgingival Microbiota During Healthy Pregnancy and Pregnancy Gingivitis. *JDR. Clin. Trans. Res.* 6 (3), 343–351. doi: 10.1177/2380084420948779

Balan, P., Chong, Y. S., Umashankar, S., Swarup, S., Loke, W. M., Lopez, V., et al. (2018). Keystone Species in Pregnancy Gingivitis: A Snapshot of Oral Microbiome During Pregnancy and Postpartum Period. *Front. Microbiol.* 9, 2360. doi: 10.3389/fmicb.2018.02360

Barak, S., Oettinger-Barak, O., Oettinger, M., Machtei, E. E., Peled, M., and Ohel, G. (2003). Common Oral Manifestations During Pregnancy: A Review. *Obstet. Gynecol. Surv.* 58 (9), 624–628. doi: 10.1097/01.OGX.0000083542.14439.CF

Beck, J., and Arbes, S. J. Jr. (2006). "Epidemiology of Gingival and Periodontal Diseases", in *Carranza's Clinical Periodontology*. Eds. M. G. Newman, H. H. Takei and P. R. Klokkevold (St. Louis: Saunders/Elsevier), 110–132.

Ben Dekhil, S. M., Peel, M. M., Lennox, V. A., Stackebrandt, E., and Sly, L. I. (1997). Isolation of *Lautropia Mirabilis* From Sputa of a Cystic Fibrosis Patient. *J. Clin. Microbiol.* 35 (4), 1024–1026. doi: 10.1128/jcm.35.4.1024-1026.1997

Boggess, K. A., Urlaub, D. M., Massey, K. E., Moos, M. K., Matheson, M. B., and Lorenz, C. (2010). Oral Hygiene Practices and Dental Service Utilization Among Pregnant Women. *J. Am. Dent. Assoc.* 141 (5), 553–561. doi: 10.14219/jada.archive.2010.0228

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37 (8), 852–857. doi: 10.1038/s41587-019-0209-9

Brown, H. K., Speechley, K. N., Macnab, J., Natale, R., and Campbell, M. K. (2015). Biological Determinants of Spontaneous Late Preterm and Early Term Birth: A Retrospective Cohort Study. *BJOG* 122 (4), 491–499. doi: 10.1111/1471-0528.13191

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-Resolution Sample Inference From Illumina Amplicon Data. *Nat. Methods* 13 (7), 581–583. doi: 10.1038/nmeth.3869

Carrillo-de-Albornoz, A., Figuero, E., Herrera, D., and Bascones-Martínez, A. (2010). Gingival Changes During Pregnancy: II. Influence of Hormonal Variations on the Subgingival Biofilm. *J. Clin. Periodontol.* 37 (3), 230–240, baq013. doi: 10.1111/j.1600-051X.2009.01514.x.

Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A., and Dewhirst, F. E. (2010). The Human Oral Microbiome Database: A Web Accessible Resource for Investigating Oral Microbe Taxonomic and Genomic Information. *Database* 2010, baq013. doi: 10.1093/database/baq013

Chung, L. H., Gregorich, S. E., Armitage, G. C., Gonzalez-Vargas, J., and Adams, S. H. (2014). Sociodemographic Disparities and Behavioral Factors in Clinical Oral Health Status During Pregnancy. *Community Dent. Oral. Epidemiol.* 42 (2), 151–159. doi: 10.1111/cdoe.12075

Cohen, D. W., Friedman, L., Shapiro, J., and Kyle, G. C. (1969). A Longitudinal Investigation of the Periodontal Changes During Pregnancy. *J. Periodontol.* 40 (10), 563–570. doi: 10.1902/jop.1969.40.10.563

Colombo, A. P., Boches, S. K., Cotton, S. L., Goodson, J. M., Kent, R., Haffajee, A. D., et al. (2009). Comparisons of Subgingival Microbial Profiles of Refractory Periodontitis, Severe Periodontitis, and Periodontal Health Using the Human Oral Microbe Identification Microarray. *J. Periodontol.* 80 (9), 1421–1432. doi: 10.1902/jop.2009.090185

Corwin, E. J., Hogue, C. J., Pearce, B., Hill, C. C., Read, T. D., Mulle, J., et al. (2017). Protocol for the Emory University African American Vaginal, Oral, and Gut Microbiome in Pregnancy Cohort Study. *BMC Pregnancy Childbirth* 17 (1), 161. doi: 10.1186/s12884-017-1357-x.

Darby, I., Hodge, P., Riggio, M., and Kinane, D. (2000). Microbial Comparison of Smoker and Non-Smoker Adult and Early-Onset Periodontitis Patients by Polymerase Chain Reaction. *J. Clin. Periodontol.* 27 (6), 417–424. doi: 10.1034/j.1600-051x.2000.027006417.x

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, A Chimera-Checked 16S rRNA Gene Database and Workbench Compatible With ARB. *Appl. Environ. Microbiol.* 72 (7), 5069–5072. doi: 10.1128/AEM.03006-05

Desouches, S. L., Walker, C. M., and Chung, J. H (2019). "Bacterial Pneumonia," in *Muller's Imaging of the Chest, 2nd ed*. Eds. C. M. Walker and J. H. Chung (Philadelphia, PA: Elsevier, Inc), 193–218.e2.

Detman, L. A., Cottrell, B. H., and Denis-Luque, M. F. (2010). Exploring Dental Care Misconceptions and Barriers in Pregnancy. *Birth* 37 (4), 318–324. doi: 10.1111/j.1523-536X.2010.00427.x

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W.-H., et al. (2010). The Human Oral Microbiome. *J. Bacteriol.* 192 (19), 5002–5017. doi: 10.1128/JB.00542-10

Eick, S., and Pfister, W. (2002). Comparison of Microbial Cultivation and a Commercial PCR Based Method Fordetection of Periodontopathogenic Species in Subgingival Plaque Samples. *J. Clin. Periodontol.* 29 (7), 638–644. doi: 10.1034/j.1600-051X.2002.290708.x

Eribe, E. R., and Olsen, I. (2017). *Leptotrichia* Species in Human Infections II. *J. Oral. Microbiol.* 9 (1), 1368848. doi: 10.1080/20002297.2017.1368848

Ewels, P., Magnusson, M., Lundin, S., and Käller, M. (2016). MultiQC: Summarize Analysis Results for Multiple Tools and Samples in a Single Report. *Bioinformatics* 32 (19), 3047–3048. doi: 10.1093/bioinformatics/btw354

Gerner-Smidt, P., Keiser-Nielsen, H., Dorsch, M., Stackebrandt, E., Ursing, J., Blom, J., et al. (1994). Lautropia Mirabilis Gen. Nov., Sp. *Nov.*, A Gram-Negative Motile Coccus With Unusual Morphology Isolated From the Human Mouth. *Microbiology* 140 (Pt 7), 1787–1797. doi: 10.1099/13500872-140-7-1787.

Giglio, J. A., Lanni, S. M., Laskin, D. M., and Giglio, N. W. (2009). Oral Health Care for the Pregnant Patient. *J. Can. Dent. Assoc.* 75 (1), 43–48.

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and Complex Bacterial Profiles in Human Periodontitis and Health Revealed by 16S Pyrosequencing. *ISME J.* 6 (6), 1176–1185. doi: 10.1038/ismej.2011.191

Guimarães, A. N., Silva-Mato, A., Miranda Cota, L. O., Siqueira, F. M., and Costa, F. O. (2010). Maternal Periodontal Disease and Preterm or Extreme Preterm Birth: An Ordinal Logistic Regression Analysis. *J. Periodontol.* 81 (3), 350–358. doi: 10.1902/jop.2009.090527

Guthmiller, J., and Novak, K. (2002). "Periodontal Disease," in *Polymicrobial Diseases*. Eds. K. Brogden and J. Guthmiller (Washington, DC: ASM Press). Available at: https://www.ncbi.nlm.nih.gov/books/NBK2496/. Chapter 8.

Henshaw, M. M., Garcia, R. I., and Weintraub, J. A. (2018). Oral Health Disparities Across the Life Span. *Dent. Clin. North Am.* 62 (2), 177–193. doi: 10.1016/j.cden.2017.12.001

Holt, S. C., and Ebersole, J. L. (2005). *Porphyromonas Gingivalis, Treponema Denticola*, and *Tannerella Forsythia*: The 'Red Complex', A Prototype Polybacterial Pathogenic Consortium in Periodontitis. *Periodontol* 38 (1), 72–122. doi: 10.1111/j.1600-0757.2005.00113.x

Hu, Y.-J., and Satten, G. A. (2020). Testing Hypotheses About the Microbiome Using the Linear Decomposition Model (LDM). *Bioinformatics* 36 (14), 4106–4115. doi: 10.1093/bioinformatics/btaa260

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., et al. (2012). Structure, Function and Diversity of the Healthy Human Microbiome. *Nature* 486 (7402), 207. doi: 10.1038/nature11234.

Hwang, S. S., Smith, V. C., McCormick, M. C., and Barfield, W. D. (2011). Racial/ethnic Disparities in Maternal Oral Health Experiences in 10 States, Pregnancy Risk Assessment Monitoring System 2004–2006. *Matern. Child Health J.* 15 (6), 722–729. doi: 10.1007/s10995-010-0643-2

Ibrahim, M., Subramanian, A., and Anishetty, S. (2017). Comparative Pan Genome Analysis of Oral *Prevotella* Species Implicated in Periodontitis. *Funct. Integr. Genomics* 17 (5), 513–536. doi: 10.1007/s10142-017-0550-3

Ide, M., and Papapanou, P. N. (2013). Epidemiology of Association Between Maternal Periodontal Disease and Adverse Pregnancy Outcomes–Systematic Review. *J. Periodontol.* 84, S181–S194. doi: 10.1902/jop.2013.134009

Illumina Inc. (2020). *16S Metagenomic Sequencing Library Preparation*. Available at: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Jared, H., and Boggess, K. A. (2008). Periodontal Diseases and Adverse Pregnancy Outcomes: A Review of the Evidence and Implications for Clinical Practice. *J. Dent. Hyg.* 82 (3), 24.

Jarjoura, K., Devine, P. C., Perez-Delboy, A., Herrera-Abreu, M., D'Alton, M., and Papapanou, P. N. (2005). Markers of Periodontal Infection and Preterm Birth. *Am. J. Obstet. Gynecol.* 192 (2), 513–519. doi: 10.1016/j.ajog.2004.07.018

Kallio, P., Nordblad, A., Croucher, R., and Ainamo, J. (1994). Self-Reported Gingivitis and Bleeding Gums Among Adolescents in Helsinki. *Community Dent. Oral. Epidemiol.* 22 (5 Pt 1), 277–282. doi: 10.1111/j.1600-0528.1994.tb02050.x

Khader, Y., Al-shishani, L., Obeidat, B., Khassawneh, M., Burgan, S., Amarin, Z. O., et al. (2009). Maternal Periodontal Status and Preterm Low Birth Weight Delivery: A Case–Control Study. *Arch. Gynecol. Obstet.* 279 (2), 165. doi: 10.1007/s00404-008-0696-2

Komine-Aizawa, S., Aizawa, S., and Hayakawa, S. (2019). Periodontal Diseases and Adverse Pregnancy Outcomes. *J. Obstet. Gynaecol. Res.* 45 (1), 5–12. doi: 10.1111/jog.13782

Kumar, R., Eipers, P., Little, R. B., Crowley, M., Crossman, D. K., Lefkowitz, E. J., et al. (2014). Getting Started With Microbiome Analysis: Sample Acquisition to Bioinformatics. *Curr. Protoc. Hum. Genet.* 18.8, 1–18.8.29. doi: 10.1002/0471142905.hg1808s82

Larsen, J. M. (2017). The Immune Response to *Prevotella* Bacteria in Chronic Inflammatory Disease. *Immunology* 151 (4), 363–374. doi: 10.1111/imm.12760

Lin, D., Moss, K., Beck, J. D., Hefti, A., and Offenbacher, S. (2007). Persistently High Levels of Periodontal Pathogens Associated With Preterm Pregnancy Outcome. *J. Periodontol.* 78 (5), 833–841. doi: 10.1902/jop.2007.060201

Liu, B., Faller, L. L., Klitgord, N., Mazumdar, V., Ghodsi, M., Sommer, D. D., et al. (2012). Deep Sequencing of the Oral Microbiome Reveals Signatures of Periodontal Disease. *PloS One* 7 (6), e37919. doi: 10.1371/journal.pone.0037919

Lombardo Bedran, T. B., Marcantonio, R. A., Spin Neto, R., Alves Mayer, M. P., Grenier, D., Spolidorio, L. C., et al. (2012). *Porphyromonas Endodontalis* in Chronic Periodontitis: A Clinical and Microbiological Cross-Sectional Study. *J. Oral. Microbiol.* 4, 10123. doi: 10.3402/jom.v4i0.10123

Lourenço, T. G., Heller, D., Silva-Boghossian, C. M., Cotton, S. L., Paster, B. J., and Colombo, A. P. (2014). Microbial Signature Profiles of Periodontally Healthy and Diseased Patients. *J. Clin. Periodontol.* 41 (11), 1027–1036. doi: 10.1111/jcpe.12302

Macedo, J., Ribeiro, R., Machado, F., Assis, N., Alves, R., Oliveira, A., et al. (2014). Periodontal Disease and Oral Health-Related Behavior as Factors Associated With Preterm Birth: A Case–Control Study in South-Eastern Brazil. *J. Periodontal. Res.* 49 (4), 458–464. doi: 10.1111/jre.12124

Magurran, A. E. (2013). *Measuring Biological Diversity* (Hoboken, NJ: Wiley-Blackwell).

Martin, J. A., Hamilton, B. E., Osterman, M. J. K., Driscoll, A. K., and Drake, P. (2018). Births: Final Data for 2017. *Natl. Vital Stat. Rep.* 67 (8), 1–50. doi:

Matthews, C. R., Joshi, V., de Jager, M., Aspiras, M., and Kumar, P. S. (2013). Host–Bacterial Interactions During Induction and Resolution of Experimental Gingivitis in Current Smokers. *J. Periodontol.* 84 (1), 32–40. doi: 10.1902/jop.2012.110662

Mavrommatis, K., Pukall, R., Rohde, C., Chen, F., Sims, D., Brettin, T., et al. (2009). Complete Genome Sequence of *Cryptobacterium Curtum* Type Strain (12-3). *Stand Genomic Sci.* 1 (2), 93–100. doi: 10.4056/sigs.12260

McInnes, P., and Cutting, M. (2010) *Manual of Procedures for Human Microbiome Project: Core Microbiome Sampling Protocol A, HMP Protocol# 07-001, Version Number: 12.0*. Available at: https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/document.cgi?study_id=phs000228.v3.p1&phd=3190

McNeil, D. W., Hayes, S. E., Randall, C. L., Polk, D. E., Neiswanger, K., Shaffer, J. R., et al. (2016). Depression and Rural Environment are Associated With Poor Oral Health Among Pregnant Women in Northern Appalachia. *Behav. Modif.* 40 (1-2), 325–340. doi: 10.1177/0145445515615353

Mejia, G. C., Elani, H. W., Harper, S., Murray Thomson, W., Ju, X., Kawachi, I., et al. (2018). Socioeconomic Status, Oral Health and Dental Disease in Australia, Canada, New Zealand and the United States. *BMC Oral. Health* 18 (1), 176. doi: 10.1186/s12903-018-0630-3

Mohanty, R., Asopa, S. J., Joseph, M. D., Singh, B., Rajguru, J. P., Saidath, K., et al. (2019). Red Complex: Polymicrobial Conglomerate in Oral Flora: A Review. *J. Family Med. Prim. Care* 8 (11), 3480–3486. doi: 10.4103/jfmpc.jfmpc_759_19

Moore, L. V., and Moore, W. E. (1994). Oribaculum Catoniae Gen. Nov., Sp. *Nov.*; Catonella Morbi Gen. Nov., Sp. *Nov.*; *Hallella Seregens Gen. Nov.*, Sp. *Nov.*; *Johnsonella Ignava Gen. Nov.*, Sp. *Nov.*; and *Dialister Pneumosintes Gen. Nov., Comb. Nov., Nom. Rev.*, Anaerobic Gram-Negative Bacilli From the Human Gingival Crevice. *Int. J. Syst. Bacteriol.* 44 (2), 187–192. doi: 10.1099/00207713-44-2-187.

Moreillon, P. (2010). "Endocarditis and Endarteritis," in *Infectious Diseases, 3rd ed*. Eds. J. Cohen, S. M. Opal and W. G. Powderly (St. Louis, MO: Mosby), 514–528.

Novak, M. J., Novak, K. F., Hodges, J. S., Kirakodu, S., Govindaswami, M., DiAngelis, A., et al. (2008). Periodontal Bacterial Profiles in Pregnant Women: Response to Treatment and Associations With Birth Outcomes in the Obstetrics and Periodontal Therapy (OPT) Study. *J. Periodontol.* 79 (10), 1870–1879. doi: 10.1902/jop.2008.070554

Offenbacher, S., Katz, V., Fertik, G., Collins, J., Boyd, D., Maynor, G., et al. (1996). Periodontal Infection as a Possible Risk Factor for Preterm Low Birth Weight. *J. Periodontol.* 67 (10s), 1103–1113. doi: 10.1902/jop.1996.67.10s.1103

Papapanou, P. N., Park, H., Cheng, B., Kokaras, A., Paster, B., Burkett, S., et al. (2020). Subgingival Microbiome and Clinical Periodontal Status in an Elderly Cohort: The WHICAP Ancillary Study of Oral Health. *J. Periodontol.* 91 (S1), S56–S67. doi: 10.1002/JPER.20-0194

Papapanou, P. N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D. H., et al. (2018). Periodontitis: Consensus Report of Workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J. Periodontol.* 89 Suppl 1, S173–S182. doi: 10.1002/JPER.17-0721

Parikh, L. I., Reddy, U. M., Männistö, T., Mendola, P., Sjaarda, L., Hinkle, S., et al. (2014). Neonatal Outcomes in Early Term Birth. *Am. J. Obstet. Gynecol.* 211 (3), 265.e1–265.e11. doi: 10.1016/j.ajog.2014.03.021

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* 41 (D1), D590–D596. doi: 10.1093/nar/gks1219

Raju, K., and Berens, L. (2021). Periodontology and Pregnancy: An Overview of Biomedical and Epidemiological Evidence. *Periodontol* 87 (1), 132–142. doi: 10.1111/prd.12394

Renvoise, A., Raoult, D., and Roux, V. (2009). *Actinomyces Massiliensis* Sp. *Nov.*, Isolated From a Patient Blood Culture. *Int. J. Syst. Evol. Microbiol.* 59 (Pt 3), 540–544. doi: 10.1099/ijs.0.001503-0

Rocha, J. S., Arima, L. Y., Werneck, R. I., Moysés, S. J., and Baldani, M. H. (2018). Determinants of Dental Care Attendance During Pregnancy: A Systematic Review. *Caries Res.* 52 (1-2), 139–152. doi: 10.1159/000481407

Rossmann, S. N., Wilson, P. H., Hicks, J., Carter, B., Cron, S. G., Simon, C., et al. (1998). Isolation of *Lautropia Mirabilis* From Oral Cavities of Human Immunodeficiency Virus-Infected Children. *J. Clin. Microbiol.* 36 (6), 1756–1760. doi: 10.1128/JCM.36.6.1756-1760.1998

Slobodkin, A. (2014). "The Family Peptostreptococcaceae," in *The Prokaryotes*. Eds. E. Rosenberg, E. F. Delong, S. Lory, E. Stackebrandt and F. Thompson (Berlin, Heidelberg: Springer). doi: 10.1007/978-3-642-30120-9_217.

Smid, M. C., Dotters-Katz, S. K., Plongla, R., and Boggess, K. A. (2015). *Leptotrichia Buccalis*: A Novel Cause of Chorioamnionitis. *Infect. Dis. Rep.* 7 (2), 5801. doi: 10.4081/idr.2015.5801

Socransky, S. S., and Haffajee, A. D. (2005). Periodontal Microbial Ecology. *Periodontol* 38 (1), 135–187. doi: 10.1111/j.1600-0757.2005.00107.x

Stadelmann, P. F., Eick, S., Salvi, G. E., Surbek, D., Mohr, S., Bürgin, W., et al. (2014). Increased Periodontal Inflammation in Women With Preterm Premature Rupture of Membranes. *Clin. Oral. Investig.* 19, 1537–1546. doi: 10.1007/s00784-014-1371-6

Usin, M. M., Tabares, S. M., Parodi, R. J., and Sembaj, A. (2013). Periodontal Conditions During the Pregnancy Associated With Periodontal Pathogens. *J. Investig. Clin. Dent.* 4 (1), 54–59. doi: 10.1111/j.2041-1626.2012.00137.x

Vettore, M. V., Leão, A. T., Leal, M., d., C., Feres, M., and Sheiham, A. (2008). The Relationship Between Periodontal Disease and Preterm Low Birthweight: Clinical and Microbiological Results. *J. Periodontal. Res.* 43 (6), 615–626. doi: 10.1111/j.1600-0765.2007.01027.x

Vielkind, P., Jentsch, H., Eschrich, K., Rodloff, A. C., and Stingu, C. S. (2015). Prevalence of Actinomyces Spp. In Patients With Chronic Periodontitis. *Int. J. Med. Microbiol.* 305 (7), 682–688. doi: 10.1016/j.ijmm.2015.08.018

Wade, W. G. (2013). The Oral Microbiome in Health and Disease. *Pharmacol. Res.* 69 (1), 137–143. doi: 10.1016/j.phrs.2012.11.006

Wu, X., Chen, J., Xu, M., Zhu, D., Wang, X., Chen, Y., et al. (2017). 16s rDNA Analysis of Periodontal Plaque in Chronic Obstructive Pulmonary Disease and Periodontitis Patients. *J. Oral. Microbiol.* 9 (1), 1324725. doi: 10.1080/20002297.2017.1324725

Wyss, C., Moter, A., Choi, B. K., Dewhirst, F. E., Xue, Y., Schüpbach, P., et al. (2004). *Treponema Putidum* Sp. *Nov.*, A Medium-Sized Proteolytic Spirochaete Isolated From Lesions of Human Periodontitis and Acute Necrotizing Ulcerative Gingivitis. *Int. J. Syst. Evol. Microbiol.* 54 (Pt 4), 1117–1122. doi: 10.1099/ijs.0.02806-0

Yang, I., Arthur, R. A., Zhao, L., Clark, J., Hu, Y., Corwin, E. J., et al. (2021). The Oral Microbiome and Inflammation in Mild Cognitive Impairment. *Exp. Gerontol.* 147, 111273. doi: 10.1016/j.exger.2021.111273

You, M., Mo, S., Leung, W. K., and Watt, R. M. (2013). Comparative Analysis of Oral Treponemes Associated With Periodontal Health and Disease. *BMC Infect. Dis.* 13, 174. doi: 10.1186/1471-2334-13-174

Zarco, M., Vess, T., and Ginsburg, G. (2012). The Oral Microbiome in Health and Disease and the Potential Impact on Personalized Dental Medicine. *Oral. Dis.* 18 (2), 109–120. doi: 10.1111/j.1601-0825.2011.01851.x

Zaura, E., Keijser, B. J., Huse, S. M., and Crielaard, W. (2009). Defining the Healthy "Core Microbiome" of Oral Microbial Communities. *BMC Microbiol.* 9, 259. doi: 10.1186/1471-2180-9-259

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 12 May 2022
doi: 10.3389/fcimb.2022.838750

Associations of *Blautia* Genus With Early-Life Events and Later Phenotype in the NutriHS

Renata G. Borges de Oliveira Nascimento Freitas[1,2], *Ana Carolina J. Vasques*[2,3], *Gabriel da Rocha Fernandes*[4], *Francieli B. Ribeiro*[2,3], *Isabela Solar*[2,3], *Marina G. Barbosa*[3], *Bianca de Almeida- Pititto*[5], *Bruno Geloneze*[2,6] *and Sandra Roberta G. Ferreira*[1*]

[1] Department of Epidemiology, School of Public Health, University of São Paulo, São Paulo, Brazil, [2] Laboratory of Investigation in Metabolism and Diabetes, Gastrocentro, School of Medical Sciences, University of Campinas, Campinas, Brazil, [3] School of Applied Sciences, University of Campinas, Campinas, Brazil, [4] Oswaldo Cruz Foundation, Belo Horizonte, Brazil, [5] Department of Preventive Medicine, Federal University of São Paulo, São Paulo, Brazil, [6] Obesity and Comorbidities Research Center, University of Campinas, Campinas, Brazil

OPEN ACCESS

Edited by:
Veeranoot Nissapatorn, Walailak University, Thailand

Reviewed by:
Alexander V. Tyakht, Institute of Gene Biology (RAS), Russia
Selvasankar Murugesan, Sidra Medicine, Qatar

***Correspondence:**
Sandra Roberta G. Ferreira
sandrafv@usp.br

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *18 December 2021*
Accepted: *11 April 2022*
Published: *12 May 2022*

Citation:
Freitas RGBdON, Vasques ACJ, Fernandes GdR, Ribeiro FB, Solar I, Barbosa MG, Pititto BdA-, Geloneze B and Ferreira SRG (2022) Associations of Blautia Genus With Early-Life Events and Later Phenotype in the NutriHS. Front. Cell. Infect. Microbiol. 12:838750. doi: 10.3389/fcimb.2022.838750

Introduction: Early-life events are associated with the risk of obesity and comorbidities later in life. The gut microbiota—whose composition is influenced by genetics and environmental factors—could be involved. Since the microbiota affects metabolism and fat storage, early-life insults could contribute to the occurrence of obesity driven, in part, by microbiota composition. We examined associations of gut bacteria with early-life events, nutritional status, and body composition in the Nutritionist's Health Study (NutriHS).

Methods: A cross-sectional study of 114 female participants examining early-life data, body composition, and biological samples was conducted. Fecal microbiota structure was determined targeting the V4 region of the 16S rRNA gene. Principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) were used to test the impact of variables on microbial diversity. Profiles were identified using the Jensen-Shannon divergence matrix and Calinski–Harabasz index. Differential abundance between the categories of exclusive breastfeeding duration and nutritional status was tested using DESeq2.

Results: In the sample [median age 28 years and body mass index (BMI) 24.5 kg/m^2], 2 microbiota profiles driven by the *Blautia* or *Prevotella* genus were identified. An estimated 9.1% of the variation was explained by the profiles ($p < 0.001$), 2.1% by nutritional status ($p = 0.004$), and 1.8% by exclusive breastfeeding ($p = 0.012$). The proportion of participants with BMI <25 kg/m^2 and who were breastfed for at least 6 months was higher in the *Blautia* profile ($p < 0.05$).

Conclusion: Findings in a *Blautia*-driven profile of healthy women reinforce that early-life events play a role in defining gut microbiota composition, confirming the importance of exclusive breastfeeding for infant gut colonization in establishing a protective profile against adiposity-related outcomes in adulthood.

Keywords: gut microbiota, early-life events, DOHAD, breastfeeding, nutritional status

INTRODUCTION

The importance of prenatal and postnatal events in long-term health outcomes has been consistently recognized (Ravelli et al., 1976; Barker, 1990; Bell et al., 2017; Block and El-Osta, 2017; Cheshmeh et al., 2020; Capra et al., 2021). Nutritional factors during intrauterine life and after birth have a major impact on infant health and later in adulthood, influencing the risk for non-communicable chronic diseases (Garmendia et al., 2014; Cadenas-Sanchez et al., 2017). Early feeding and infant growth rate have been associated with the risk of obesity and cardiometabolic diseases later in life (Kelishadi and Farajian, 2014; Kapourchali and Cresci, 2020). Important underlying mechanisms of these associations involve the gut microbiome (Bouter et al., 2017; Meijnikman et al., 2018). Gut colonization of the newborn starts at birth by bacteria from the mother and the environment. Major determinants of gut microbiota composition in early life are type of delivery, lactation, antibiotic use, and sanitary conditions (Biasucci et al., 2010; Martin et al., 2016; Le Doare et al., 2018; Cheng and Ning, 2019). Evidence indicates that these factors shape the gut microbiota throughout life (Rodríguez et al., 2015; Cheng and Ning, 2019) and that adult microbiota composition shows slight fluctuations around a core of stable colonizers.

Despite similar counts of human cells and microbes throughout the gastrointestinal tract, the gut microbiome contains 100 times more genes (Qin et al., 2010; Shen et al., 2013; Sender et al., 2016). This indicates that microbial communities play vital roles in the host and that an unbalanced microbiota can deteriorate regulatory functions, triggering immune and metabolic disturbances (Levy et al., 2017; Sommer et al., 2017). Factors such as aging (Rodríguez et al., 2015; Cheng and Ning, 2019; Fan and Pedersen, 2021), diet (David et al., 2014), nutritional status, and exercise induce changes (Rodríguez et al., 2015; Mailing et al., 2019) in microbiota composition, hampering understanding of the involvement of this complex ecosystem in pathophysiological processes. Arumugam et al. (2011) proposed analyzing the gut microbiota based on microbial profiles driven by discriminative genera referred to as enterotypes. Long-term dietary patterns have been linked to enterotypes in populations. A carbohydrate-based or vegetarian diet was found to be associated with *Prevotella*, while the typical Western diet was associated with *Bacteroides* enterotype (De Filippo et al., 2010; Wu et al., 2011; de Moraes et al., 2017). However, further studies have questioned such discrete profiles, given that these microbial communities proved not to be recurrent across diverse human populations (Gorvitovskaia et al., 2016). Despite controversies, it is clear that the risk or protection against non-communicable chronic diseases conferred by lifestyle is modulated by the gut microbiota, which affects nutrient acquisition, energy regulation, and fat storage (Rosenbaum et al., 2015; Wu et al., 2021). This could be a plausible pathway by which early-life exposures are associated with later body phenotypes.

Our group has been conducting the Nutritionist's Health Study (NutriHS) involving nutrition undergraduates and nutritionists (Folchetti et al., 2016). This represents a unique opportunity to collect reliable nutrition-related data, accurate body composition measurements, and biological samples to test associations with early-life events and current lifestyle potentially mediated by the gut microbiota. The aim of the present study was to examine associations of gut bacteria with early-life events, current nutritional status, and body composition in NutriHS participants.

MATERIALS AND METHODS

Study Design and Participants

This cross-sectional analysis was part of the multicenter NutriHS conducted at the School of Public Health of the University of São Paulo State, Brazil, to investigate markers of cardiometabolic diseases (Folchetti et al., 2016). Current data were collected at the University of Campinas (UNICAMP), located in Campinas city in the interior of São Paulo state. The NutriHS was approved by the local research ethics committee, and volunteers signed an electronic informed consent form available on the *e*-NutriHS system (www.fsp.usp.br/nutrihs). Recruitment of volunteers took place between 2018 and 2019.

Eligibility criteria were female undergraduates or nutritionists aged 19–44 years, body mass index (BMI) between 18.5 and 39.9 kg/m², and individuals whose mothers were alive. Pregnant and nursing women or individuals with diabetes, kidney, heart, and liver diseases, or other severe systemic diseases, in use of medications affecting glucose metabolism and/or body adiposity, or in use of probiotics or antibiotics in the last 3 months were excluded. Participants filled out online structured validated questionnaires. Respondents were then invited to schedule a face-to-face visit for physical examination and collection of biological samples. A total of 248 women answered the questionnaires, 127 met the inclusion criteria, and 114 concluded the full protocol (**Figure S1**).

Early-Life and Current Data

Regarding information about early-life events, participants were advised to consult birth cards and seek assistance from their mothers. Maternal data collected were pre-pregnancy age, education levels (<11; ≥11 years) and BMI, and gestational

diabetes, hypertension, or other complications (yes; no), parity (0; ≥1 pregnancy), tobacco, alcohol, and/or drug use (no; yes), and type of delivery (vaginal; C-section). Maternal gestational weight gain and participants' birth weight were obtained as continuous variables. Continuous data on participant birth weight and duration of exclusive breastfeeding were further categorized into <2.5 kg, 2.5–4.0 kg, or ≥4.0 kg and into <6 months or ≥6 months, respectively.

Current data collected were skin color (white; non-white), age, family income (<6; ≥6 minimum wages), and engagement in leisure time physical activity (no; yes). Physical activity was assessed using the short version of the International Physical Activity Questionnaire (Craig et al., 2003) validated for use in Brazil (Matsudo et al., 2001). Dietary intake was estimated using a validated food frequency questionnaire for the adult population living in São Paulo, with the previous year as the time frame (Selem et al., 2014). The questionnaire comprised 101 food items, and food equivalents in the USDA National Nutrient Database for Standard Reference were employed (Haytowitz et al., 2019).

Clinical and Body Composition Assessment

Body weight was obtained using a digital scale, and height was measured using a fixed stadiometer. BMI was calculated, and nutritional status was classified according to the WHO standards (WHO, 2015). Adequate nutritional status was defined as BMI >18.5 and <25 kg/m^2. Waist circumference was measured at the midpoint between the last rib and iliac crest using an inelastic tape.

Body composition was assessed using dual-energy x-ray absorptiometry (DXA) (GE Lunar iDXA® with EnCore software, Madison, WI, USA) by a trained researcher. Instrument quality control was checked routinely according to the manufacturer's instructions. Parameters of interest were measurements of total fat and visceral fat mass and of total and appendicular lean mass.

Biochemical Analyses

After a 12-h overnight fast, blood samples were collected for biochemical determinations. Glucose and lipid profile [total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides] were measured using the glucose oxidase and enzymatic colorimetric methods, respectively. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedwald equation. Plasma insulin was obtained using an automated two-site chemiluminescent immunometric assay (Immulite 1000 System, Siemens Health Diagnostics, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated (Matthews et al., 1985). High-sensitivity C-reactive protein was determined by nephelometry using a BN ProSpec System (Siemens, Marburg, Germany).

Plasma concentrations of short-chain fatty acids (SCFAs: acetate, propionate, and butyrate) were measured by gas chromatography (Wang et al., 2019). Briefly, ethanol, n-hexane, and an internal standard (caprylic acid) were added to serum. Samples were centrifuged and transferred to specific vials, and pH was adjusted to 4.0. A calibration curve with 0.015–0.1 mg/ml SCFA was used in the quantification. Chromatographic analyses were performed using a gas chromatograph-mass spectrometer (model Coupled QP2010 Plus; Shimadzu®, Kyoto, Japan) and a fused-silica capillary Stabilwax column (Restec Corporation, USA) with dimensions of 30 m × 0.25 mm internal diameter and coated with a 0.25-μm-thick layer of polyethylene glycol. Samples were injected at 250°C using a 25:1 split ratio for feces or splitless. High-grade pure helium was used as the carrier gas with a constant flow rate of 1.0 ml/min. Mass conditions were as follows: ionization voltage, 70 eV; ion source temperature, 200°C; full scan mode in the 35–500 mass range with 0.2 s/scan velocity. The butyrate columns did not appear, since the concentration of the acid was not detectable in the samples.

Gut Microbiota Analysis

Fecal samples were refrigerated within 24 h after collection, and aliquots were stored at -80°C until analysis. According to the manufacturer's instructions, DNA was extracted using the Maxwell® 16 DNA purification kit and the protocol was carried out on the Maxwell® 16 Instrument (Promega, Madison, WI, USA). We used the primers and workflow to generate the amplicon from the V4 region of the 16s rRNA gene according to Penington et al. (2018). The amplicon library produced was sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA), according to the manufacturer's instructions.

The raw read files were processed in the R environment using the dada2 package [10.1038/nmeth.3869] (Ombrello, 2020). The forward and reverse sequences were trimmed to 150 bases. Reads containing more than two expected errors were removed. Errors in filtered sequences were corrected by the algorithm and joined to form the amplicon sequence variants (ASVs). The chimeric sequences were also removed, and a sample count table was generated. The taxonomic classification was done with the *tag.me* package [10.1101/263293] using the model 515F-806R (Pires et al., 2018).

Statistical Analyses

All data were recorded, edited, and entered using the Statistical Package for the Social Sciences (SPSS version 20; IBM, NY, USA) and the R package for microbiota analyses. Level of significance was set at a p-value of 5%. Descriptive data were expressed as means [standard deviations (SDs)] or medians {q25–q75 ranges [interquartile range (IQR)]}. The Kolmogorov–Smirnov test was used to test data normality. Parametric tests (Pearson's correlation coefficient and Student's t test) and non-parametric tests (Spearman's correlation coefficient and Mann–Whitney) were applied according to the distribution of variables.

The beta diversity was calculated using principal coordinates analysis (PCoA) and the ade4 R package for each library (Dray and Dufour, 2007). Permutational multivariate analysis of variance (PERMANOVA) was performed using 999 permutations to test the impact of categorical variables on beta diversity. Distance-based redundancy analysis (dbRDA) highlights variables with some association with the individual microbiota dissimilarities (Legendre and Anderson, 1999). Profiles were identified based on the Jensen-Shannon divergence matrix and using the Partitioning Around Medoids (PAM) algorithm, and the optimal number of clusters was determined by the Calinski–Harabasz index. The alpha diversity was measured by the Shannon and Simpson indexes.

Differential abundance between profiles according to the categories of exclusive breastfeeding duration and nutritional status was tested using DESeq2, leaving genus with at least 50%-fold change and present in half of the samples (Love et al., 2014).

Macronutrient intakes were expressed as percentage of total energy intake (TEI) and fatty acid intake in grams. Correlations between dietary components and SCFA concentrations and body adiposity parameters were tested using Spearman's coefficient.

RESULTS

The sample of 114 participants had a median age of 28 (IQR 24–31) years; 41.6% were undergraduates and 58.4% were nutritionists. Sixty-one percent of the participants engaged in moderate physical activity regularly, none was a professional athlete, and 51.0% had normal BMI. Regarding maternal characteristics, 35% had higher-level education and 90% were normal weight before pregnancy and had no clinical complications during the pregnancy. In the total sample, there was a predominance of cesarean delivery (66%) and normal birth weight (90%), 94% were breastfed, and 18% were exclusively breastfed for at least 6 months. In addition, 30% of participants reported overweight/obesity in childhood or adolescence.

The dbRDA (**Figure 1**) results show that exclusive breastfeeding and adequate nutritional status are located to the right and adiposity parameters to the left of the plot. The relative abundance of *Blautia*, *Anaerostipes*, and *Lachnoclostridium* increased directly on the X-axis representing both breastfeeding and adequate nutritional status (**Figure 1**). Conversely, inverse relationships for *Ruminococcaceae* were observed. The results of the redundancy analysis for the most abundant bacteria are shown in the supplementary material (**Figure S2**).

Beta diversity analysis of the microbiota revealed two bacterial profiles in the samples driven by the *Blautia* or *Prevotella* genus. Fifty-six participants were assigned to *Blautia* and 58 to *Prevotella* profiles. The PERMANOVA analysis on the Jensen-Shannon divergence values estimated that 9.1% of the variation among the samples was explained by the profiles ($p < 0.001$), 2.1% by nutritional status ($p = 0.004$), and 1.8% by exclusive breastfeeding ($p = 0.012$). Proportions of participants with BMI <25 kg/m^2 and of those breastfed for at least 6 months were significantly ($p < 0.05$) higher in the *Blautia*-driven profile (**Figure 2**). A schematic interpretation of the main findings is provided in **Figure 3**. The proportion categorized by type of delivery (vaginal or cesarean section) or birth weight (adequate or inadequate) did not differ between the 2 groups (not shown in figures).

The differential abundance analysis identified genus drivers used to describe the bacterial composition in the profiles. The candidates present in at least 50% of the fecal samples are shown in the supplementary material (**Figure S3**), and bacteria differentially abundant between the 2 profiles were listed in **Table S1**.

Differences in some abundances between the profiles are depicted in **Figure 4**. *Lachnoclostridium* (*Lachnospiraceae* family, *Clostridiales* order, *Clostridia* class) abundance was higher in the *Blautia* profile, whereas several genera from *Ruminococcaceae* and *Christensenellaceae* families (both from *Clostridiales* order, *Clostridia* class) were predominant in the *Prevotella* profile.

The main characteristics of participants by profile are given in **Table 1**. Gestational weight gain, type of delivery, and birth weight did not differ between the groups, but the rate of exclusive breastfeeding ≥6 months was higher in the *Blautia*- than that in the *Prevotella*-driven profile (21.4% vs. 6.9%, respectively, $p = 0.04$). Clinical and body composition variables of both groups were within normal ranges. Butyrate concentrations were undetectable for the whole sample.

Dietary data of participants such as total energy and macronutrient and fatty acid intakes as a percentage of total energy did not differ, but median intakes of total, soluble, and insoluble fibers were higher in the *Prevotella*- than those in the *Blautia*-driven profile (**Table 2**). The dbRDA showed that the percentage of variance explained by diet was low (X-axis with 4.5% and Y-axis with 1.5%), being 94% explained by other factors. No significant correlation of fiber intake with SCFA concentrations and metabolic or body adiposity variables was detected.

DISCUSSION

This study explores the discussion regarding the influence of early-life events on gut microbiota composition in adulthood. A specific sample of women with literacy in nutrition was investigated. The associations suggested that longer breastfeeding impacts both microbiota composition and nutritional status in adulthood. By using a clustering approach to define microbiota profiles, in one profile driven by the genus *Blautia*, the same associations were confirmed. Both *Blautia*- and *Prevotella*-driven profiles are consistent with a healthy diet rich in fibers with an adequate macronutrient distribution and were therefore expected in the individuals studied. The findings in this homogeneous sample revealed the presence of macrostructures in the gut microbiota dominated by *Blautia* or *Prevotella*, SCFA-producing genera associated with beneficial metabolic effects. Interestingly, *Blautia* was associated with exclusive breastfeeding, whose relevance for gut colonization and body systems programming has been previously reported, as well as its health implications throughout the life span. Our findings not only reinforce the relevance of early feeding but also suggest an impact on gut colonization that persists into adulthood, contributing to a beneficial microbiota pattern. Furthering this knowledge could help in the prevention of chronic diseases.

For the overall sample, direct associations of some genera (*Blautia*, *Anaerostipes*, and *Lachnoclostridium*) of the family *Lachnospiraceae* with the recommended practice of long breastfeeding to prevent chronic diseases (including those related to excess body adiposity) were suggested. In fact, the profile analyses showed that the same genera were also associated with adequate nutritional status in adulthood. These findings

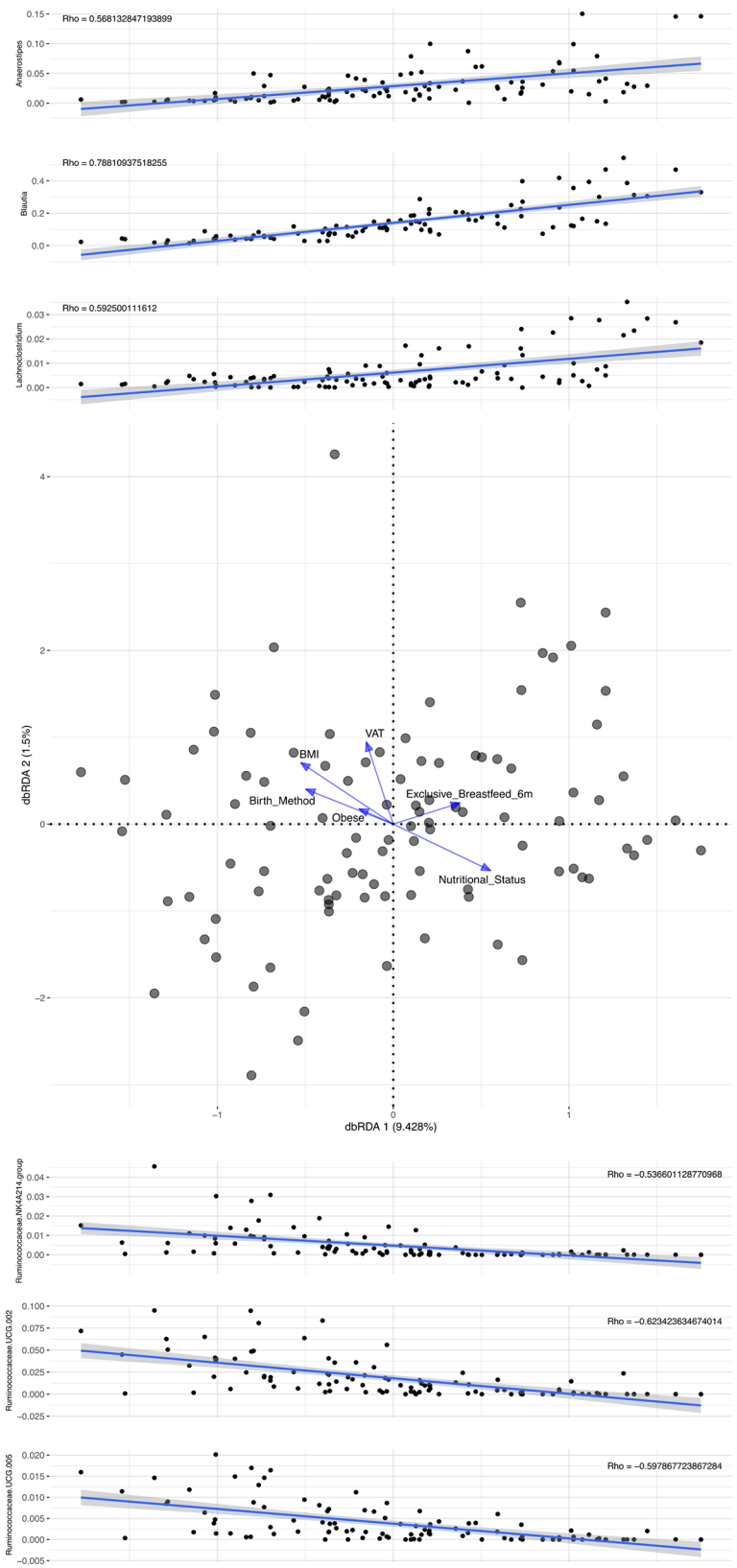


FIGURE 1 | Distance-based redundancy analysis identified in 114 participants.

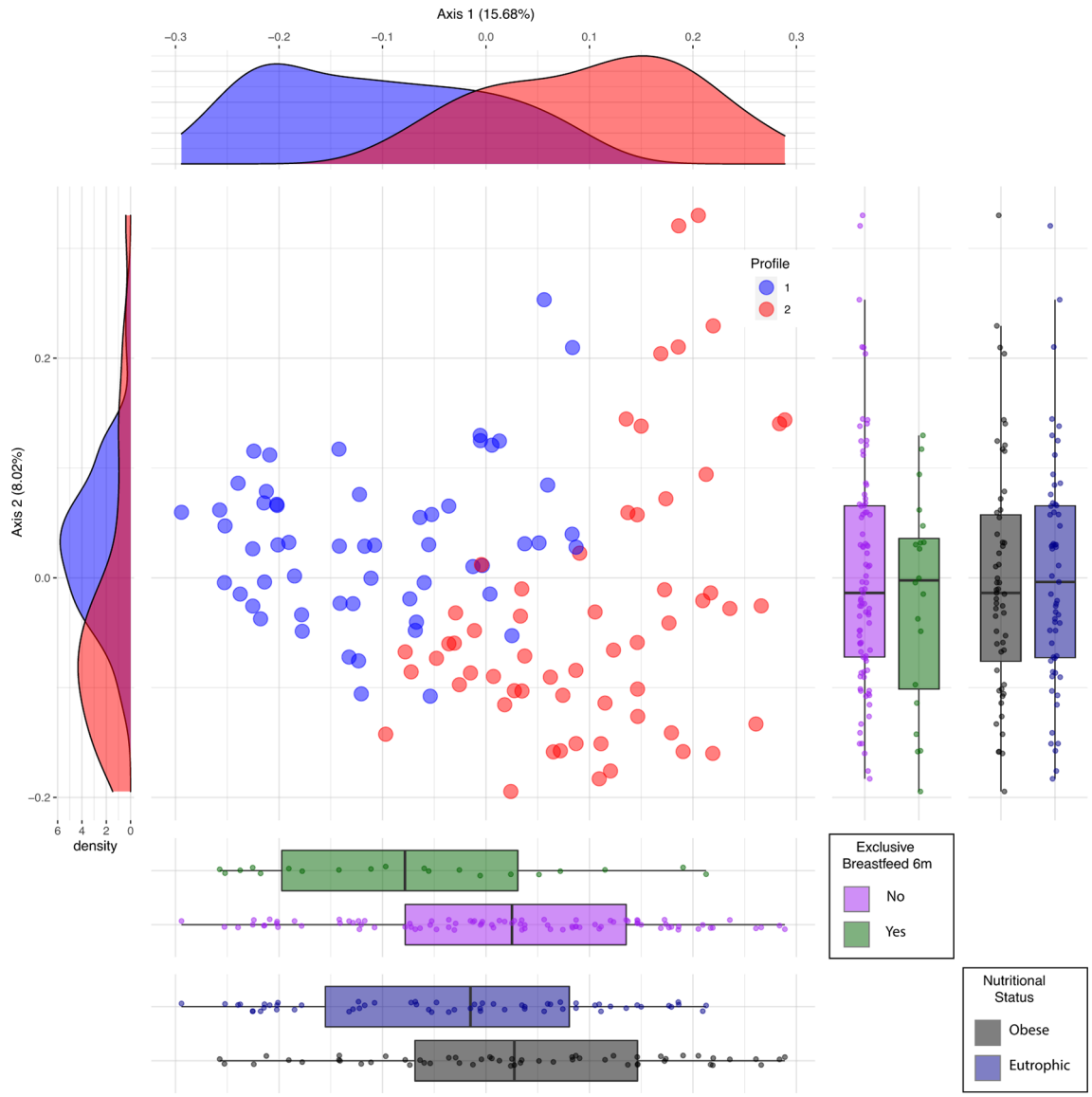


FIGURE 2 | Profiles driven by *Blautia* (#1) and *Prevotella* (#2) identified by principal coordinates analysis (PCoA). #1 in blue is driven by *Blautia*; #2 in red is driven by *Prevotella*. Vertical boxplots represent the distribution of participants according to categories of breastfeeding and nutritional status ($p < 0.05$). Horizontal boxplots show the distribution of participants into profiles stratified by these categories.

contrast with previous reports of an association of *Lachnoclostridium* species with adiposity (Zhao L. et al., 2017; Sun et al., 2020; Nogal et al., 2021) but are in line with other studies in which *Anaerostipes* abundance was associated with a lower risk of type 2 diabetes (Yang et al., 2018). With regard to the family *Ruminococcaceae*, the present dbRDA initially suggested a relationship with unfavorable body adiposity distribution that was not confirmed when correlations to visceral adipose tissue (VAT) were tested. Although *Ruminococcus*, *Anaerostipes*, and *Blautia* produce SCFA (Vital et al., 2014; Koh et al., 2016), which have beneficial metabolic actions (Kasubuchi et al., 2015; Zhao et al., 2018; Müller et al., 2019), other controversial associations have been reported. In Mexican children, these genera were directly associated with obesity (Vazquez-Moreno et al., 2021). Inconsistencies have highlighted the need of improving knowledge about the intestinal bacteria assemblages of individuals from different geographical regions.

Using a clustering approach, early-life events and current characteristics of the sample were compared to verify associations suggesting underlying mechanisms of diseases. It is noteworthy that most participants engaged in physical activity regularly and consumed a fiber-rich diet, factors known to impact microbiota composition. These conditions have also

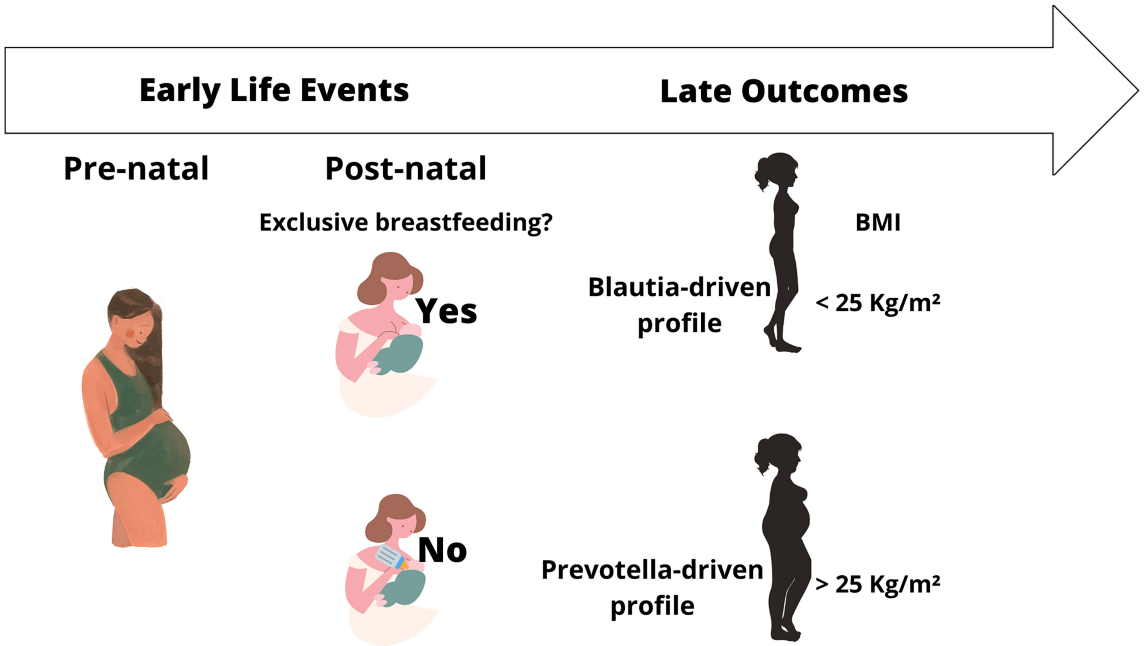


FIGURE 3 | Impact of longer breastfeeding on microbiota composition and adult nutritional status. Credit: Figure produced using Canva graphic design platform (https://www.canva.com/) and brgfx/Freepik.

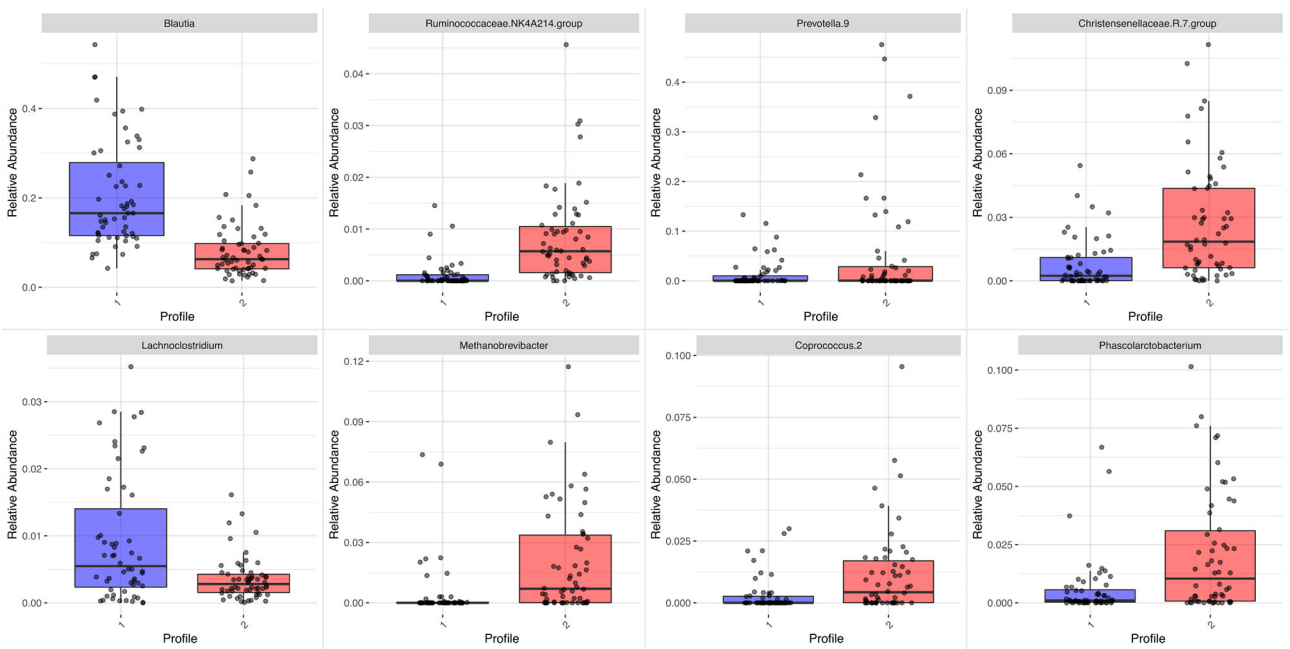


FIGURE 4 | Boxplot of differential abundances of selected genera by profile (#1 in blue is driven by *Blautia*; #2 in red is driven by *Prevotella*). Adjusted p-value <0.05.

been associated with anti-inflammatory status and favorable clinical profile (Hemmingsen et al., 2017; Nyberg et al., 2020). Normal mean values of C-reactive protein and insulin resistance index (HOMA-IR) indicated a low risk for metabolic disturbances in participants of both profiles. The findings of high abundances of *Blautia* and *Prevotella* were expected, since these genera belong to *Lachnospiraceae* and *Prevotellaceae* families that have the ability to degrade complex

TABLE 1 | Means (standard deviation) or medians (interquartile range) for clinical variables and body composition parameters of the 114 participants according to profile.

	***Blautia* profile N = 56**	***Prevotella* profile N = 58**	**p-value**
•Early-life data			
Pre-pregnancy maternal BMI (kg/m^2)	21.8 ± 2.2	22.0 ± 2.8	0.77
Gestational weight gain (kg)	14.0 (9.0; 20.0)	12.0 (9.0; 16.0)	0.42
Type of delivery			0.89
- Normal, n (%)	36 (64.3)	36 (62.1)	
- Cesarean, n (%)	18 (32.1)	19 (32.8)	
Birth weight (kg)	3.2 ± 0.5	3.2 ± 0.4	0.92
Exclusive breastfeeding ≥6 months			0.04
- No, n (%)	39 (69.6)	43 (77.1)	
- Yes, n (%)	12 (21.4)	4 (6.9)	
• Clinical data			
Body mass index (kg/m^2)	23.9 (20.9; 28.1)	25.7 (21.7; 28)	0.25
Waist circumference (cm)	76.5 (71.1; 86.1)	79.1 (73.5; 91.0)	0.14
Fasting glucose (mg/dl)	82.9 ± 5.8	81.6 ± 5.8	0.26
HOMA-IR	1.2 (0.9; 1.7)	0.9 (0.7; 1.6)	0.12
HDL cholesterol (mg/dl)	58 (50; 67.5)	55 (49; 67)	0.29
Triglycerides (mg/dl)	79 (61; 103.5)	70 (59; 103)	0.45
C-reactive protein (mg/L)	1.2 (0.6; 2.7)	1.2 (0.6; 3.2)	0.59
Total short-chain fatty acids[a] (mg/ml)	0.15 (0.10; 0.19)	0.13 (0.11; 0.20)	0.22
Acetate (mg/ml)	0.14 (0.09; 0.17)	0.11 (0.08; 0.14)	0.24
Propionate (mg/ml)	0.003 (0.002; 0.012)	0.004 (0.002; 0.011)	0.57
• DXA measurements			
Total lean mass (kg)	38.1 ± 5.1	38.9 ± 5.1	0.44
Appendicular skeletal muscle mass (kg)	16.8 ± 2.8	17.2 ± 2.7	0.40
Total fat mass (%)	37.9 ± 6.6	38.5 ± 7.8	0.65
Android fat (%)	34.7 (29.4; 46.1)	35.2 (26.3; 47.4)	0.89
Gynoid fat (%)	43.1 ± 6.6	43.9 ± 7.4	0.54
Visceral adipose tissue (g)	141 (85; 435)	156 (87; 544)	0.49

Continuous variables were compared using Student's t test or Mann–Whitney test, and data were expressed as mean ± standard deviation or median and q25–q75 ranges in parentheses. Categorical variables were compared using chi-square test.

[a]*Total short-chain fatty acid = acetate + propionate.*

HOMA-IR, Homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; DXA, dual-energy x-ray absorptiometry; BMI, Body mass index; HOMA-IR, Homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; DXA, dual-energy x-ray absorptiometry.

TABLE 2 | Medians (interquartile range) of total energy intake (TEI) and dietary data of the 114 participants according to profile.

	***Blautia* profile**	***Prevotella* profile**	**p-value**
Total energy intake (kcal)	1,958 (1,639; 2,223)	2,011 (1,593; 2,685)	0.51
Carbohydrate (% TEI)	47.0 (40.3; 52.2)	47.0 (41.2; 53.5)	0.43
Protein (% TEI)	16.0 (14.5; 18.9)	17.0 (13.9; 19.3)	0.97
Total fat (% TEI)	37.0 (33.0; 40.5)	36.0 (30.9; 39.3)	0.72
SFA (g)	27.7 (23.2; 35.2)	28.0 (20.7; 37.1)	0.78
MUFA (g)	25.3 (21.6; 32.2)	26.0 (19.5; 33.9)	0.87
PUFA (g)	17.0 (11.7; 21.6)	16.3 (11.3; 22.4)	0.86
Total fiber (g)	20.8 (16.1; 26.1)	23.7 (19.5; 34.3)	0.02
Soluble fiber (g)	5.6 (4.6; 7.5)	6.7 (5.0; 9.6)	0.04
Insoluble fiber (g)	15.3 (11.5; 19)	16.9 (14.1; 24.7)	0.02

Variables were compared using the Mann–Whitney test.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

polysaccharides into SCFAs (Biddle et al., 2013; Eren et al., 2015). Measurements of SCFA in feces and blood have represented an indirect way of assessing the effect of fermentable carbohydrates' intake. The higher the intake of these carbohydrates, the higher the SCFA concentration (So et al., 2018), but, in addition to the substrate availability, SCFA production is affected by intestinal transit time and microbiota composition (Macfarlane and Macfarlane, 2003). Characteristics of the microbiota of our participants should be contributing to improve the status of inflammation and insulin sensitivity, desirable for the prevention of cardiometabolic diseases (Kasubuchi et al., 2015; Zhao et al., 2018; Canfora et al., 2019). However, in the present study, no significant correlation was detected among fiber intake, SCFA concentrations, body adiposity, or metabolic variables. Additionally, the dbRDA showed a low percentage of variance explained by diet. The homogeneity and healthy characteristics

of the sample as a whole may have precluded the detection of significant associations between these variables, as well as differences between participants from each profile.

Interestingly, comparisons of early-life events between the profiles showed that participants in the *Blautia* group had a higher rate of longer duration of exclusive breastfeeding. Considering the importance of gut colonization during this stage of life and given that these microorganisms coexist with the host throughout the life span (Milani et al., 2017), the association found might prove relevant. The first 1,000 days of life are considered a critical developmental window for programming systems and influencing the risk for long-term outcomes (Gluckman et al., 2005; Capra et al., 2021). In addition to the mode of delivery, growing evidence points to the role of early-life nutrition in shaping the offspring's microbiota (Arrieta et al., 2014; Rodríguez, 2014). Bacteria are transferred through human milk and influence immune and metabolic homeostasis. Our results suggest that longer exposure to human milk might be associated with abundance of the *Blautia* genus. Despite limitations of linking distant factors with the gut microbiota of grown-up children and young adults, the hypothesis raised is feasible, considering the beneficial effects attributed to these bacteria. Breast milk composition is complex, containing nutrients, bacteria, and many other compounds. Oligosaccharides—present in human milk but not in most formula—serve as prebiotics, i.e., substrates for fermentation favoring the growth of beneficial bacteria such as the *Bifidobacterium* genus, which uses them to produce SCFA (Bridgman et al., 2017). The breastfed participants may have had their microbiota shaped to favor an abundance of certain commensal genera over others. In this respect, *Blautia* genus shares properties with *Bifidobacterium* in producing SCFAs and improving gut barrier functions. An interesting finding of our group previously suggested that breastfeeding duration could influence the offspring's adherence to a prudent dietary pattern and metabolic parameters in adulthood (Eshriqui et al., 2019; Eshriqui et al., 2020). Another latent factor that could underlie the microbiota variability is the maternal and paternal BMI before conception (Eshriqui et al., 2021; Freitas et al., 2021), but, according to the PERMANOVA adjustments, there was no association between these maternal variables and the offspring's microbiota structure.

A variety of exposures throughout life should have a role in modulating the microbiota of our participants. The current healthy lifestyle of individuals from the *Blautia*-driven profile may be contributing to an adequate BMI and normal biochemical profile. It is known that exercise-induced cardiometabolic benefits are in part gut microbiota-mediated (Chen et al., 2018), but there is also evidence on the associations of early-life events with obesity and related diseases (Ptashne, 2007; Garmendia et al., 2014; Cadenas-Sanchez et al., 2017). Lack of breastfeeding and exposure to formula were shown to increase the risk of obesity in infancy and adulthood (Dietz, 2001; Kelishadi and Farajian, 2014), with clear involvement of gut microbiota in this association. There was a predominance of participants with BMI <25 kg/m^2 in the *Blautia* profile. This finding is congruent with evidence that butyrate (Berni Canani et al., 2016; Takahashi et al., 2016; Wang et al., 2018) and acetate produced by *Blautia* contribute to reduce obesity by regulating G-protein-coupled receptors (Kimura et al., 2013; Liu et al., 2021). In animals, weight gain prevention by SCFA supplementation (Lu et al., 2016) raises the possibility of a novel strategy for controlling human obesity. Our data are also in agreement with previous studies conducted in Spanish children (Benítez-Páez et al., 2020) and in Japanese adults (Ozato et al., 2019). A growing body of evidence indicates the potential on a deeper understanding of the "gut microbiota–host metabolism" interplay for managing prevalent diseases in different populations.

In some respects, the differential abundance analysis showed unexpected results. In the *Blautia*-driven profile, characterized by a higher proportion of lean individuals, *Methanobrevibacter* was less abundant. A previous study addressing this genus reported opposite results; however, the study in question involved an older sample of both sexes and had different purposes and methodological approaches (Schwiertz et al., 2010). Acetate-producing *Lachnoclostridium* was more abundant, in contrast with associations found for diet-induced obesity in animals (Zhao et al., 2017; Sun et al., 2020) and with VAT in female twins (Nogal et al., 2021). Some investigators have speculated that *Lachnoclostridium* could also be a Trimethylamine (TMA)-producing bacteria and, *via* the Trimethylamine N-oxide (TMAO) pathway, may increase the cardiometabolic risk (Schugar et al., 2017). In the *Prevotella*-driven profile, there was a higher abundance of acetate and butyrate-producing bacteria. The *Christensenellaceae* R7 group, *Ruminococcaceae* NK4A214, and *Phascolarctobacterium* have been associated with a favorable cardiometabolic profile. The *Christensenellaceae* R7 group was associated with less VAT and more lean mass in elderly people (Tavella et al., 2021), while the *Christensenellaceae* R7 group, *Ruminococcaceae* NK4A214, and *Phascolarctobacterium* were inversely correlated to glucose metabolism disturbance (Naderpoor et al., 2019; Chen et al., 2021). In our study, *Coprococcus_2* was more abundant in the *Prevotella*-driven than the *Blautia*-driven profile. A high abundance of this genus has been described in women with polycystic ovary syndrome (Zhou et al., 2020) and high lifetime cardiovascular disease risk (Kelly et al., 2016). Therefore, it can be concluded that both bacterial profiles identified in the gut microbiota of healthy Brazilian women may include both beneficial and harmful bacteria. Rather than investigating the role of isolated bacteria for risk prediction, a better strategic approach might be to prevent diseases by focusing on the microbial balance and interactions in the host, submitted to multiple exposures during the life course in different habitats.

This study has limitations related to the sample size due to strict inclusion criteria and composition. The sample comprised highly educated women with a healthy clinical profile, precluding generalizing our results to other samples with different characteristics. The sample homogeneity likely led to the detection of fewer differences between profiles, despite using an accurate technique for assessing body compartments. Our study was not designed to establish a causal relationship between exposure and long-term outcomes. Memory bias was also a concern. In order to minimize this type of error, the study included only participants whose mothers were alive, since the evidence shows that mothers are able to report the early life of

their offspring with acceptable precision almost 30 years later (Chin et al., 2017). Another limitation was the lack of information regarding several risk factors such as antibiotic use and stressful conditions known to influence microbiota composition from birth to adulthood.

In conclusion, findings in a bacterial profile driven by *Blautia* present in healthy Brazilian women reinforce that early-life events play a role in defining gut microbiota profile. While acknowledging the need for investigations with appropriate design to further explore this hypothesis, we highlight the relevance of exclusive breastfeeding for gut colonization in early life to guide the establishment of a protective microbiota against adiposity-related outcomes throughout life.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB49536.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa UNICAMP (CAAE 79775817.4.1001.5404). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Contributed to conception and design: RB, AV, GR, BA-P, and SF. Contributed to acquisition, analysis, or interpretation: RB, AV, GR, FR, IS, MB, BG, and SF. Drafted the article: RB and SF. Critically revised the article: RB, AV, GR, BG, and SF. Gave final approval and agreed to be accountable for all aspects of work, ensuring integrity and accuracy: RB, AV, GR, FR, IS, MB, BA-P, BG, and SF.

FUNDING

This work was supported by the Foundation for Research Support of the State of São Paulo–FAPESP (grant 2018/11433-9, 2018/11401-0).

ACKNOWLEDGMENTS

We thank the Clinical Pathology Laboratory of "Hospital das Clínicas," UNICAMP, and Growth and Body Composition Laboratory, Center for Investigation in Pediatrics, UNICAMP, for the scientific partnership. We also thank Vinícius Santos for assisting with clinical examinations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.838750/full#supplementary-material

Supplementary Figure 1 | Infographic work of the overall methodology.

Supplementary Figure 2 | Redundancy analysis (RDA) highlighting profile drivers.

Supplementary Figure 3 | Boxplot of differential abundances of genera by profile (#1 in blue is driven by *Blautia*; #2 in red is driven by *Prevotella*). Adjusted p-value <0.05.

Supplementary Table 1 | Differential abundances of bacteria by profile.

REFERENCES

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., and Finlay, B. (2014). The Intestinal Microbiome in Early Life: Health and Disease. *Front. Immunol.* 5. doi: 10.3389/fimmu.2014.00427

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., et al. (2011). Enterotypes of the Human Gut Microbiome. *Nature* 473 (7346), 174–180. doi: 10.1038/nature09944

Barker, D. J. (1990). The Fetal and Infant Origins of Adult Disease. *BMJ* 301 (6761), 1111. doi: 10.1136/bmj.301.6761.1111

Bell, K. A., Wagner, C. L., Feldman, H. A., Shypailo, R. J., and Belfort, M. B. (2017). Associations of Infant Feeding With Trajectories of Body Composition and Growth. *Am. J. Clin. Nutr.* 106 (2), 491–498. doi: 10.3945/ajcn.116.151126

Benítez-Páez, A., Gómez Del Pugar, E. M., López-Almela, I., Moya-Pérez, Á., Codoñer-Franch, P., and Sanz, Y. (2020). Depletion of *Blautia* Species in the Microbiota of Obese Children Relates to Intestinal Inflammation and Metabolic Phenotype Worsening. *mSystems* 5 (2), e00857–e00819. doi: 10.1128/mSystems.00857-19

Berni Canani, R., Sangwan, N., Stefka, A. T., Nocerino, R., Paparo, L., Aitoro, R., et al. (2016). *Lactobacillus Rhamnosus* GG-supplemented Formula Expands Butyrate-Producing Bacterial Strains in Food Allergic Infants. *ISME J.* 10 (3), 742–750. doi: 10.1038/ismej.2015.151

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., and Retetangos, C. (2010). Mode of Delivery Affects the Bacterial Community in the Newborn Gut. *Early Hum. Dev.* 86 Suppl 1, 13–15. doi: 10.1016/j.earlhumdev.2010.01.004

Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. *Diversity* 5 (3), 627–640. doi: 10.3390/d5030627

Block, T., and El-Osta, A. (2017). Epigenetic Programming, Early Life Nutrition and the Risk of Metabolic Disease. *Atherosclerosis* 266, 31–40. doi: 10.1016/j.atherosclerosis.2017.09.003

Bouter, K. E., van Raalte, D. H., Groen, A. K., and Nieuwdorp, M. (2017). Role of the Gut Microbiome in the Pathogenesis of Obesity and Obesity-Related Metabolic Dysfunction. *Gastroenterology* 152 (7), 1671–1678. doi: 10.1053/j.gastro.2016.12.048

Bridgman, S. L., Azad, M. B., Field, C. J., Haqq, A. M., Becker, A. B., Mandhane, P. J., et al. (2017). Fecal Short-Chain Fatty Acid Variations by Breastfeeding Status in Infants at 4 Months: Differences in Relative Versus Absolute Concentrations. *Front. Nutr.* 4. doi: 10.3389/fnut.2017.00011

Cadenas-Sanchez, C., Henriksson, P., Henriksson, H., Delisle Nyström, C., Pomeroy, J., Ruiz, J. R., et al. (2017). Parental Body Mass Index and its Association With Body Composition, Physical Fitness and Lifestyle Factors in

Their 4-Year-Old Children: Results From the MINISTOP Trial. *Eur. J. Clin. Nutr.* 71 (10), 1200–1205. doi: 10.1038/ejcn.2017.62

Callahan, A. K. (2016). *Encycl. Med. Immunol.* 13 (7), 1–7.

Canfora, E. E., Meex, R., Venema, K., and Blaak, E. E. (2019). Gut Microbial Metabolites in Obesity, NAFLD and T2DM. Nature Reviews. *Endocrinology* 15 (5), 261–273. doi: 10.1038/s41574-019-0156-z

Capra, M. E., Pederiva, C., Viggiano, C., De Santis, R., Banderali, G., and Biasucci, G. (2021). Nutritional Approach to Prevention and Treatment of Cardiovascular Disease in Childhood. *Nutrients* 13 (7), 2359. doi: 10.3390/nu13072359

Cheng, M., and Ning, K. (2019). Stereotypes About Enterotype: The Old and New Ideas. *Genomics Proteomics Bioinf.* 17 (1), 4–12. doi: 10.1016/j.gpb.2018.02.004

Chen, J., Guo, Y., Gui, Y., and Xu, D. (2018). Physical Exercise, Gut, Gut Microbiota, and Atherosclerotic Cardiovascular Diseases. *Lipids Health Dis.* 17 (1), 17. doi: 10.1186/s12944-017-0653-9

Chen, Z., Radjabzadeh, D., Chen, L., Kurilshikov, A., Kavousi, M., Ahmadizar, F., et al. (2021). Association of Insulin Resistance and Type 2 Diabetes With Gut Microbial Diversity: A Microbiome-Wide Analysis From Population Studies. *JAMA network Open* 4 (7), e2118811. doi: 10.1001/jamanetworkopen.2021.18811

Cheshmeh, S., Nachvak, S. M., Rezvani, N., and Saber, A. (2020). Effects of Breastfeeding and Formula Feeding on the Expression Level of *FTO*, *CPT1A* and *PPAR-α* Genes in Healthy Infants. *Diabetes, Metabolic Syndrome and Obesity:. Targets Ther.* 13, 2227–2237. doi: 10.2147/DMSO.S252122

Chin, H. B., Baird, D. D., McConnaughey, D. R., Weinberg, C. R., Wilcox, A. J., and Jukic, A. M. (2017). Long-Term Recall of Pregnancy-Related Events. *Epidemiology* 28 (4), 575–579. doi: 10.1097/EDE.0000000000000660

Craig, C. L., Marshall, A. L., Sjöström, M., Bauman, A. E., Booth, M. L., Ainsworth, B. E., et al. (2003). International Physical Activity Questionnaire: 12-Country Reliability and Validity. *Med. Sci. sports exercise.* 35 (8), 1381–1395. doi: 10.1249/01.MSS.0000078924.61453.FB

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet Rapidly and Reproducibly Alters the Human Gut Microbiome. *Nature* 505 (7484), 559–563. doi: 10.1038/nature12820

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of Diet in Shaping Gut Microbiota Revealed by a Comparative Study in Children From Europe and Rural Africa. *Proc. Natl. Acad. Sci. United States America* 107 (33), 14691–14696. doi: 10.1073/pnas.1005963107

de Moraes, A. C., Fernandes, G. R., da Silva, I. T., Almeida-Pititto, B., Gomes, E. P., Pereira, A. D., et al. (2017). Enterotype may Drive the Dietary-Associated Cardiometabolic Risk Factors. *Front. Cell. Infection Microbiol.* 7. doi: 10.3389/fcimb.2017.00047

Dietz, W. H. (2001). Breastfeeding may Help Prevent Childhood Overweight. *Jama* 285 (19), 2506–2507. doi: 10.1001/jama.285.19.2506

Dray, S., and Dufour, A. B. (2007). The Ade4 Package: Implementing the Duality Diagram for Ecologists. *J. Stat. Software* 22 (4), 1–20. doi: 10.18637/jss.v022.i04

Eren, A. M., Sogin, M. L., Morrison, H. G., Vineis, J. H., Fisher, J. C., Newton, R. J., et al. (2015). A Single Genus in the Gut Microbiome Reflects Host Preference and Specificity. *ISME J.* 9 (1), 90–100. doi: 10.1038/ismej.2014.97

Eshriqui, I., Folchetti, L. D., Valente, A., de Almeida-Pititto, B., and Ferreira, S. (2019). Breastfeeding Duration Is Associated With Offspring's Adherence to Prudent Dietary Pattern in Adulthood: Results From the Nutritionist's Health Study. *Journal of Developmental Origins of Health and Disease* 11 (2), 136–145. doi: 10.1017/S204017441900031X

Eshriqui, I., Valente, A., Folchetti, L. D., de Almeida-Pititto, B., and Ferreira, S. (2021). Pre-Pregnancy BMI Is Associated with Offspring Body Composition in Adulthood Before Adiposity-Related Disorders: A Retrospective Cohort. *Public Health Nutrition* 24 (6), 1296–1303. doi: 10.1017/S1368980020005285

Eshriqui, I., Folchetti, L. D., Valente, A., de Almeida-Pititto, B., and Ferreira, S. (2020). Pre-Pregnancy BMI Is Associated With Offspring Body Composition in Adulthood Before Adiposity-Related Disorders: A Retrospective Cohort. *Public Health Nutrition* 24 (6), 1296–1303. doi: 10.1017/S1368980020005285

Fan, Y., and Pedersen, O. (2021). Gut Microbiota in Human Metabolic Health and Disease. Nature Reviews. *Microbiology* 19 (1), 55–71. doi: 10.1038/s41579-020-0433-9

Folchetti, L. G., Silva, I. T., Almeida-Pititto, B. D., and Ferreira, S. R. (2016). Nutritionists' Health Study Cohort: A Web-Based Approach of Life Events, Habits and Health Outcomes. *BMJ Open* 6 (8), e012081. doi: 10.1136/bmjopen-2016-012081

Freitas, R., Vasques, A., Ribeiro, F. B., Solar, I., Hanada, A. S., Barbosa, M. G., et al (2021). Maternal and Paternal Obesity Are Associated With Offspring Obestatin Levels in the Nutritionists' Health Study. *Nutrition (Burbank, Los Angeles County, Calif.)* 83, 111067. doi: 10.1016/j.nut.2020.111067

Garmendia, M. L., Corvalan, C., and Uauy, R. (2014). Assessing the Public Health Impact of Developmental Origins of Health and Disease (DohaD) Nutrition Interventions. *Ann. Nutr. Metab.* 64 (3-4), 226–230. doi: 10.1159/000365024

Gluckman, P. D., Cutfield, W., Hofman, P., and Hanson, M. A. (2005). The Fetal, Neonatal, and Infant Environments-the Long-Term Consequences for Disease Risk. *Early Hum. Dev.* 81 (1), 51–59. doi: 10.1016/j.earlhumdev.2004.10.003

Gorvitovskaia, A., Holmes, S. P., and Huse, S. M. (2016). Interpreting *Prevotella* and *Bacteroides* as Biomarkers of Diet and Lifestyle. *Microbiome* 4, 15. doi: 10.1186/s40168-016-0160-7

Haytowitz, D. B., Ahuja, J. K. C., Wu, X., Somanchi, M., Nickle, M., Nguyen, Q. A., et al. (2019). *Usda National Nutrient Database for Standard Reference, Legacy Release* (Beltsville: Nutrient Data Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA). Available at: https://data.nal.usda.gov/dataset/usda-national-nutrient-database-standard-reference-legacy-release.

Hemmingsen, B., Gimenez-Perez, G., Mauricio, D., Roqué I Figuls, M., Metzendorf, M. I., and Richter, B. (2017). Diet, Physical Activity or Both for Prevention or Delay of Type 2 Diabetes Mellitus and its Associated Complications in People at Increased Risk of Developing Type 2 Diabetes Mellitus. *Cochrane Database Systematic Rev.* 12 (12), CD003054. doi: 10.1002/14651858.CD003054.pub4

Kapourchali, F. R., and Cresci, G. (2020). Early-Life Gut Microbiome-the Importance of Maternal and Infant Factors in its Establishment. *Nutr. Clin. Practice: Off. Publ. Am. Soc. Parenteral Enteral Nutr.* 35 (3), 386–405. doi: 10.1002/ncp.10490

Kasubuchi, M., Hasegawa, S., Hiramatsu, T., Ichimura, A., and Kimura, I. (2015). Dietary Gut Microbial Metabolites, Short-Chain Fatty Acids, and Host Metabolic Regulation. *Nutrients* 7 (4), 2839–2849. doi: 10.3390/nu7042839

Kelishadi, R., and Farajian, S. (2014). The Protective Effects of Breastfeeding on Chronic non-Communicable Diseases in Adulthood: A Review of Evidence. *Advanced Biomed. Res.* 3, 3. doi: 10.4103/2277-9175.124629

Kelly, T. N., Bazzano, L. A., Ajami, N. J., He, H., Zhao, J., Petrosino, J. F., et al (2016). Gut Microbiome Associates With Lifetime Cardiovascular Disease Risk Profile Among Bogalusa Heart Study Participants. *Circulation Res.* 119 (8), 956—964. doi: 10.1161/CIRCRESAHA.116.309219

Kimura, I., Ozawa, K., Inoue, D., Imamura, T., Kimura, K., Maeda, T., et al. (2013). The Gut Microbiota Suppresses Insulin-Mediated Fat Accumulation *Via* the Short-Chain Fatty Acid Receptor GPR43. *Nat. Commun.* 4, 1829. doi: 10.1038/ncomms2852

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From Dietary Fiber to Host Physiology: Short-chain Fatty Acids as Key Bacterial Metabolites. *Cell* 165 (6), 1332–1345. doi: 10.1016/j.cell.2016.05.041

Le Doare, K., Holder, B., Bassett, A., and Pannaraj, P. S. (2018). Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.00361

Legendre, P., and Anderson, M. J. (1999). Distance-Based Redundancy Analysis: Testing Multispecies Responses in Multifactorial Ecological Experiments. *Ecol. Monogr.* 69 (1), 1–24. doi: 10.1890/0012-9615(1999)069[0001:DBRATM]2.0.CO;2

Levy, M., Kolodziejczyk, A. A., Thaiss, C. A., and Elinav, E. (2017). Dysbiosis and the Immune System. Nature Reviews. *Immunology*. 17 (4), 219–232. doi: 10.1038/nri.2017.7

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., et al. (2021). *Blautia - A* New Functional Genus With Potential Probiotic Properties? *Gut Microbes* 13 (1), 1–21. doi: 10.1080/19490976.2021.1875796

Love, M. I., Huber, W., and Anders, S. (2014). Moderated Estimation of Fold Change and Dispersion for RNA-seq Data With Deseq2. *Genome Biol.* 15 (12), 550. doi: 10.1186/s13059-014-0550-8

Lu, Y., Fan, C., Li, P., Lu, Y., Chang, X., and Qi, K. (2016). Short Chain Fatty Acids Prevent High-Fat-Diet-Induced Obesity in Mice by Regulating G Protein-Coupled Receptors and Gut Microbiota. *Sci. Rep.* 6, 37589. doi: 10.1038/srep37589

Macfarlane, S., and Macfarlane, G. T. (2003). Regulation of Short-Chain Fatty Acid Production. *Proc. Nutr. Soc.* 62 (1), 67–72. doi: 10.1079/PNS2002207

Mailing, L. J., Allen, J. M., Buford, T. W., Fields, C. J., and Woods, J. A. (2019). Exercise and the Gut Microbiome: A Review of the Evidence, Potential

Mechanisms, and Implications for Human Health. *Exercise Sport Sci. Rev.* 47 (2), 75–85. doi: 10.1249/JES.0000000000000183

Martin, R., Makino, H., Cetinyurek Yavuz, A., Ben-Amor, K., Roelofs, M., Ishikawa, E., et al. (2016). Early-Life Events, Including Mode of Delivery and Type of Feeding, Siblings and Gender, Shape the Developing Gut Microbiota. *PloS One* 11 (6), e0158498. doi: 10.1371/journal.pone.0158498

Matsudo, S., Araújo, T., Marsudo, V., Andrade, D., Andrade, E., and Braggion, G. (2001). Questionário Internacional De Atividade Fisica (IPAQ): Estudo De Validade E Reprodutibilidade No Brasil. *Rev. Bras. Atividade Física e Saúde* 6 (2), 5–18. doi: 10.12820/rbafs.v.6n2p5-18

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., and Turner, R. C. (1985). Homeostasis Model Assessment: Insulin Resistance and Beta-Cell Function From Fasting Plasma Glucose and Insulin Concentrations in Man. *Diabetologia* 28 (7), 412–419. doi: 10.1007/BF00280883

Meijnikman, A. S., Gerdes, V. E., Nieuwdorp, M., and Herrema, H. (2018). Evaluating Causality of Gut Microbiota in Obesity and Diabetes in Humans. *Endocrine Rev.* 39 (2), 133–153. doi: 10.1210/er.2017-00192

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., et al. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol. Mol. Biol. Reviews: MMBR.* 81 (4), e00036-17. doi: 10.1128/MMBR.00036-17

Müller, M., Hernández, M., Goossens, G. H., Reijnders, D., Holst, J. J., Jocken, J., et al. (2019). Circulating But Not Faecal Short-Chain Fatty Acids are Related to Insulin Sensitivity, Lipolysis and GLP-1 Concentrations in Humans. *Sci. Rep.* 9 (1), 12515. doi: 10.1038/s41598-019-48775-0

Naderpoor, N., Mousa, A., Gomez-Arango, L. F., Barrett, H. L., Dekker Nitert, M., and de Courten, B. (2019). Faecal Microbiota is Related to Insulin Sensitivity and Secretion in Overweight or Obese Adults. *J. Clin. Med.* 8 (4), 452. doi: 10.3390/jcm8040452

Nogal, A., Louca, P., Zhang, X., Wells, P. M., Steves, C. J., Spector, T. D., et al. (2021). Circulating Levels of the Short-Chain Fatty Acid Acetate Mediate the Effect of the Gut Microbiome on Visceral Fat. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.711359

Nyberg, S. T., Singh-Manoux, A., Pentti, J., Madsen, I., Sabia, S., Alfredsson, L., et al. (2020). Association of Healthy Lifestyle With Years Lived Without Major Chronic Diseases. *Jama* 180 (5), 760–768. doi: 10.1001/jamainternmed.2020.0618

Ozato, N., Saito, S., Yamaguchi, T., Katashima, M., Tokuda, I., Sawada, K., et al. (2019). *Blautia* Genus Associated With Visceral Fat Accumulation in Adults 20-76 Years of Age. *NPJ Biofilms Microbiomes.* 5 (1), 28. doi: 10.1038/s41522-019-0101-x

Penington, J. S., Penno, M., Ngui, K. M., Ajami, N. J., Roth-Schulze, A. J., Wilcox, S. A., et al. (2018). Influence of Fecal Collection Conditions and 16S rRNA Gene Sequencing at Two Centers on Human Gut Microbiota Analysis. *Sci. Rep.* 8 (1), 4386. doi: 10.1038/s41598-018-22491-7

Pires, D. E. V., Oliveira, F. S., Correa, F. B., Morais, D. K., and Fernandes, G. R. (2018). Tag. ME: Taxonomic Assignment of Genetic Markers for Ecology. *BioRxiv*, 263293. doi: 10.1101/263293

Ptashne, M. (2007). On the Use of the Word 'Epigenetic'. *Curr. biology: CB* 17 (7), R233–R236. doi: 10.1016/j.cub.2007.02.030

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing. *Nature* 464 (7285), 59–65. doi: 10.1038/nature08821

Ravelli, G. P., Stein, Z. A., and Susser, M. W. (1976). Obesity in Young Men After Famine Exposure *In Utero* and Early Infancy. *New Engl. J. Med.* 295 (7), 349–353. doi: 10.1056/NEJM197608122950701

Rodríguez, J. M. (2014). The Origin of Human Milk Bacteria: Is There a Bacterial Entero-Mammary Pathway During Late Pregnancy and Lactation? *Adv. Nutr.* 5 (6), 779–784. doi: 10.3945/an.114.007229. (Bethesda, Md.).

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., et al. (2015). The Composition of the Gut Microbiota Throughout Life, With an Emphasis on Early Life. *Microbial Ecol. Health Dis.* 26, 26050. doi: 10.3402/mehd.v26.26050

Rosenbaum, M., Knight, R., and Leibel, R. L. (2015). The Gut Microbiota in Human Energy Homeostasis and Obesity. *Trends Endocrinol. Metab.* 26 (9), 493–501. doi: 10.1016/j.tem.2015.07.002

Schwiertz, A., Taras, D., Schäfer, K., Beijer, S., Bos, N. A., Donus, C., et al. (2010). Microbiota and SCFA in Lean and Overweight Healthy Subjects. *Obesity (Silver Spring, Md.)* 18 (1), 190–195. doi: 10.1038/oby.2009.167

Selem, S. S., Carvalho, A. M., Verly-Junior, E., Carlos, J. V., Teixeira, J. A., Marchioni, D. M., et al. (2014). Validity and Reproducibility of a Food Frequency Questionnaire for Adults of São Paulo, Brazil. *Braz. J. Epidemiol.* 17 (4), 852–859. doi: 10.1590/1809-4503201400040005

Sender, R., Fuchs, S., and Milo, R. (2016). Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PloS Biol.* 14 (8), e1002533. doi: 10.1371/journal.pbio.1002533

Shen, J., Obin, M. S., and Zhao, L. (2013). The Gut Microbiota, Obesity and Insulin Resistance. *Mol. Aspects Med.* 34 (1), 39–58. doi: 10.1016/j.mam.2012.11.001

Schugar, R. C., Shih, D. M., Warrier, , Helsley, M. R.N., Burrows, A., Ferguson, D., et al (2017). The TMAO-Producing Enzyme Flavin-Containing Monooxygenase 3 Regulates Obesity and the Beiging of White Adipose Tissue. *Cell Reports* 19 (12), 2451—61. doi: 10.1016/j.celrep.2017.05.077

Sommer, F., Anderson, J. M., Bharti, R., Raes, J., and Rosenstiel, P. (2017). The Resilience of the Intestinal Microbiota Influences Health and Disease. Nature Reviews. *Microbiology* 15 (10), 630–638. doi: 10.1038/nrmicro.2017.58

So, D., Whelan, K., Rossi, M., Morrison, M., Holtmann, G., Kelly, J. T., et al. (2018). Dietary Fiber Intervention on Gut Microbiota Composition in Healthy Adults: A Systematic Review and Meta-Analysis. *Am. J. Clin. Nutr.* 107 (6), 965–983. doi: 10.1093/ajcn/nqy041

Sun, X., Zhao, H., Liu, Z., Sun, X., Zhang, D., Wang, S., et al. (2020). Modulation of Gut Microbiota by Fucoxanthin During Alleviation of Obesity in High-Fat Diet-Fed Mice. *J. Agric. Food Chem.* 68 (18), 5118–5128. doi: 10.1021/acs.jafc.0c01467

Takahashi, K., Nishida, A., Fujimoto, T., Fujii, M., Shioya, M., Imaeda, H., et al. (2016). Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. *Digestion* 93 (1), 59–65. doi: 10.1159/000441768

Tavella, T., Rampelli, S., Guidarelli, G., Bazzocchi, A., Gasperini, C., Pujos-Guillot, E., et al. (2021). Elevated Gut Microbiome Abundance of Christensenellaceae, Porphyromonadaceae and Rikenellaceae Is Associated With Reduced Visceral Adipose Tissue and Healthier Metabolic Profile in Italian Elderly. *Gut Microbes* 13 (1), 1–19. doi: 10.1080/19490976.2021.1880221

Vazquez-Moreno, M., Perez-Herrera, A., Locia-Morales, D., Dizzel, S., Meyre, D., Stearns, J. C., et al. (2021). Association of Gut Microbiome With Fasting Triglycerides, Fasting Insulin and Obesity Status in Mexican Children. *Pediatr. Obes.* 16 (5), e12748. doi: 10.1111/ijpo.12748

Vital, M., Howe, A. C., and Tiedje, J. M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)Genomic Data. *mBio* 5 (2), e00889. doi: 10.1128/mBio.00889-14

Wang, R., Fan, C., Fan, X., Zhao, Y., Wang, Y., Li, P., et al. (2019). A Fast and Accurate Way to Determine Short Chain Fatty Acids in Human Serum by GC–MS and Their Distribution in Children With Digestive Diseases. *Chromatographia* 83 (2), 273–286. doi: 10.1007/s10337-019-03831-9

Wang, Z., Zolnik, C. P., Qiu, Y., Usyk, M., Wang, T., Strickler, H. D., et al. (2018). Comparison of Fecal Collection Methods for Microbiome and Metabolomics Studies. *Front. Cell. Infection Microbiol.* 8. doi: 10.3389/fcimb.2018.00301

World Health Organization (2015). Obesity: Preventing and Managing the Global Epidemic. *Report of a WHO Consulation (WHO Technical Report Series 894)* 2000. Available at: https://www.who.int/nutrition/publications/obesity/WHO_TRS_894/en/.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., et al. (2011). Linking Long-Term Dietary Patterns With Gut Microbial Enterotypes. *Science* 334 (6052), 105–108. doi: 10.1126/science.1208344

Wu, J., Wang, K., Wang, X., Pang, Y., and Jiang, C. (2021). The Role of the Gut Microbiome and its Metabolites in Metabolic Diseases. *Protein Cell.* 12 (5), 360–373. doi: 10.1007/s13238-020-00814-7

Yang, Q., Lin, S. L., Kwok, M. K., Leung, G. M., and Schooling, C. M. (2018). The Roles of 27 Genera of Human Gut Microbiota in Ischemic Heart Disease, Type 2 Diabetes Mellitus, and Their Risk Factors: A Mendelian Randomization Study. *Am. J. Epidemiol.* 187 (9), 1916–1922. doi: 10.1093/aje/kwy096

Zhao, Y., Liu, J., Hao, W., Zhu, H., Liang, N., He, Z., et al. (2017). Structure-Specific Effects of Short-Chain Fatty Acids on Plasma Cholesterol Concentration in Male Syrian Hamsters. *J. Agric. Food Chem.* 65 (50), 10984–10992. doi: 10.1021/acs.jafc.7b04666

Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y. Y., Wang, X., et al. (2018). Gut Bacteria Selectively Promoted by Dietary Fibers Alleviate Type 2 Diabetes. *Science* 359 (6380), 1151–1156. doi: 10.1126/science.aao5774

Zhou, L., Ni, Z., Cheng, W., Yu, J., Sun, S., Zhai, D., et al. (2020). Characteristic Gut Microbiota and Predicted Metabolic Functions in Women With PCOS. *Endocrine Connections* 9 (1), 63–73. doi: 10.1530/EC-19-0522

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 13 May 2022
doi: 10.3389/fcimb.2022.883798

Accurate 16S Absolute Quantification Sequencing Revealed Vaginal Microecological Composition and Dynamics During Mixed Vaginitis Treatment With Fufang FuRong Effervescent Suppository

Meng Li[1,2†], Zhen Zeng[2†], Huijun Feng[2], Yang Cao[2], Qiongqiong Zhang[1,2], Tao Lv[2], Xingsheng Yang[3], Dianrong Song[4], Ping Li[5], Lina Hu[6], Shangrong Fan[7], Ruifang An[8], Bei Zhang[9], Lei Zhang[2] and Qinping Liao[2*]*

[1] School of Clinical Medicine, Tsinghua University, Beijing, China, [2] Department of Obstetrics and Gynecology, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China, [3] Department of Obstetrics and Gynecology, Qilu Hospital of Shandong University, Jinan, China, [4] Gynecological Department, The First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China, [5] Department of Obstetrics and Gynecology, The Affiliated Obstetrics and Gynecology Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, Nanjing, China, [6] Department of Gynecology, The Second Hospital Affiliated to Chongqing Medical University, Chongqing, China, [7] Department of Obstetrics and Gynecology, Peking University Shenzhen Hospital, Shenzhen, China, [8] Department of Obstetrics and Gynecology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China, [9] Department of Obstetrics and Gynaecology, Xuzhou Central Hospital, Affiliated Xuzhou Clinical College of Xuzhou Medical University, Xuzhou, China

Background: The diagnosis and treatment of mixed vaginitis are more complicated than single pathogenic infections, and there may be adverse reactions and several contraindications to conventional antibiotic therapy. Therefore, this study aimed to evaluate the preliminary effects of Fufang Furong Effervescent Suppository for the management of aerobic vaginitis (AV) mixed with bacterial vaginosis (BV) using Accurate 16S absolute quantification sequencing (Accu16S).

Methods: In the present randomized, blind, multi-center clinical trial, women (20 to 55 years) who had received a diagnosis of AV+BV were randomly assigned into clindamycin positive control (n = 41) and Fufang Furong Effervescent Suppository (n = 39) groups. The follow-up occurred in three time periods (V1: -2~0 days; V2: 15-17 days; V3: 40 ± 3 days). At each visit, two vaginal swabs, one for clinical evaluation and one for laboratory examination, were taken from each patient. The Nugent score, Donders' score, drug-related complications, recurrence rates, and microecological changes of vaginal swabs were assessed in the time three periods.

Results: At baseline, the two groups were similar in frequency of presentation with vaginal burning, odor, abnormal discharge, and itching. No meaningful differences in Nugent and Donders' scores were detected between the two groups at stage V2 (Nugent: $p = 0.67$; Donders': $p = 0.85$) and V3 (Nugent: $p = 0.97$; Donders: $p = 0.55$). The Furong group

OPEN ACCESS

Edited by:
Veeranoot Nissapatorn, Walailak University, Thailand

Reviewed by:
Supriya Dinesh Mehta, University of Illinois at Chicago, United States
Barbara Giordani, University of Bologna, Italy

***Correspondence:**
Qinping Liao
13701124527@163.com
Lei Zhang
18601130459@163.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *25 February 2022*
Accepted: *19 April 2022*
Published: *13 May 2022*

Citation:
Li M, Zeng Z, Feng H, Cao Y, Zhang Q, Lv T, Yang X, Song D, Li P, Hu L, Fan S, An R, Zhang B, Zhang L and Liao Q (2022) Accurate 16S Absolute Quantification Sequencing Revealed Vaginal Microecological Composition and Dynamics During Mixed Vaginitis Treatment With Fufang FuRong Effervescent Suppository. Front. Cell. Infect. Microbiol. 12:883798. doi: 10.3389/fcimb.2022.883798

presented fewer complications compared to the Clindamycin group. However, this difference was not statistically significant (p = 0.15). Additionally, Accu16S indicated that the total abundance of bacteria in both groups sharply decreased in stage V2, but slightly increased in V3. In stage V3, the absolute abundance of *Lactobacillus* in the Furong group was considerably higher compared to untreated samples ($p < 0.05$). On the other hand, no momentous increase was detected in the Clindamycin group ($p > 0.05$).

Conclusion: Fufang Furong Effervescent Suppository can be as effective as clindamycin cream in the management of AV+BV while may restore the vagina microecosystem better.

Keywords: accurate 16S absolute quantification sequencing, mixed vaginitis, microbiome, antibiotic, traditional Chinese medicine

INTRODUCTION

Vaginitis is one of the most common causes for women of different ages to visit a health care provider, representing an important concern for public health (Mills, 2017). The most common types of vaginitis affecting women during their reproductive age can be divided based on the pathological agent, including bacterial vaginitis (BV), trichomonal vaginitis (TV), vulvovaginal candidiasis (VVC), and aerobic vaginitis (AV) (Paavonen and Brunham, 2018). Mixed vaginitis refers to the simultaneous presence of at least two vaginal pathogens, both contributing to an abnormal vaginal milieu and the corresponding clinical symptoms and signs (Sobel et al., 2013). Mixed vaginitis is more complicated than single pathogen infections and its prevalence has been poorly documented across the world (Qi et al., 2021). Data from several studies have suggested that BV+TV is the most common mixed vaginitis (37.8%), followed by BV+VVC (14.9%) and BV+VVC+TV (4.1%) (Ozyurt et al., 2001; Rivers et al., 2011). Since the term 'aerobic vaginitis' was first introduced by Donders in 2002, increasing studies have shown a high incidence of AV-related mixed vaginitis. Currently, the rate of AV-related mixed infections is the most noteworthy in Chinese women, with 36.9% of AV+BV, 38.1% of AV+VVC, and 25% of AV +trichomoniasis (Fan et al., 2013).

Although the knowledge about mixed vaginitis has increased, its standard treatment has not been standardized yet. Currently, oral metronidazole, vaginal clindamycin cream, or metronidazole gel are the main BV therapies (Donders et al., 2014). The CDC (Centers for Disease Control and Prevention) Sexually Transmitted Infection Treatment Guidelines of 2021 also suggests these treatments (Workowski et al., 2021). For AV, topical vaginal clindamycin or kanamycin or oral other antibacterial drugs that are active against aerobic bacteria such as cefuroxime or moxifloxacin are recommended (Mendling et al., 2016; Wang et al., 2016; Feng et al., 2018). The European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline of 2018 also recommends topical clindamycin as the primary treatment for AV (Sherrard et al., 2018). Therefore, clindamycin is also effective in women with mixed AV and BV and was selected as a positive control in the present study.

Women diagnosed with AV+BV would empirically receive antibiotic treatment, which can unbalance the vaginal ecosystem. Additionally, local vaginal burning or irritation and clindamycin resistance (due to the extensive use of antibiotics) have become concerning problems. (Javed et al., 2019). Hence, Traditional Chinese Medicine (TCM) has been considered as an alternative treatment for AV+BV. Clinical research has revealed that the treatment of vaginitis using vaginal lavage with TCM demonstrated satisfactory efficacy (Liu et al., 2014; Li et al., 2016). Treatments for vaginitis based on TCM, such as Fufang Furong Effervescent Suppository, can not only disinfect and relieve itching but also relatively reduce the destruction of the vaginal microbiome. Fufang FuRong Effervescent Suppository (Shaanxi Momentum Qixuehe Pharmaceutical Co., Ltd., China) is comprised of 6 herbs (1000g suppositories): *Sophorae Flavescentis Radix* (320g), *Cnidii Fructus* (230g), *Phellodendri Chinensis Cortex* (110g), *Hibisci Mutabilis Folium* (130g), *Artemisiae Argyi Folium* (120g), *Alumen* (90g).

The advence in high-throughput sequencing has greatly accelerated the study of the vaginal microbiome and gynecological diseases, enabling researchers to map the composition and function of the microbiome in high-resolution and culture-independent models (Almeida and Shao, 2018). However, most of these techniques are relative quantification 16S-seq (RQS) and do not consider absolute bacterial abundances. Inappropriate interpretations based on relative quantifications can mislead some researches (Stämmler et al., 2016).

Thus, accurate absolute quantification of bacterial populations is necessary. Therefore, in the present study, we adopted Accurate 16S absolute quantification sequencing (Accu16S) (Wei et al., 2021) to determine the absolute and relative abundances of the vaginal microbiome and evaluate the preliminary efficacy of Fufang Furong Effervescent Suppository in the treatment of mixed vaginitis.

MATERIALS AND METHODS

Study Design

From August 2019 to June 2021, we conducted a multicenter, randomized, blind, positive parallel controlled trial to assess the efficacy of Fufang Furong Effervescent Suppository for the

treatment of AV combined with BV. The trial received ethical approval from the ethics committee of the Beijing Tsinghua Changgung Hospital (19190-0-02). In addition, the protocol was registered in the Chinese Clinical Trial Registry (ChiCTR1900027616). Patients were recruited from gynecological centers of 27 public hospitals in China. In this study, we only analyzed clinical data and vaginal swabs from patients at seven hospitals where specimen preservation conditions were available (distributed in the east, south, west, north and central parts of China), including Peking University Shenzhen Hospital, The First Affiliated Hospital of Xi'an Jiaotong University, Second Affiliated Hospital of Chongqing Medical University, Shandong University Qilu Hospital, Xuzhou Central Hospital, The First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine and Nanjing Maternal and Child Health Care Hospital. The CONSORT Checklist and CONSORT Flow Diagram in supplementary documents. All eligible patients provided written informed consent or legal guardian consent before participating in this study. The first, second, and last authors vouch for the integrity and accuracy of the data and the fidelity of the trial to the protocol.

Participants

Eligible participants represented women between 20 to 55 years who regularly menstruated and had a sexual history. The inclusion and exclusion criteria are presented in **Table 1**. Currently, the gold standard for BV diagnosis is the Nugent score (Nugent et al., 1991). In this case, a scale of 0-10 is used to assess the disease severity, with 7–10 indicating a positive BV diagnosis. The diagnosis of AV is based on the Donders' score. The highest AV score is 10, with scores under 3 corresponding to 'no signs of AV', 3-4 to 'light AV', 5-6 to 'moderate AV' and > 6 to 'severe AV' (Donders et al., 2017). Only AV patients with light to moderate symptoms were included in this study. Additionally, all participants underwent vaginal microbiota functional tests, such as vaginal pH, H_2O_2 concentration, sialidase activity, and leucocyte esterase (LE) activity (Mårdh et al., 2003), as described previously (An et al., 2016). All tests mentioned above were performed three days after menstruation. Furthermore, all qualified patients were also assessed for TCM-related clinical symptoms, including increased vaginal discharge, leucorrhea yellowish or odors. Meanwhile, some basic vital signs and laboratory examinations, such as blood routine, urine routine, liver, and kidney function, were analyzed considering the normal limits.

Procedures

Patients were randomly assigned into two groups: positive control group (clindamycin phosphate cream - Clindamycin group) and experimental group (Fufang Furong Effervescent Suppository - Furong group). Allocation was based on a random permutation table. The screening period was set as the baseline (-2-0 days - stage V1). Before drug administration, all of the participants were trained to insert vaginal tablets. Then, patients in both groups were put on medication for 12 consecutive days (one tablet once a night), during which they were told to abstain from vaginal intercourse. Next, they were asked to return to the clinic twice, at 3-5 days after the treatment period (15-17 days - stage V2), and 28 ± 3 days after the treatment period (40 ± 3 days - stage V3). At each visit, participants underwent routine laboratory examinations and two vaginal swabs were retrieved, one for assessment of the Nugent and Donders' scores, and the other was retained at -80°C for further Accu16S. Each participant was assessed for compliance and reported for co-medication and other special circumstances during the period. Moreover, all women were asked to record any side effects of the treatment in a checklist and were asked to bring it in the follow-up visits.

Accu16STM Assay

Accu16S was performed by Genesky Biotechnologies Inc., Shanghai, 201315 (China). Briefly, total genomic DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN BIOTECH, Beijing, China) according to the manufacturer's instructions. The integrity of genomic DNA was detected through agarose gel electrophoresis, and the concentration and purity of genomic DNA were detected through the Nanodrop2000 (Thermo Fisher Scientific, Massachusetts, USA) and Qubit3.0 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Multiple spike-ins with identical conserved regions to natural 16S rRNA genes and

TABLE 1 | Inclusion and exclusion criteria.

Inclusion	Exclusion
Premenopausal women aged 20-55;	Combined with other vaginitis such as TV, VVC;
Sexual history;	Combined with other serious gynecological diseases, such as gynecological malignant tumor and pelvic inflammatory disease;
Conforms to the diagnostic criteria of BV+AV;	Combined With serious primary diseases of heart, brain, liver, kidney, blood system and endocrine system (ALT, AST≥normal upper limit 2 times;Scr≥normal upper limit);
Donders scores ≥3 and ≤6;	Received any oral or topical medication for the disease within one week;
Nugent score ≥7;	Hypersensitivity to the investigated product or clindamycin;
Volunteer to participate and signed an informed consent form;	Pregnant and Lactation women or recently planned pregnancy;
	Combined with neuropsychiatric disorders and suspected or confirmed history of alcohol and drug abuse;
	Participate in other clinical trials within 1 month;

variable regions replaced by random sequence with ~40% GC contents were artificially synthesized. Then, appropriate proportion of spike-ins mixture with known gradient copy numbers were added to the sample DNA. The V3-V4 hypervariable regions of the 16S rRNA gene and spike-ins were amplified with the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') and then sequenced using Illumina NovaSeq 6000 sequencer (Illumina, California, USA).

Illumina Read Data Processing and Analysis

The raw read sequences were processed in QIIME2 (Bolyen et al., 2019, 2). The adaptor and primer sequences were trimmed using the cutadapt plugin (Version 0.5.0, Babraham Bioinformatics, UK). DADA2 plugin was used for quality control and to identify amplicon sequence variants (ASVs) (Callahan et al., 2016). Taxonomic assignments of ASV representative sequences were performed at a confidence threshold of 80% by Mothur (Version 1.41.1, https://www.mothur.org/) with the command classify.seqs based on the RDP (Version 11.5) database. Then the spike-in sequences were identified and reads were counted. Standard curve for each sample was generated based the read-counts versus spike-in copy number, and the absolute copy number of each ASV in each sample was calculated by using the read-counts of the corresponding ASV. Since the spike-in sequence is not a component of the sample flora, the spike-in sequence needs to be removed in the subsequent analysis (Jiang et al., 2019).

Statistical Analyses

Data were gathered at the baseline, 15-17 days, and 40 ± 3 days after treatments. The IBM SPSS 23.0 (IBM Corporation, USA) and R (version 3.5.1) were used for statistical analyses. Descriptive statistics included *N* (%), means, and standard deviations (SD). To compare categorical and quantitative variables between the two groups, χ^2, and independent *t*-tests were used, respectively. The heatmap, α-diversity, coordinate analysis plots (PCoA), and boxplot figures were constructed using R. A $p < 0.05$ was considered significant.

RESULTS

Clinical Data

A total of 80 patients were enrolled from the seven public hospitals mentioned above, and their specific distribution is shown in **Table S1**. No significant differences were detected for the baseline features between the two groups. Moreover, both groups presented a similar rate of vaginal clinical symptoms before therapy (**Table 2**). Clinical efficacy assessment was based on the Nugent and Donders' scores during follow-up. The prognosis of AV and BV in both groups was notably improved (**Figure 1**). The comparable and sharp decline of the Nugent score is shown in **Figure 1A**. Besides, most patients presented an abrupt decline in their Donders' scores (**Figure 1B**). No significant differences were detected for the Nugent and Donders' scores between the two groups at each follow-up period (**Tables 3**, **4**). In stage V2, 20 patients (51.3%) in the Clindamycin group were defined as BV-negative (Nugent 0-3) and 12 (30.8%) as BV intermediate (Nugent 4-6). In the Fufang Furong Effervescent Suppository group, 19 (48.7%) patients were BV-negative, and 13 (33.4%) were BV intermediate ($p = 0.67$). In stage V3, 16 (48.5%) patients in the Clindamycin group were BV-negative and 4 (12.1%) were BV intermediate. In the Furong group, 19 (54.3%) patients were BV-negative and 3 (8.6%) were BV intermediate ($p = 0.97$) (**Table 3**). Only 9 patients in both groups were diagnosed with mild to moderate AV at stage V2 ($p = 0.85$; **Table 4**). At stage V3, 9 mild and 2 moderate AV patients were identified in the Furong group. Meanwhile, only 10 patients in the Clindamycin group had mild AV. However, this difference between the treatment groups was not statistically significant.

The relapse rate was also diagnosed based on the Nugent and Donders' scores (**Table 5**). The Clindamycin group had fewer relapse patients than the Furong group, but it was not significant (AV: $p = 0.22$; BV: $p = 0.20$). On the other hand, more patients in the Clindamycin group did not respond to the treatment compared to the Furong group but without any substantial difference (AV: $p = 0.18$; BV: $p = 0.26$). Then, we analyzed treatment side effects in these two groups. The side effects reported mainly involved VVC, urticaria, vaginal burning, and

TABLE 2 | Sample characteristics and vaginal clinical symptoms at the baseline.

Variable	Clindamycin Group (n=41)	FuRong Group (n=39)	P value
Characters (mean ± SD)			
Age (years)	37.07 ± 8.74	35.67 ± 8.36	0.46
Body mass index (kg/m^2)	21.05 ± 2.59	21.77 ± 3.46	0.30
Pregnancy	2.17 ± 1.52	2.38 ± 1.53	0.53
Parity	1.15 ± 0.85	1.21 ± 0.73	0.74
Clinical symptoms n, (%)			0.65
Itching	11 (26.8)	13 (33.3)	
Bad odor	20 (51.3)	24 (61.5)	
Burning	2 (5.13)	3 (7.6)	
Abnormal vaginal discharge	30 (73.2)	30 (76.9)	
Diagnosed First Time n, (%)			0.33
Yes	38 (92.7)	38 (97.4)	
No	3 (7.3)	1 (2.6)	

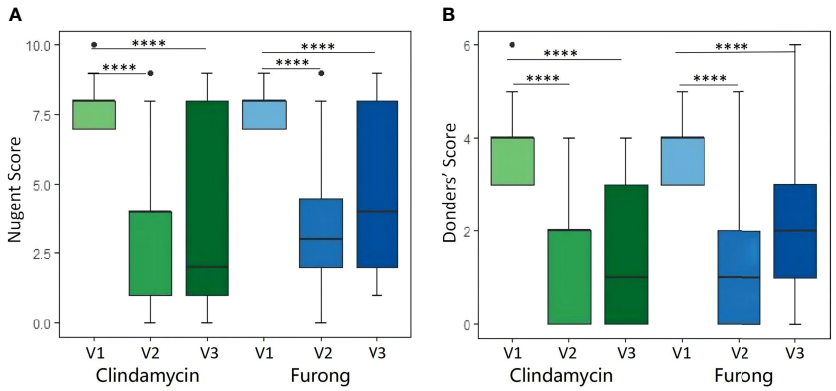


FIGURE 1 | Nugent score **(A)** and Donders score **(B)** before and after Clindamycin and Furong treatments. (****P value < 0.0001).

liver function damage. In the Clindamycin group, one patient developed severe urticaria 1 h after medication, which was later improved by antiallergic therapy. Adverse reactions occurred in 9 (22.0%) patients in the Clindamycin group and 7 (17.9%) in the Furong group (p = 0.65). Among them, 6 (14.6%) cases in the Clindamycin group and 2 (5.1%) in the Furong group had drug-related complications, but this difference was not significant (p = 0.15). Two patients who developed severe VVC after clindamycin treatment were excluded from the follow-up due to antifungal therapy.

Absolute Quantification of Vaginal Swabs

A total of 170 qualified samples were used for Accu16S (**Figure 2**). After the removal of ambiguous, short, low-quality reads and singleton OTUs, a total of 34108020 reads remained for community analysis of 170 samples. Spike-in reads accounted for 27.67 ± 9.0% (18.67-36.67%) of total reads in a given library, similar to the expected proportion of 30%. The α-diversity indicators Shannon and Simpson (Both of them are commonly used to reflect α-diversity. The larger the Shannon value, the higher the community diversity. The greater the Simpson index value, the lower the community diversity.) for the vagina microbiota in both groups are shown in **Figures 3A, B**. The diversity of bacteria in these samples abruptly decreased compared to initial untreated samples (p = 0.00). The PCoA based on weighted Unifrac matrix clustering data of microbial β-diversity among the 170 samples is shown in **Figure 3C** (relative abundance) and 3D (absolute abundance). Vaginal samples from stage V1 in both groups were gathered and separated from those in stages V2 and V3. Moreover, the vaginal microbiota composition was similar in stages V2 and V3, similar to the clinical data. The absolute and relative quantification did not influence the PCoA.

After treatment, the total abundance of bacteria in both groups sharply decreased in stage V2, but slightly increased in V3, especially in the Furong group (**Figure 4A**). The maximal loss of the total bacteria amount was detected in stage V2 of

TABLE 3 | Nugent score during the three follow-up periods.

Nugent Score	Clindamycin Group (n=41)	FuRong Group (n=39)	P value
V1 n, (%)			0.19
7	15 (36.6)	15 (38.5)	
8	20 (48.8)	23 (59.0)	
9	3 (7.3)	1 (2.6)	
10	3 (7.3)	0	
V2 n, (%)			0.67
0-3	19 (48.7)	20 (51.3)	
4-6	13 (33.4)	12 (30.8)	
7	1 (2.6)	4 (10.3)	
8	5 (12.8)	1 (2.6)	
9	1 (2.6)	2 (5.1)	
V3 n, (%)			0.97
0-3	19 (54.3)	16 (48.5)	
4-6	3 (8.6)	4 (12.1)	
7	3 (8.6)	1 (3.0)	
8	9 (25.7)	10 (30.3)	
9	1 (2.9)	2 (6.1)	

TABLE 4 | Donders' score during the three follow-up periods.

Donders' Score	Clindamycin Group (n=41)	FuRong Group (n=39)	P value
V1 n, (%)			0.63
3-4	33 (80.5)	33 (84.6)	
5-6	8 (19.5)	6 (15.4)	
V2 n, (%)			0.85
<3	30 (76.9)	30 (76.9)	
3-4	9 (23.1)	8 (20.5)	
5-6	0	1 (2.6)	
V3 n, (%)			0.55
<3	25 (71.4)	22 (66.7)	
3-4	10 (28.6)	9 (27.3)	
5-6	0	2 (6.0)	

TABLE 5 | Recurrence and persistence 28 ± 3 days after medications.

	Clindamycin Group (n=41)	FuRong Group (n=39)	P value
Relapse			
AV	6	10	0.22
BV	5	9	0.20
Persist			
AV	4	1	0.18
BV	5	2	0.26

clindamycin treatment. *Gardnerella*, *Prevotella*, *Atopobium*, *Sneathia*, and *Megasphaera* were the dominant bacteria before the treatment (**Figure 4A**). The absolute quantification of these five bacteria without treatment did not differ between the two groups and harshly decreased after treatment (**Figure 5**). Despite the strong sterilization promoted by clindamycin, the absolute abundance of *Lactobacillus* in the vagina was still slightly increased. Interestingly, in the Furong group, the absolute

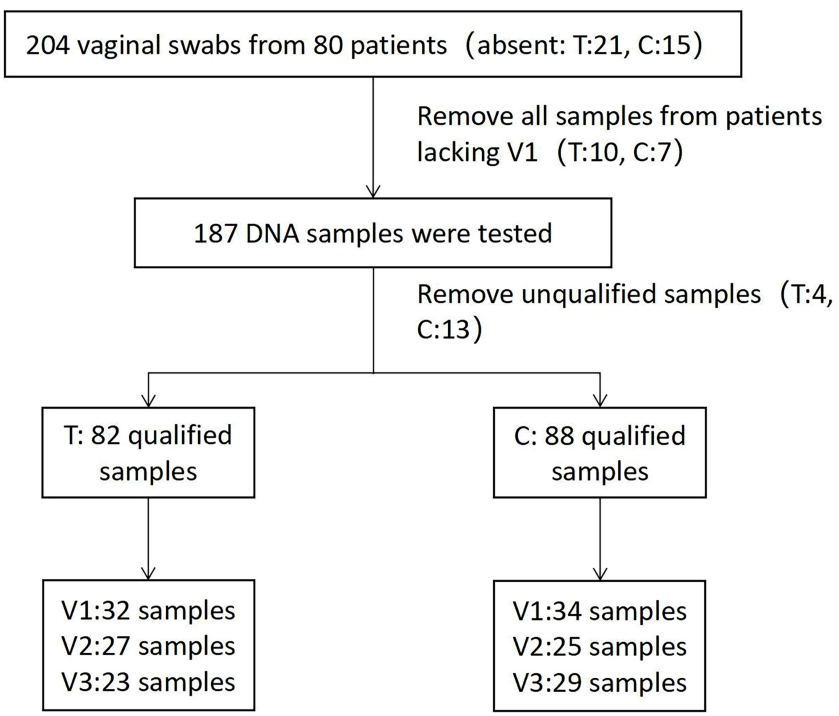


FIGURE 2 | Flowchart of Accu16S qualified sample screening.

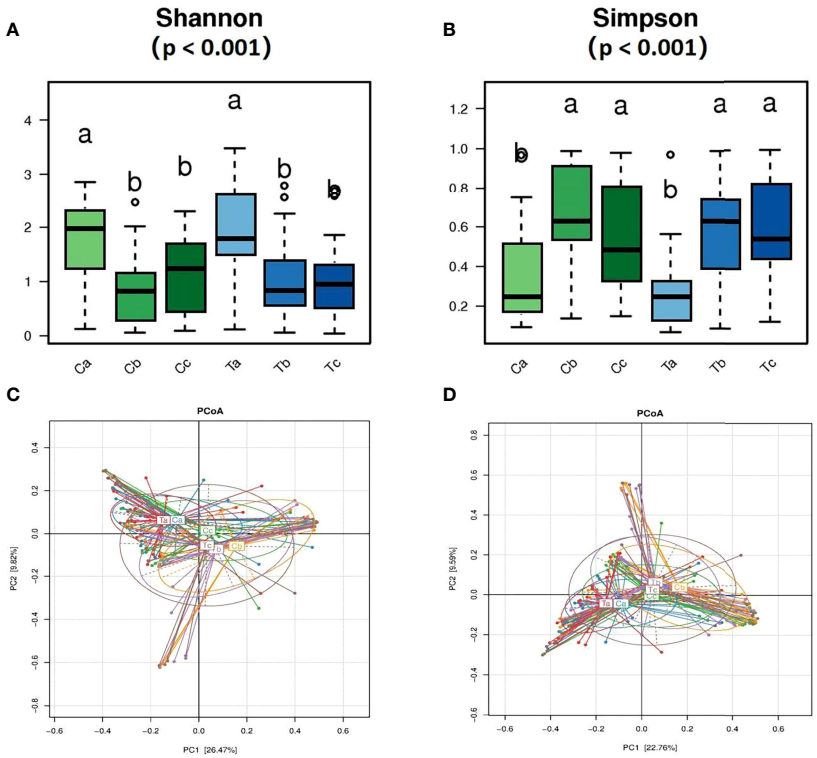


FIGURE 3 | Comparisons of the vagina microbiota α diversity **(A, B)** and relative **(C)** and absolute **(D)** abundances in the principal coordinate analysis (PCoA) of bacterial β diversity. **(A, B)** rank all the mean values from highest to lowest using significant difference letter notation. The largest mean is marked with the letter a and compared with other means, where the difference is not significant, the letter a is marked until an average with a significant difference is marked with the letter b.

abundance of *Lactobacillus* also notably increased while pathogenic bacteria diminished. The relative abundance of dominant bacteria in stage V1 was similar to their absolute abundance in both groups (**Figure 4A**). However, RQS considerably amplified the quantity of *Lactobacillus* in stages V2 and V3, which might mislead the judgment. The comparison between relative and absolute quantifications of several important bacterial communities during the entire course of treatment is presented in **Figure S1**. Overall, the relative quantification can reflect the changing trend of bacteria to a certain extent, but it can distort the therapeutic effects due to inaccurate quantification.

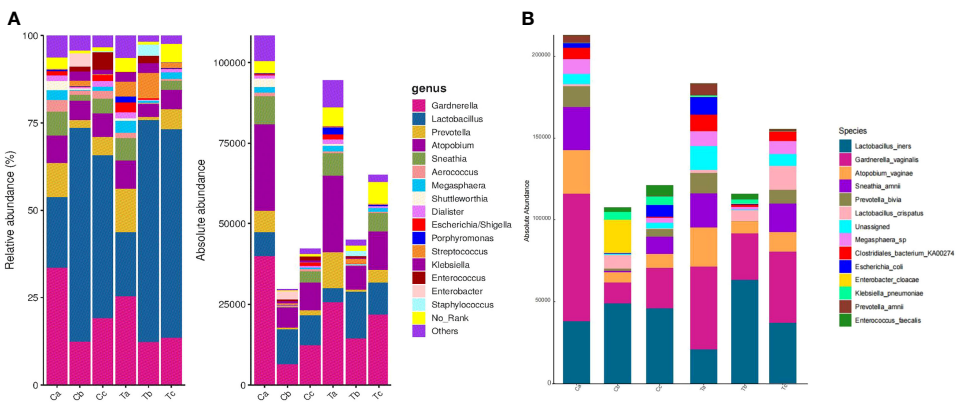


FIGURE 4 | Relative and absolute abundances of the major taxa at the genus **(A)** and the species **(B)** levels in Clindamycin and Furong groups.(Ta, Tb, and Tc represent stages V1, V2, and V3 of the Furong group, respectively; Ca, Cb, and Cc represent stages V1, V2, and V3 of the Clindamycin group, respectively).

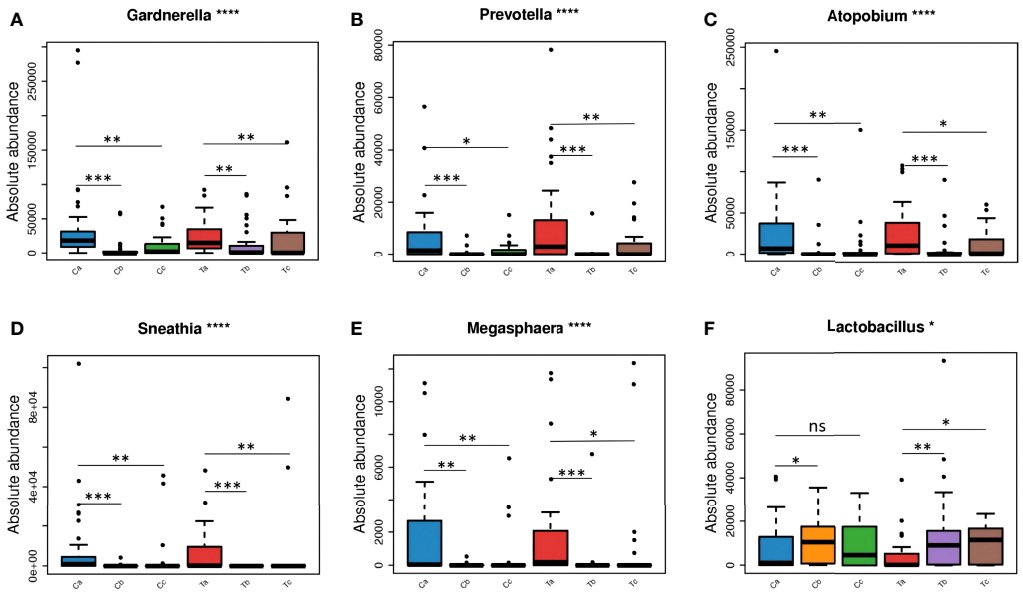


FIGURE 5 | The changes of dominant microorganisms between the two groups at each visit: *Gardnerella* **(A)**, *Prevotella* **(B)**, *Atopobium* **(C)**, *Sneathia* **(D)**, *Megasphaera* **(E)**, and *Lactobacillus* **(F)**. (Ta, Tb, and Tc represent stages V1, V2, and V3 of the Furong group, respectively; Ca, Cb, and Cc represent stages V1, V2, and V3 of the Clindamycin group, respectively; ns, nonsense; *0.01≤P value < 0.05; **0.001≤P value < 0.01; *** 0.0001≤P value < 0.001; ****P value < 0.0001).

Three types of bacteria (*Gardnerella*, *Prevotella*, and *Atopobium*) had a faint increment during stage V3 in both groups (**Figure 4A**). Therefore, we conducted a Wilcoxon rank-sum test for these bacteria in stages V1 and V3 for each group. The absolute abundance of pathogenic bacteria in stage V3 was greatly lower compared to before either Clindamycin or Furong treatments (**Figure S2**). Meanwhile, the absolute abundance of *Lactobacillus* in the Furong group was considerably higher compared to untreated samples ($p < 0.05$) but did not differ from the Clindamycin group ($p > 0.05$). Since 16S analyses can not efficiently classify the annotation results at the species level, we manually compared the sequences in NCBI (National Center for Biotechnology Information to obtain the results in **Figure 4B**. In both groups, *Lactobacillus iners* was dominant in *Lactobacillus* spp., before or after treatments. Its changes were similar to the overall trend of *Lactobacillus*, of which both showed an increase in the total amount after the treatment, especially in the Furong group. Another important increase in *Lactobacillus* species was represented by *Lactobacillus crispatus*. Interestingly, *L. crispatus* only had a transient increase in stage V2 in the Clindamycin group and did not differ 28 ± 3 days after management. Meanwhile, *L. crispatus* continued to increase after Furong therapy with a measurable centrality ($p < 0.05$). Furthermore. the species level analyses (**Figure 4B**) confirmed *Gardnerella vaginalis, Atopobium vaginae, Sneathia amnii*, and *Prevotella bivia* as the dominant pathogenic bacteria. At the genus level (heatmap - **Figure 6**), a cluster pattern of the bacterial community composition was identified, similar to the grouping pattern observed using PCoA. First, *Lactobacillus* became the dominant bacteria in vaginal microbiota at stages V2 and V3 in both groups. Second, a preponderance of *Gardnerella, Prevotella*, and *Atopobium* was detected among pre-treatment patients, consistent with our previous findings.

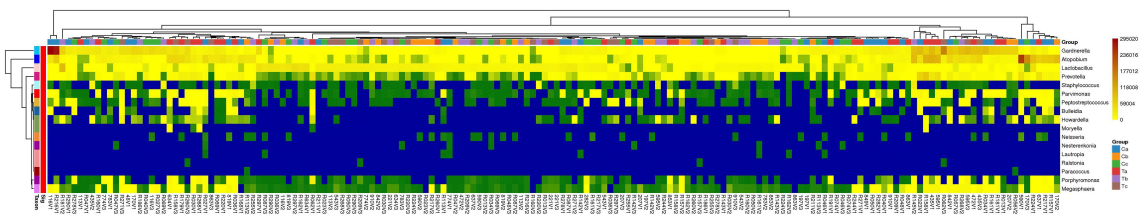


FIGURE 6 | Heatmap of the distributions of the 17 most abundant bacterial genera present in the vaginal swab samples. (The abscissa represents the sample, the ordinate represents different species at the genus level. The absolute abundances of bacterial genera are indicated by color intensity. Ta, Tb, and Tc represent stages V1, V2, and V3 of the Furong group, respectively; Ca, Cb, and Cc represent stages V1, V2, and V3 of the Clindamycin group, respectively).

DISCUSSION

Vaginal infections, such as BV, TV, VVC, and AV, affect the health and quality of life of women and can lead to adverse gynecological complications and reproductive outcomes (Marnach et al., 2022). Several reports have shown that incident BV is associated with an initial decrease in the number of healthy-associated *Lactobacillus* species *(L.crispatus, L.iners, L.jensenii*, and *L.gasseri)* and a subsequent increase in the abundance of *Gardnerella*, *Prevotella*, *Atopobium*, and other anaerobic bacteria (Muzny et al., 2018), consistent with our current results. At the species level, *L.iners* was the dominant *Lactobacillus* in AV+BV patients, and the main pathogenic bacteria were *Gardnerella vaginalis, Atopobium vaginae, Sneathia amnii*, and *Prevotella bivia* (**Figure 4B**), similar to previous studies (Muzny et al., 2018). BV is characterized by dysbiosis of the vaginal microbiota and often occurs with another vaginitis, especially TV and AV. In China, due to the high incidence of combined AV and BV (accounted for 65.35% in mixed vaginitis), more attention should be directed to these infections (Wang et al., 2022). Although AV and BV share some similarities, such as a decrease or absence of *Lactobacillus*, an increase in vaginal secretions, odor, and pH increment (usually more pronounced in AV), there are meaningful differences between them. Different from BV patients, AV patients have increased aerobic bacteria, mostly *Escherichia coli*, *group B streptococci*, and *Staphylococcus aureus* (Donders et al., 2017; Wang et al., 2020). In our current study, no notably changes related to these aerobic bacteria were observed, which might be related to the fact that most patients recruited had light, or mild to moderate AV (**Table 4**). Clindamycin is active against staphylococci and streptococci as well as anaerobes. Antibiotics are a vital resource for treating genital infections, but the rise of antibiotic-resistant bacteria has become a public health problem. Due to antibiotic resistance, 30% recurrence rate for BV occurs within the first month, 59% within 6 months, and 69% within 12 months of treatment (Bradshaw et al., 2006). Moreover, many AV-related bacteria have developed clear clindamycin resistance (Asghar et al., 2020; Genovese et al., 2020). The reduced susceptibility to antibiotics in recent years raised concerns about their use as alternatives. Traditional Chinese Medicine is an important part of Complementary and Alternative Medicine. TCM has been widely used to prevent and treat diseases in China and other parts of East Asia for thousands of years (Zhang et al., 2019). For the past few years, there have been increasing studies regarding the application of TCM in the treatment of genital infections (Zhao et al., 2021). In the TCM theory, vaginitis is usually classified as the area of "leukorrheal diseases," and its causative pathogens can be classified as heat, damp, cold, and toxin (Lin et al., 2019). Generally, TCM treatment of gynecological infections can be used in two ways: oral and external use, which can be applied independently or in combination. The Fufang Furong Effervescent Suppository is a vaginal topical medication, which encompasses a great therapeutic effect on vaginitis. Its main component, *Sophora flavescens*, has many effects, including antibacterial and anti-inflammatory, insecticidal and anti-pruritic, anti-viral, and analgesic (He et al., 2015). Many studies have confirmed that the pharmacological mechanism of *sophora flavescens* may be *via* the activation or suppression of key molecules in certain cellular signaling pathways, such as nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) and transforming growth factor-β/mothers against decapentaplegic homolog (TGF-β/Smad) (Lin et al., 2022; Tang et al., 2022). Furthermore, other herbs in the formula also have certain bactericidal and anti-inflammatory effects, which complement each other. In the present study, we demonstrated the effect of Fufang Furong Effervescent Suppository in the treatment of AV+BV and that it did not differ from clindamycin. No significant differences in the cure rates were detected between the two groups at 3-5 and 28 ± 3 days after BV treatment (**Table S2**). For AV, there was also no significant difference in scores between the two groups at stages V2 and V3. Overall, no discrepancy was observed in the treatment effect of AV+BV between the two groups.

Drug-related complications occurred in six patients in the Clindamycin group (VVC occurred in three cases, liver damage in one case, severe allergic reaction in one case, and vulvar irritation in one case) and two patients in the Furong group (one case of vaginal burning and one of contact bleeding). Probably due to the small sample size, no remarkable difference in adverse drug reactions was observed between these two groups. Although the number and extent of complications caused by antibiotics were much more severe than those caused by the Furong treatment, we need a larger volume of data to verify these hypotheses. The most interesting aspect of this study was that 28 ± 3 days after the end of treatment, the recovery of the vaginal microbiome in the Furong group was better than that in the Clindamycin group (**Figures S2D–H**). This corresponded to the minimum amount of absolute quantitative bacteria in stage V2 in the Clindamycin group (**Figure 4A**), indicating that clindamycin has a strong bactericidal ability and a big destructive force to the vaginal microbiome, finally requiring more time for vaginal microbiome recovery. This was better verified at the species level: *L. crispatus* is widely accepted as a mainstay in maintaining vaginal microecological balance in healthy women and we found that it significantly increased at stage V3 in the Furong group (**Figure 4B**). Recently, the studies on the faster recovery of vaginal flora have mainly focused on the addition of probiotics during or after treatment (Ho et al., 2016; Superti and De Seta, 2020). However, the effectiveness of adding probiotics remains controversial. A randomized controlled trail showed that oral probiotic *Lacticaseibacillus rhamnosus GR-1* and *Limosilactobacillus reuteri RC-14* adjunctive treatment did not increase the cure rate of BV patients compared to metronidazole alone (Zhang et al., 2021). Another meta-analysis indicated that probiotics used after antibiotic treatment were only effective in the short term (Wang et al., 2019). In the best scenario, TCM replacement therapy can restore the vaginal flora quickly while the therapeutic effect is not inferior to antibiotics.

Moreover, RQS can reflect the proportion and trends of microorganisms to some extent, but it might generate spurious results and can not reveal the variation in absolute abundances of individual microrganisms (Props et al., 2017; Guo et al., 2020). Comparing microbial abundances between different samples in

various temporal dimensions is important in the prognosis and clinical medication guidance. The RQS also provides a deep insight into the composition of bacterial communities in vaginal swabs. However, it can not provide dynamic information on the amount of bacterial DNA before and after treatments with different medications (**Figure 4**). More recently, Tkacz et al. (2018) developed absolute quantitation of amplicon families using synthetic chimeric DNA spikes, which has been shown to uncover the comprehensive dynamics of bacterial communities (Jiang et al., 2019). Here, we aimed to use absolute quantification to identify the therapeutic effect and compare it with relative quantification. Both quantitative methods showed that the three pathogenic bacteria closely related to BV were slightly elevated in stage V3 (**Figure 4**), consistent with the fact that BV is prone to relapse in clinical practice (Bostwick et al., 2016). Nevertheless, absolute quantification more accurately displayed the trend of pathogenic bacteria and could better predict recurrence. In the present study, the proportion of *Lactobacillus* was considerably increased in both groups after treatment by relative qualification, which was inconsistent with the absolute data. This apparent relative increase in the proportion of *Lactobacillus* was caused by a decrease in pathogenic bacteria such as *Gardnerella*, *Prevotella*, *Atopobium*. Similarly, relative quantification also overstates the proportion of pathogenic bacteria compared to absolute quantification in stages V2 and V3, which might affect the judgment regarding medication efficacy (**Figure S1**). Additionally, both absolute and relative quantifications showed an outstanding increase of *Lactobacillus* in the vagina after 28 ± 3 days after Furong treatment (**Figures S3A, B**). However, in the Clindamycin group, the absolute quantification indicated that the total amount of *Lactobacillus* in stage V3 did not significantly change ($p > 0.05$), while the overall sum of *Lactobacillus* under relative quantification was notably higher compared to untreated samples ($p < 0.05$) (**Figures S3C, D**). From the absolute quantification, we can conclude that Fufang Furong Effervescent Suppository seems to be better restore the vaginal microbiome than clindamycin, but the relative quantification covered this result. Altogether, these results indicated that relative quantification can sometimes obscure important results and lead to improper conclusions.

Our study also has some limitations. First, the integrity of the experiment was compromised by the loss of some vaginal swabs and unqualified samples during follow-up. Second, Accu16S does not have sufficient resolution to identify microbiomes at the species or strain level, which might result in the omission of some microbial taxa. In the future, quantitative metagenomic sequencing might be a good alternative to better understand the dynamics of the microbiome during disease treatment.

CONCLUSION

Overall, the Nugent and Donders' scores improved in Furong and Clindamycin groups after treatment, and no significant difference was detected between the two groups regarding the theraputic efficiency. Moreover, Accu16S was superior to RQS to accurately assess the dynamics of the vaginal microbiome and evaluate drug effects during treatment. Finally, we demonstrated that Fufang Furong Effervescent Suppository has the same therapeutic effect as clindamycin in the treatment of AV+BV, and may restore the vaginal microecology better.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA809916.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Beijing Tsinghua Changgung Hospital (19190-0-02). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QL and LZ designed and conducted the research. QZ, TL, XY, DS, PL, LH, SF, RA and BZ carried out the clinical trail. ML, HF, and YC analyzed the data and wrote the paper. ZZ provided critical revisions of the article for intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This trial was funded by National Key Research and Development Program (2018YFC1707404), Beijing Natural Science Foundation (7202239), Beijing Traditional Chinese Medicine Science and Technology Development Fund Project (JJ-2020-09), Ministry of Science and Technology of China Biotechnology Development Center, National Key Research and Development Program of TCM Modernization Research key project (2018YFC1707410-04).

ACKNOWLEDGMENTS

We thank Shaanxi Momentum Qixuehe Pharmaceutical Co., Ltd, China and Genesky Biotechnologies Inc., Shanghai, 201315. We also thank the principal investigators and researchers, as well as the women who participated in the trial, who contributed time and effort to make this study a success.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.883798/full#supplementary-material

REFERENCES

Almeida, A., and Shao, Y. (2018). Genome Watch: Keeping Tally in the Microbiome. *Nat. Rev. Microbiol.* 16, 124. doi: 10.1038/nrmicro.2018.13

An, R., Zhang, D., Liu, Z., Xue, F., and Liao, Q. (2016). Expert Consensus on Clinical Application of Vaginal Microecological Assessment. *Chin. J. Obstet. Gynecol.* 51, 721–723. doi: 10.3760/cma.j.issn.0529-567x.2016.10.001

Asghar, S., Khan, J. A., Mahmood, M. S., and Arshad, M. I. (2020). A Cross-Sectional Study of Group B Streptococcus-Associated Sepsis, Coinfections, and Antibiotic Susceptibility Profile in Neonates in Pakistan. *Adv. Neonatal. Care* 20, E59–E69. doi: 10.1097/ANC.0000000000000701

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Bostwick, D. G., Woody, J., Hunt, C., and Budd, W. (2016). Antimicrobial Resistance Genes and Modelling of Treatment Failure in Bacterial Vaginosis: Clinical Study of 289 Symptomatic Women. *J. Med. Microbiol.* 65, 377–386. doi: 10.1099/jmm.0.000236

Bradshaw, C. S., Morton, A. N., Hocking, J., Garland, S. M., Morris, M. B., Moss, L. M., et al. (2006). High Recurrence Rates of Bacterial Vaginosis Over the Course of 12 Months After Oral Metronidazole Therapy and Factors Associated With Recurrence. *J. Infect. Dis.* 193, 1478–1486. doi: 10.1086/503780

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-Resolution Sample Inference From Illumina Amplicon Data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., and Vieira-Baptista, P. (2017). Aerobic Vaginitis: No Longer a Stranger. *Res. Microbiol.* 168, 845–858. doi: 10.1016/j.resmic.2017.04.004

Donders, G. G. G., Zodzika, J., and Rezeberga, D. (2014). Treatment of Bacterial Vaginosis: What We Have and What We Miss. *Expert Opin. Pharmacother.* 15, 645–657. doi: 10.1517/14656566.2014.881800

Fan, A., Yue, Y., Geng, N., Zhang, H., Wang, Y., and Xue, F. (2013). Aerobic Vaginitis and Mixed Infections: Comparison of Clinical and Laboratory Findings. *Arch. Gynecol. Obstet.* 287, 329–335. doi: 10.1007/s00404-012-2571-4

Feng, Y., Zhang, L., Zhu, Y., Chen, R., Huang, Z., and Liao, Q. (2018). Evaluation the Therapeutic Effect of Two Kinds of Methods Treating Simple Aerobic Vaginitis. *Chin. J. Prac. Gynecol. Obstet.* 09, 1055–1057. doi: 10.19538/j.fk2018090126

Genovese, C., D'Angeli, F., Di Salvatore, V., Tempera, G., and Nicolosi, D. (2020). *Streptococcus Agalactiae* in Pregnant Women: Serotype and Antimicrobial Susceptibility Patterns Over Five Years in Eastern Sicily (Italy). *Eur. J. Clin. Microbiol. Infect. Dis.* 39, 2387–2396. doi: 10.1007/s10096-020-03992-8

Guo, X., Zhang, X., Qin, Y., Liu, Y.-X., Zhang, J., Zhang, N., et al. (2020). Host-Associated Quantitative Abundance Profiling Reveals the Microbial Load Variation of Root Microbiome. *Plant Commun.* 1, 100003. doi: 10.1016/j.xplc.2019.100003

He, X., Fang, J., Huang, L., Wang, J., and Huang, X. (2015). *Sophora Flavescens* Ait.: Traditional Usage, Phytochemistry and Pharmacology of an Important Traditional Chinese Medicine. *J. Ethnopharmacol.* 172, 10–29. doi: 10.1016/j.jep.2015.06.010

Ho, M., Chang, Y.-Y., Chang, W.-C., Lin, H.-C., Wang, M.-H., Lin, W.-C., et al. (2016). Oral *Lactobacillus Rhamnosus* GR-1 and *Lactobacillus Reuteri* RC-14 to Reduce Group B Streptococcus Colonization in Pregnant Women: A Randomized Controlled Trial. *Taiwan. J. Obstet. Gynecol.* 55, 515–518. doi: 10.1016/j.tjog.2016.06.003

Javed, A., Parvaiz, F., and Manzoor, S. (2019). Bacterial Vaginosis: An Insight Into the Prevalence, Alternative Treatments Regimen and It's Associated Resistance Patterns. *Microb. Pathog.* 127, 21–30. doi: 10.1016/j.micpath.2018.11.046

Jiang, S.-Q., Yu, Y.-N., Gao, R.-W., Wang, H., Zhang, J., Li, R., et al. (2019). High-Throughput Absolute Quantification Sequencing Reveals the Effect of Different Fertilizer Applications on Bacterial Community in a Tomato Cultivated Coastal Saline Soil. *Sci. Total. Environ.* 687, 601–609. doi: 10.1016/j.scitotenv.2019.06.105

Lin, W. X., Du, X., Yang, L. L., Chen, S. Y., Qiu, W. Y., Wu, H. W., et al. (2019). Differences in the Composition of Vaginal Microbiota Between Women Exhibiting Spleen-Deficiency Syndrome and Women With Damp-Heat Syndrome, Two of the Most Common Syndromes of Vaginitis in Traditional Chinese Medicine. *Evid. Based. Complement. Alternat. Med.* 2019, 5456379. doi: 10.1155/2019/5456379

Lin, Y., He, F., Wu, L., Xu, Y., and Du, Q. (2022). Matrine Exerts Pharmacological Effects Through Multiple Signaling Pathways: A Comprehensive Review. *Drug Des. Devel. Ther.* 16, 533–569. doi: 10.2147/DDDT.S349678

Li, T., Niu, X., Zhang, X., Wang, S., and Liu, Z. (2016). Baofukang Suppository Promotes the Repair of Vaginal Epithelial Cells in Response to Candida Albicans. *AMB. Express.* 6, 109. doi: 10.1186/s13568-016-0281-1

Liu, C., Zhang, Y., Kong, S., Tsui, I., Yu, Y., and Han, F. (2014). Applications and Therapeutic Actions of Complementary and Alternative Medicine for Women With Genital Infection. *Evid. Based. Complement. Alternat. Med.* 2014, 658624. doi: 10.1155/2014/658624

Mårdh, P.-A., Novikova, N., Niklasson, O., Bekassy, Z., and Skude, L. (2003). Leukocyte Esterase Activity in Vaginal Fluid of Pregnant and non-Pregnant Women With Vaginitis/Vaginosis and in Controls. *Infect. Dis. Obstet. Gynecol.* 11, 19–26. doi: 10.1155/S1064744903000036

Marnach, M. L., Wygant, J. N., and Casey, P. M. (2022). Evaluation and Management of Vaginitis. *Mayo. Clin. Proc.* 97, 347–358. doi: 10.1016/j.mayocp.2021.09.022

Mendling, W., Weissenbacher, E. R., Gerber, S., Prasauskas, V., and Grob, P. (2016). Use of Locally Delivered Dequalinium Chloride in the Treatment of Vaginal Infections: A Review. *Arch. Gynecol. Obstet.* 293, 469–484. doi: 10.1007/s00404-015-3914-8

Mills, B. B. (2017). Vaginitis: Beyond the Basics. *Obstet. Gynecol. Clin. North Am.* 44, 159–177. doi: 10.1016/j.ogc.2017.02.010

Muzny, C. A., Blanchard, E., Taylor, C. M., Aaron, K. J., Talluri, R., Griswold, M. E., et al. (2018). Identification of Key Bacteria Involved in the Induction of Incident Bacterial Vaginosis: A Prospective Study. *J. Infect. Dis.* 218, 966–978. doi: 10.1093/infdis/jiy243

Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991). Reliability of Diagnosing Bacterial Vaginosis is Improved by a Standardized Method of Gram Stain Interpretation. *J. Clin. Microbiol.* 29, 297–301. doi: 10.1128/jcm.29.2.297-301.1991

Ozyurt, E., Toykuliyeva, M. B., Danilyans, I. L., Morton, O., and Baktir, G. (2001). Efficacy of 7-Day Treatment With Metronidazole+Miconazole (Neo-Penotran®) - A Triple-Active Pessary for the Treatment of Single and Mixed Vaginal Infections. *Int. J. Gynecol. Obstet.* 74, 35–43. doi: 10.1016/S0020-7292(01)00388-5

Paavonen, J., and Brunham, R. C. (2018). Bacterial Vaginosis and Desquamative Inflammatory Vaginitis. *N Engl. J. Med.* 379, 2246–2254. doi: 10.1056/NEJMra1808418

Props, R., Kerckhof, F.-M., Rubbens, P., De Vrieze, J., Hernandez Sanabria, E., Waegeman, W., et al. (2017). Absolute Quantification of Microbial Taxon Abundances. *ISME. J.* 11, 584–587. doi: 10.1038/ismej.2016.117

Qi, W., Li, H., Wang, C., Li, H., Zhang, B., Dong, M., et al. (2021). Recent Advances in Presentation, Diagnosis and Treatment for Mixed Vaginitis. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.759795

Rivers, C. A., Adaramola, O. O., and Schwebke, J. R. (2011). Prevalence of Bacterial Vaginosis and Vulvovaginal Candidiasis Mixed Infection in a Southeastern American STD Clinic. *Sex. Transm. Dis.* 38, 672–674. doi: 10.1097/OLQ.0b013e31820fc3b8

Sherrard, J., Wilson, J., Donders, G., Mendling, W., and Jensen, J. S. (2018). 2018 European (IUSTI/WHO) International Union Against Sexually Transmitted Infections (IUSTI) World Health Organisation (WHO) Guideline on the Management of Vaginal Discharge. *Int. J. STD. AIDS* 29, 1258–1272. doi: 10.1177/0956462418785451

Sobel, J. D., Subramanian, C., Foxman, B., Fairfax, M., and Gygax, S. E. (2013). Mixed Vaginitis-More Than Coinfection and With Therapeutic Implications. *Curr. Infect. Dis. Rep.* 15, 104–108. doi: 10.1007/s11908-013-0325-5

Stämmler, F., Gläsner, J., Hiergeist, A., Holler, E., Weber, D., Oefner, P. J., et al. (2016). Adjusting Microbiome Profiles for Differences in Microbial Load by Spike-In Bacteria. *Microbiome* 4, 28. doi: 10.1186/s40168-016-0175-0

Superti, F., and De Seta, F. (2020). Warding Off Recurrent Yeast and Bacterial Vaginal Infections: Lactoferrin and Lactobacilli. *Microorganisms* 8, 130. doi: 10.3390/microorganisms8010130

Tang, Q., Liu, Y., Peng, X., Wang, B., Luan, F., and Zeng, N. (2022). Research Progress in the Pharmacological Activities, Toxicities, and Pharmacokinetics of Sophoridine and its Derivatives. *Drug Des. Devel. Ther.* 16, 191–212. doi: 10.2147/DDDT.S339555

Tkacz, A., Hortala, M., and Poole, P. S. (2018). Absolute Quantitation of Microbiota Abundance in Environmental Samples. *Microbiome* 6, 110. doi: 10.1186/s40168-018-0491-7

Wang, C., Fan, A., Li, H., Yan, Y., Qi, W., Wang, Y., et al. (2020). Vaginal Bacterial Profiles of Aerobic Vaginitis: A Case-Control Study. *Diagn. Microbiol. Infect. Dis.* 96, 114981. doi: 10.1016/j.diagmicrobio.2019.114981

Wang, C., Han, C., Geng, N., Fan, A., Wang, Y., Yue, Y., et al. (2016). Efficacy of Oral Moxifloxacin for Aerobic Vaginitis. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 95101. doi: 10.1007/s1009601525138

Wang, Z., He, Y., and Zheng, Y. (2019). Probiotics for the Treatment of Bacterial Vaginosis: A Meta-Analysis. *Int. J. Environ. Res. Public Health* 16, 3859. doi: 10.3390/ijerph16203859

Wang, X., Zhang, L., Sun, X., Liao, Q., Liu, Y., Wang, Y., et al. (2022). Analysis on Vaginal Micro-Ecology of 32046 Outpatients. *Chin. J. Clin. Obstet. Gynecol.* 23, 158. doi: 10.13390/j.issn.1672-1861.2022.02.00

Wei, M., Li, C., Dai, Y., Zhou, H., Cui, Y., Zeng, Y., et al. (2021). High-Throughput Absolute Quantification Sequencing Revealed Osteoporosis-Related Gut Microbiota Alterations in Han Chinese Elderly. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.630372

Workowski, K. A., Bachmann, L. H., Chan, P. A., Johnston, C. M., Muzny, C. A., Park, I., et al. (2021). Sexually Transmitted Infections Treatment Guideline. *MMWR. Recommendations. Rep.* 70, 1. doi: 10.15585/mmwr.rr7004a1

Zhang, Y., Lyu, J., Ge, L., Huang, L., Peng, Z., Liang, Y., et al. (2021). Probiotic *Lacticaseibacillus Rhamnosus* GR-1 and *Limosilactobacillus Reuteri* RC-14 as an Adjunctive Treatment for Bacterial Vaginosis do Not Increase the Cure Rate in a Chinese Cohort: A Prospective, Parallel-Group, Randomized, Controlled Study. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.669901

Zhang, R., Zhu, X., Bai, H., and Ning, K. (2019). Network Pharmacology Databases for Traditional Chinese Medicine: Review and Assessment. *Front. Pharmacol.* 10. doi: 10.3389/fphar.2019.00123

Zhao, H., Zhao, L., Wu, F., and Shen, L. (2021). Clinical Research on Traditional Chinese Medicine Treatment for Bacterial Vaginosis. *Phytother. Res.* 35, 4943–4956. doi: 10.1002/ptr.7123

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 13 May 2022
doi: 10.3389/fcimb.2022.854164

EasyMap - An Interactive Web Tool for Evaluating and Comparing Associations of Clinical Variables and Microbiome Composition

Ehud Dahan[1], *Victoria M. Martin*[2] *and Moran Yassour*[1,3]*

[1] Microbiology and Molecular Genetics, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel, [2] Department of Pediatrics, Massachusetts General Hospital, Boston, MA, United States, [3] School of Computer Science & Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel

OPEN ACCESS

Edited by:
Elisabetta Caselli, University of Ferrara, Italy

Reviewed by:
Luke R. Thompson, Atlantic Oceanographic and Meteorological Laboratory (NOAA), United States
Ornella Cominetti, Nestlé Research Center, Switzerland

***Correspondence:**
Moran Yassour
moranya@mail.huji.ac.il

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *13 January 2022*
Accepted: *05 April 2022*
Published: *13 May 2022*

Citation:
Dahan E, Martin VM and Yassour M (2022) EasyMap - An Interactive Web Tool for Evaluating and Comparing Associations of Clinical Variables and Microbiome Composition. Front. Cell. Infect. Microbiol. 12:854164. doi: 10.3389/fcimb.2022.854164

One of the most common tasks in microbiome studies is comparing microbial profiles across various groups of people (e.g., sick vs. healthy). Routinely, researchers use multivariate linear regression models to address these challenges, such as linear regression packages, MaAsLin2, LEfSe, etc. In many cases, it is unclear which metadata variables should be included in the linear model, as many human-associated variables are correlated with one another. Thus, multiple models are often tested, each including a different set of variables, however the challenge of selecting the metadata variables in the final model remains. Here, we present EasyMap, an interactive online tool allowing for (1) running multiple multivariate linear regression models, on the same features and metadata; (2) visualizing the associations between microbial features and clinical metadata found in each model; and (3) comparing across the various models to identify the critical metadata variables and select the optimal model. EasyMap provides a side-by-side visualization of association results across the various models, each with additional metadata variables, enabling us to evaluate the impact of each metadata variable on the associated feature. EasyMap's interface enables filtering associations by significance, focusing on specific microbes and finding the robust associations that are found across multiple models. While EasyMap was designed to analyze microbiome data, it can handle any other tabular data with numeric features and metadata variables. EasyMap takes the common task of multivariate linear regression to the next level, with an intuitive and simple user interface, allowing for wide comparisons of multiple models to identify the robust microbial feature associations. EasyMap is available at http://yassour.rcs.huji.ac.il/easymap.

Keywords: microbiome, multivariate linear regression, clinical association, interactive, webtool

INTRODUCTION

Examining microbiome differences in the context of clinical changes has become a widely-popular task in many academic and industry contexts (Belkaid and Hand, 2014; Borbet et al., 2019; Niu et al., 2021; Sorbara and Pamer, 2022). The ease of collecting stool samples (compared to blood or biopsy samples), together with the growing evidence of the microbiome's contribution to human health (Becattini et al., 2016), makes the gut-microbiome case/control cohort design even more commonly used in the field (Zhu et al., 2013; Duvallet et al., 2017; Huang et al., 2021).

While animal-studies are conducted in a well controlled environment, they often do not represent human health in sufficient accuracy (Nguyen et al., 2015; Nagpal et al., 2018; Ma, 2021). On the other hand, in human cohorts we have the great challenge of dealing with all the additional characteristics that vary in the human population, such as age, diet, lifestyle, which are known confounders of the gut microbiome, and can bias our results (Vujkovic-Cvijin et al., 2020; "De-Confounding Microbiome Association Studies" n.d.; Devkota, 2016; Bartolomaeus et al., 2020). In an attempt to address this inherent bias in human studies, the field always strives to establish as large cohorts as possible, such as the UK biobank, LifeLines, and the TEDDY cohort ("UK Biobank - UK Biobank" n.d.; TEDDY Study Group, 2008; Davidson-Pilon, 2019). However, it is very difficult and costly to establish and manage large cohorts, and not all clinical manifestations enable such large cohorts, and even in these large numbers, computational methods that take into account the confounding factors are much in need (Jessica and Hanson, 2020).

An additional challenge in microbiome case/control studies is the interoperability of the results. While some machine-learning algorithms perform well on large datasets (Chen et al., 2020; Gou et al., 2021; Carrieri et al., 2021), they are often discriminative in the case/control task without revealing additional information on the underlying reason for the success of their method. Alternatively, the results will highlight specific microbial features that may play a role in the examined clinical manifestation (Oh and Zhang, 2021), which can be further studied from a medical- or a basic-science perspective, as the basis for further studies understanding the mechanisms underlying this association (Aasmets et al., 2021).

A common approach in all case/control studies is the use of multivariate linear regression models that take into account the variables of interest (i.e., microbiome composition) together while accounting for the confounding variables mentioned above (Ramette, 2007; Xia and Sun, 2017; Bodein et al., 2019; Raimondi et al., 2021). There are many packages (R, python, and independent tools) that perform this task, and one of the most-popular tools in the context of microbiome studies is MaAsLin2 (Mallick et al., 2021; S. Ma et al., n.d.; Ma et al., 2021; Zhang et al., 2021). It is especially useful in microbiome studies due to the data transformation (arcsine square-root transformation, Methods), outlier removal, presentation of results and its overall ease of use. Oftentimes, researchers will run multiple models in an attempt to find the ideal model that explains the data best, without overfitting. However, the routine task of comparing MaAsLin results across multiple models is challenging. First in running the multiple models, but more importantly in interpreting the subtle differences in their results.

Here, we present EasyMap, a user-friendly interactive web-based tool that enables running multiple linear regression models and comparing across their results in a graphical manner. While EasyMap was designed to analyze microbiome data, it can handle any other tabular data with numeric features and metadata variables. EasyMap enables the users to upload their own data, construct multiple models, and run the analyses using MaAsLin2, regardless of their computational background and expertise. EasyMap also improves the usability of viewing the significant results, in an interactive high- and low-level visualization of the results. Most importantly, EasyMap provides an easy framework for comparing across models, stratifying the linear regression results by additional variables, and eventually assisting in choosing the optimal model for the data.

METHODS AND IMPLEMENTATION

Tool Implementation

The EasyMap web tool was developed using the *shiny* R package (version 1.6.0), and it uses the *MaAsLin2* R package (version 1.4.0) for multivariate linear regression. All code is available for download on the Yassour lab git repository (Dahan, 2021). EasyMap is available for public use at https://yassour.rcs.huji.ac.il/easymap.

Multivariate Linear Model

EasyMap provides an easy web-based system to perform a multivariable association analysis between microbial features changes to clinical measurement (metadata). Analysis modules include preprocessing, normalisation and transformation and produce a statistically significant output including correction for multiple tests (see below). All user choices are processed to MaAsLin2 format, and are run with the default MaAsLin2 parameters.

Variables in the model are defined as either random- or fixed-effect, based on the user definition (**Figure 1**, Step 2). Briefly, fixed variables impact all samples equally, while random variables impact samples differentially, based on the value of the random-effect variable (Srinivasjois, 2021; Kanters, 2022).

Preprocessing and Normalization

After loading the input data, total sum scaling normalization is applied to each sample, and then microbial features with a total normalized sum of less than 0.0001 were removed. Next, abundance data were transformed with the arcsine square root-transformation (AST) (Biometry: The Principles and Practice of Statistics in Biological Research, 1981). Microbiome data is often sparse and zero-inflated, thus the arcsine square root transformation is an ideal choice to spread the abundance values, but maintain zero abundance. By default, MaAsLin2 uses the na.exclude function, which excludes the Not available (NA) values from the multivariate linear regression calculations, but

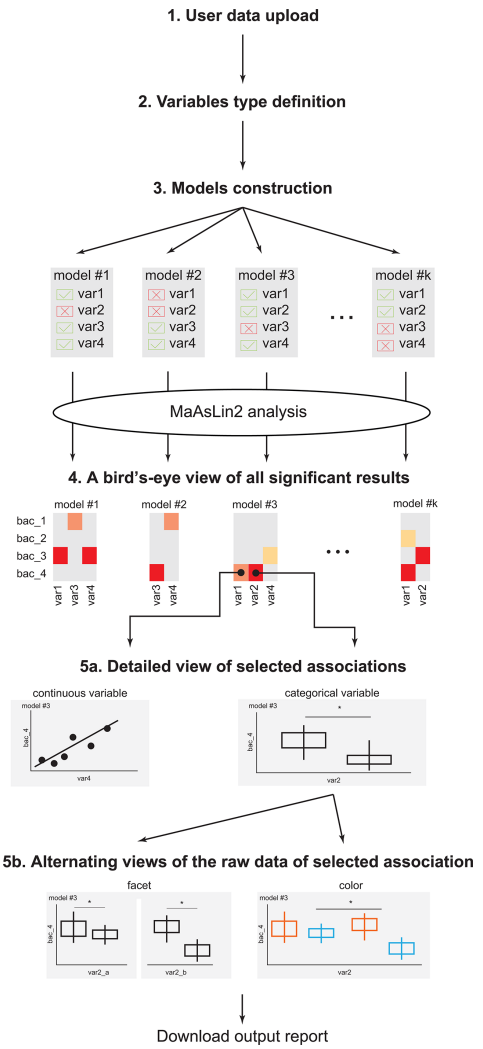


FIGURE 1 | EasyMap workflow. A schematic representation of the EasyMap workflow. Step 1 - User data upload: Enable users to upload their separator based files (comma, tab, etc.). The case study data can be loaded using the 'example' button. Step 2 - Variables type definition: Define the variables in the data as categorical or continuous, and as random or fixed effects for the linear model. Step 3 - Model construction: Include or exclude variables for each model, and select the reference value for each variable. Step 4 - A birds-eye view of all significant results: An interactive heatmap presenting all significant results (calculated by running MaAsLin2 on the constructed models). The results from the various models are displayed side-by-side for optimal comparisons, and the display parameters (such as the specific microbial features, models, and significance threshold) are shown on the left. Step 5a - Detailed view of selected associations: Once the user clicks on a specific association in the heatmap, a detailed view of that result is displayed below. Associations with categorical or continuous variables are displayed as box plots, or scatter plots, respectively. Plots include q-values extracted from MaAsLin2, with brackets indicating the specific comparisons. Step 5b - Alternating views of the raw data of selected association: The detailed plots can be further stratified in two options: (a) coloring by a specific variable (keeping the x-axis as before); and (b) faceting by a specific variable, which splits the x-axis based on the values of the selected variable. Download output report: The heatmap and the presented detailed plots can be downloaded as a pdf file. * = statistical value (p or q value) is less than statistic threshold.

keeps these values for the association visualization (will appear as a separate category, without any statistical calculation). Specifically, if the model includes the variable which has an NA value for some samples, these will not be included in the statistical analysis, but will be presented in the visualization.

Statistical Analysis

Nominal p-values across all associations in the resulting heatmap were adjusted using Benjamini-Hochberg FDR method performed by MaAsLin2, and the coefficient and resulting q-values appear in the box plots with the corresponding brackets. After 'faceting' the box plot, the tool presents the p-values calculated by a two-sided t-test (using the *ggpubr* R package; version 0.4.0), based on all the samples that appear in the plot (without any MaAsLin2 filtering).

Box Plots

In the box plots each dot represents one sample, the middle line represents the median of the distribution, and the box boundaries represent the first and third quartiles. The y-axis represents the transformed relative abundance of a microbial feature bacteria (AST, Methods) and the x-axis is the selected effect variable. All box plots were generated by the ggplot2 R package (version 3.3.3). See statistical analysis Methods section above for q-values and p-values description.

Case Study Data

From the GMAP prospective observational healthy infant cohort, selected infants diagnosed with food protein-induced allergic proctocolitis (FPIAP) who had a minimum of 4 longitudinal stool samples and selected matched controls for each who met the same sampling criteria. 16S rRNA gene libraries targeting the V4 region of the 16S rRNA gene were sequenced on an Illumina MiSeq 300 (raw sequencing data can be found on NCBI BioProject PRJNA730851). Total of 954 samples remain for further analysis.

Running EasyMap on Other, Non-Microbiome, Data

We developed EasyMap to assist us in analyzing microbiome data. However, it can also be used in additional contexts where multivariate linear models are commonly used, maintaining all its added value. The input data format is described below (see Step 1: Input data upload), yet specific attention should be paid to the AST transformation that is applied automatically on the feature data input, which is not optimal for all datasets. The user can choose to not apply this transformation on the uploaded data.

WORKFLOW OF EASYMAP + CASE STUDY EXAMPLE

Cohort and Data Description

Here, we describe the step-by-step flow of the EasyMap interactive tool, available at http://yassour.rcs.huji.ac.il/easymap (also presented in **Figure 1**). To demonstrate the performance of

EasyMap and make it easier to use, we carried out a case study (Martin et al., 2021) and added a short explanation at the end of each step. This case study examines the early childhood microbiome in allergic infants. In this project, 160 infants were longitudinally sampled during the first year of life (6 time points). During this period, 81 infants were diagnosed with food-protein induced allergic proctocolitis (FPIAP), specifically to cow's milk proteins. To characterize the microbial profiles, 16S sequencing was performed on all infant stool samples (N=954). Here, we demonstrate the advantage of using the EasyMap web tool to investigate statistical significant associations between microbial features and clinical data (i.e., allergic diagnosis), using multivariate linear regression models.

Step 1: Input Data Upload

The first step is uploading the user input data, which is a separator based file (csv, tsv etc.) including all relevant data: clinical metadata variables and taxonomic features' abundance for each sample (**Figure 1**). This file should follow MaAsLin (Morgan et al., 2012) format (largely described below) and should have a header line. Suppose there are n metadata variables and m taxonomic features, the input file will have three sections of columns: (a) The first column will contain the sample ID, which is a unique identifier of samples; (b) The next n columns will contain the metadata variables, where each of the variables can be either all strings or all numeric but not mixture. These can include clinical measurements and also other information, such as subject ID, or collecting clinic; (c) The last m columns will contain the abundance of the taxonomic features (relative or absolute). All abundance data will be normalized by total sum scaling (TSS) normalization (MaAsLin2 default) and then will be transformed by the arc-sinus transformation (AST, Methods). The user can choose not to apply the AST transformation on the uploaded data by unchecking the AST checkbox in Step 3 (model construction).

Users can upload their separator based files (comma, tab) through the 'Upload Files' tab. Once uploaded, all the identified columns will appear and the user can click the "Submit" button and continue with the analysis. If there is any problem with parsing the file, the error will be presented to the user.

Step 1 - Case Study

In this case study, we considered six clinical variables that were collected in our cohort, and are also known to have an impact on gut microbiome composition: mode of delivery (vaginal or C-section), age (at time of visit), use of probiotics in the first year of life, infant diet at each time point (breastfed, formula-fed, mixed); and finally the disease status (case/control). In this study we are searching for microbial features that are associated with the disease status, taking into account all other clinical variables (top of this file is presented in **Figure 2A**). Clicking on the 'example' button loads a sample of the case study data.

Step 2: Variables Type Definition

After uploading the data, it is necessary to define the type of all clinical metadata variables (**Figure 1**). First, the user selects the column that represents the unique sample ID. Second, the user selects the variables that will be used as *random effects* in the linear model (see Methods). The model will account for these variables, but will not search for associations between the random variables and the microbial features. Next, the user selects the fixed effect variables, which can be assigned as either continuous or categorical. All variables that remain unselected are automatically defined as the microbial features, thus all clinical variables must be selected as either random or fixed effect variables.

Categorical variables are automatically sorted alphabetically (for example, always, never, sometimes), however, if the user has a specific relevant order, the variable values can include a prefix to maintain this order (like, a_never, b_sometimes, c_always). Numeric variables that have four or less unique values will be treated as categorical variables. Once all variables are defined an "approve" button will appear at the bottom of the screen.

Step 2 - Case Study

In this case study there were 954 samples from 160 different infants. We defined the infant ID as a random variable such that multiple samples from the same infants will be accounted for together, and not as independent measurements. Next, we defined delivery mode, disease status, probiotic use and infant diet as categorical variables. All variables other than diet have two values, and diet has three (breastfed, mixed or formula). Finally, we defined the age at the time of visit as a continuous variable, and all remaining variables are left as the microbial features (the relative abundance of each bacteria in each of the samples; **Figure 2A**).

Step 3: Model Construction

When searching for statistical-significant associations, we first need to choose the clinical variables that our model should account for. These variables are usually chosen based on prior understanding of the clinical situation, and also including factors that are known to impact the microbial community composition. Naively, one can include all collected variables in the model, however, including too many variables would lead to overfitting the data, and diluting the signal across too many variables, potentially missing the significant association altogether. Oftentimes, we choose multiple models, each containing a different set of examined variables, with the aim to compare the results across these models. EasyMap was built to enable a comprehensive comparison across various models, thus highlighting the strong, consistent associations across multiple models.

After defining and approving the variables (as described in step 2) the user will next move to selecting the variables to be used in the first model (**Figure 1**). In the case of categorical variables, the user can also specify the reference value to be used for each variable. For example, if delivery_mode has two possible values: "C-section" or "vaginal", the user can specify that "vaginal" will be the reference value. By default, the tool sorts the values alphabetically and the first value is used as reference. In the example above this would have been "C-section".

A

Unique identifier	Random effect	Categoric Fixed effects			Continous Fixed effects	Bacteria abundance		
sample IDs	participant IDs	disease_state	diet	probiotics use	age	bac_1	Bac2	Bac3
1	a	Yes	F	0	0.1	0.1	0.08	0.1
2	a	Yes	F	1	0.8	0.1	0.04	0.4
3	b	Yes	M	0	0.1	0.4	0.05	0.55
4	b	No	M	0	2.1	0.2	0.1	0.3
5	c	No	M	1	1	0.4	0.4	0.2
6	d	No	B	0	1.5	0.05	0.8	0.15

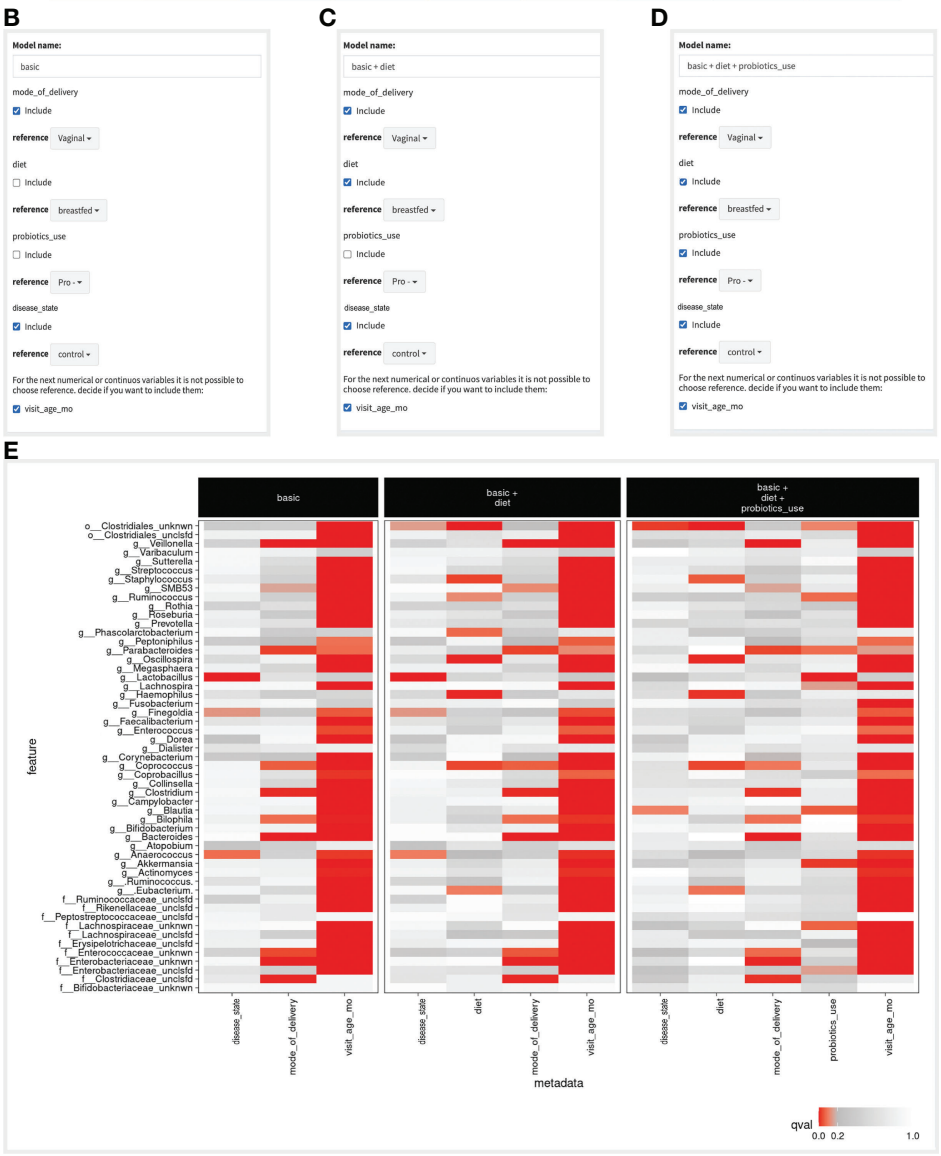


FIGURE 2 | Defining the variables, constructing the models and comparing the results. **(A)** Input file example, where column colors represent different variable types (appear at the top of the table), and the first row is the table's header. **(B–D)** Screenshots of the model construction step. Checkbox values next to each variable indicate if it is included in each model, and the reference value for each variable is selected in the dropbox. The last variable, named 'visit_age_mo' is defined as a continuous variable, therefore its reference is assigned as zero. **(E)** A heatmap showing the birds-eye view of all significant results across all models, from the MaAsLin2 analysis. Each box represents a model, rows are microbial features, and columns are model variables. Each entry in the heatmap is colored by the significance of the association of the specific microbial feature with the specific model variable.

Additional models can be added by clicking on the "add new set" button, and repeating this selection step for each model. By default, the new model is initiated with the selection of variables of the most recently defined model. While many models can be added and compared across, it impacts the total running time and the ease of results viewing in the next step, thus comparing 3-5 models seems ideal.

Example Step 3 - Case Study

In this case study we wanted to find a microbial feature that is associated with disease status. We wanted to examine the contribution of a specific clinical variable to the associations we found. Here, we focused on the impact of infant diet and probiotics use on the microbial associations with disease status. Therefore we considered three models: (1) Including the case/control, mode of delivery and visit age variables as a base model. (2) model 1 variables + infant diet; and (3) model 2 variables + probiotic use (**Figures 2B–D**). We were interested to see whether associations found in model 1 remained when adding the diet and probiotic use variables, which will be revealed in the next step.

Output Description

The output of the EasyMap is composed of two sections: A heatmap of all significant results and a detailed view of selected associations (for example using box plots), with the ability to facet and color the raw data. All the results that are shown on the screen (heatmap with the detailed plots) can be exported to a pdf file.

Step 4: A Birds-Eye View of All Significant Results

The first step in comparing the models is a high-level comparison of all microbial associations that were found to be significant in at least one model. Heatmap color represents the significance, and by default, the FDR q-value threshold is set to be 0.2 (only associations that pass this threshold appear in color). The user can further filter the presented microbial features, using the drop-down menus on the left. The user can select a different threshold, and also choose which models to include in the heatmap (**Figure 1**).

Example Step 4 - Case Study

When examining the bird-eye view of significant results of our three models (**Figure 2E**), the first clear observation is that infant age was strongly correlated with most microbial features (**Figure 2B**). When examining the disease variables/column in the basic model, we found three significant associations (*g:Lactobacillus*, *g:Finegoldia* & *g:Anaerococcus*). However, in the two additional models (models 2 & 3), these associations are not significant anymore, and additional significant associations are detected (*o:Clostridiales_unknwn* in models 2 & 3, and *g:Blautia* in model 3). To enable a simpler comparison we have subset the heatmap to display only the microbial features mentioned above (**Figure 3A**). Interestingly, in the case of *g:Lactobacillus*, there was still a significant association in model 3, only with the probiotic use variable (**Figure 3A**). This shift indicated that once we added the probiotic use to the model, it better explains the different *g:Lactobacillus* abundance across the disease groups.

Step 5a: Detailed View of Selected Associations

One unique and useful feature of EasyMap is the ability to toggle quickly between the bird's eye view of all associations in the heatmap and zooming in on specific associations of interest (**Figure 1**). When the user hovers on a single cell in the heatmap, the cell is highlighted, and the relevant microbial feature together with the selected model, and associated clinical variable appear as text at the bottom of the panel. When the user clicks on a certain cell in the heatmap, the bottom panel is populated with a detailed plot showing the relative abundance (AST, if it was transformed, Methods) of the selected microbial feature by the selected clinical variable (this can be either a box plot for a categorical variable or a scatter plot for a continuous clinical variable). Note that if the relative abundance values (y-axis) are arc-sinus transformed thus can exceed 1, and range in [0, 1.57079]. The detailed plot also displays the q-values that are outputted by MaAsLin2 for all tested associations in this variable (using brackets comparing each value to the selected reference). Significance analysis appears for all possible comparisons between the reference and other values, with their respective q values, even for the non-significant comparisons.

Step 5b: Alternating Views of the Raw Data of Selected Associations

Finally, to include additional metadata to the existing plot, the user can facet the box plot and/or color the dots, by a specific variable (**Figure 1**). When the plot is faceted, the MaAsLin2 q-values are removed from the plot, and instead a t-test is performed, and p-values are presented. Finally, the user can color the dots based on the categorical variables of the model, and add labels to the dots, based on the random variables of the model.

Example Step 5 - Case Study

To further examine the case of *g:Lactobacillus*, we clicked on the square that corresponds to the *g:Lactobacillus* row in the disease column on model 1, which displayed the box plot on the bottom panel (**Figure 3B**). The MaAsLin2 q-value was 1.65e-06, indicating that *g:Lactobacillus* is highly correlated with disease status. However, when clicking on the square that corresponds to the *g:Lactobacillus* row in the disease column on *model 3* (**Figure 3C**), the presented q-value was 2.44e-01, which did not pass our default significance threshold ($q<0.2$), indicating that adding the probiotic use to our model decreases the significance of the disease association. Furthermore, we noted that in model 3, probiotic use was significantly associated with *g:Lactobacillus* abundance, suggesting we investigate this transition further.

Indeed, many of the allergic infants received probiotics in their first year of life, thus the more significant association of *g:Lactobacillus* is with probiotics. To further investigate the contribution of probiotic use in this case, the user can stratify

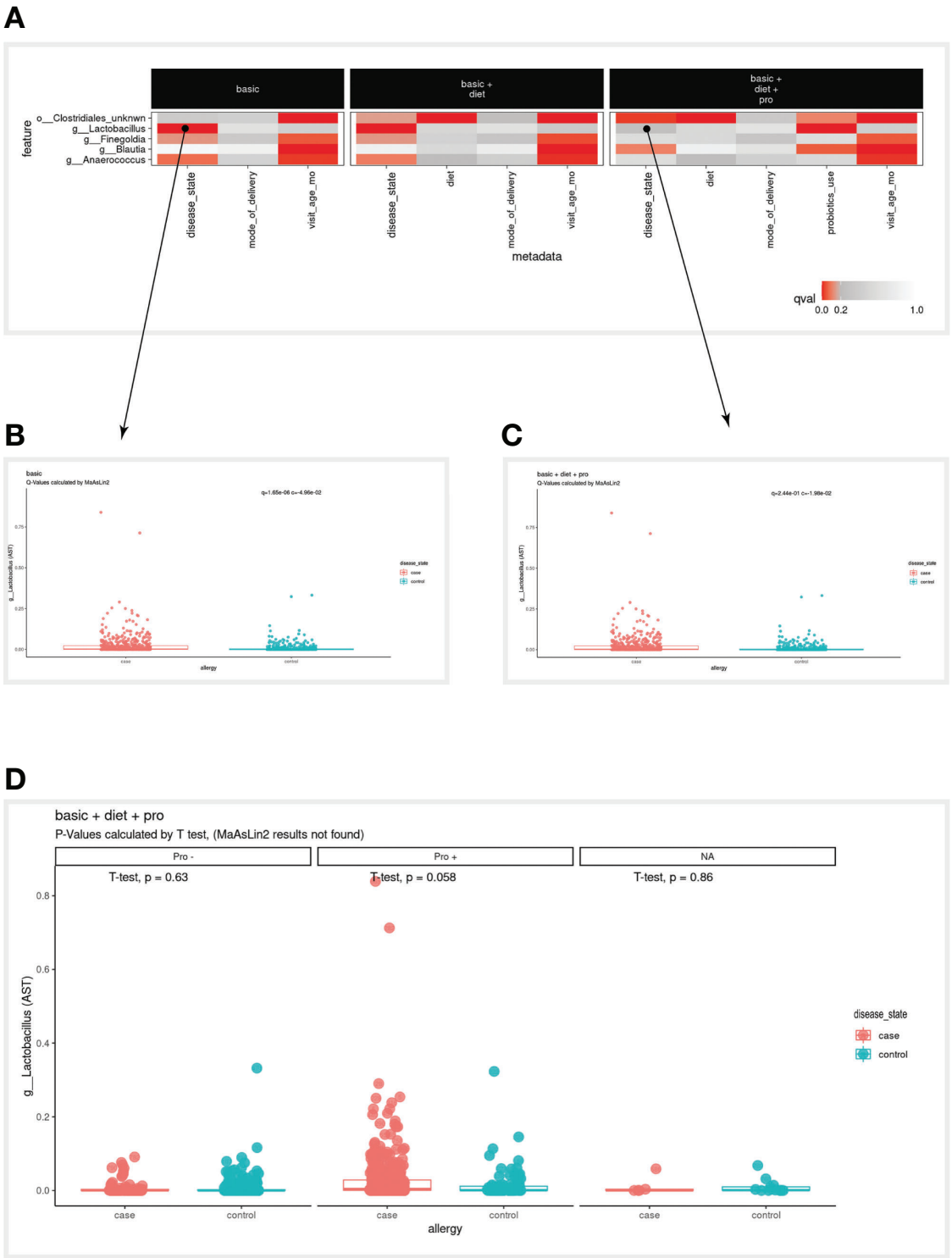


FIGURE 3 | Case study results. **(A)** A subset of the full heatmap, showing the three tested models with only five selected microbial features (rows). **(B, C)** Clicking on the heatmap entries marked with a circle, generates these box plots, displaying the linear association of *g:Lactobacillus* with the disease variable, in the 'basic' model **(B)** and from 'basic + diet + pro' model **(C)**. In the box plots, each dot represents a sample, where the y-axis is the relative abundance of the microbial feature (AST, see Methods), and the x-axis is the values of the disease variable. **(D)** Box plot as in **(C)** stratified by values of the probiotics_use variable (Pro-, Pro+, NA). NA, Not available.

the association between disease and *g:Lactobacillus* by probiotic use, using the "facet by" option of the boxplot. Once the user selects probiotics as the faceted variable, the association between disease and *g:Lactobacillus* can be studied within the context of probiotics (with/without; **Figure 3D**). Once again, note that in the stratified view, the statistical analysis is using t-test in this specific context, rather than the MaAsLin2 systematic q-value (Methods).

DISCUSSION

A common goal of microbial community studies related to human epidemiology is to identify associations between microbial features and clinical variables. These studies must take into account additional factors, of clinical or environmental nature, that also impact the microbiome composition. Often, researchers turn to multivariate linear regression models to find the clinical associations while accounting for other measured confounding effects. Here, we present EasyMap, an interactive web-based tool that enables uploading custom input data, defining multiple such models, running the linear regression (using MaAsLin2 (Mallick et al., 2021)) and comparing the results across all tested models. Comparing the results allows for a better selection of model variables, without overfitting the data.

EasyMap can be run as an online webtool, and the full code is also available on github (Dahan, 2021), making the tool useful for researchers with varying levels of computational backgrounds. Currently, the web-based tool has a few hard-coded settings (such as the common data transformation; Methods), which are helpful in maintaining its ease of use, however user requests from github will be accommodated upon popular demand.

We developed EasyMap to assist us in analyzing data from our lab's studies. It was built as a wrapper for MaAsLin2, with added visualization and comparison abilities, tailored for microbiome studies. However, it can be used in many additional contexts where multivariate linear models are commonly used, maintaining all its added value. EasyMap is also extremely useful for sharing results with collaborators, and enabling all participants to dig deeper in the analysis of their data.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data and scripts can be found here: https://github.com/yassourlab/EasyMap. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Massachusetts General Hospital Institutional Review Board (IRB). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ED developed the tool, VM evaluated the tool, MY guided the work, and ED and MY wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Funding for this project is provided in part by the Azrieli Foundation and the Israeli Science Foundation.

REFERENCES

Aasmets, O., Lüll, K., Lang, J. M., Pan, C., Kuusisto, J., Fischer, K., et al. (2021). Machine Learning Reveals Time-Varying Microbial Predictors With Complex Effects on Glucose Regulation. *mSystems* 6 (1), 1–12. doi: 10.1128/mSystems.01191-20

Bartolomaeus, T.U.P., Till, B., Bartolomaeus, H., Löber, U., Avery, E. G., Mähler, A., et al. (2020). Quantifying Technical Confounders in Microbiome Studies. *Cardiovasc. Res.* 117 (3), 863–875. doi: 10.1093/cvr/cvaa128

Becattini, S., Taur, Y., and Pamer, E. G. (2016). Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. *Trends Mol. Med.* 22 (6), 458–478. doi: 10.1016/j.molmed.2016.04.003

Belkaid, Y., and Hand, T. W. (2014). Role of the Microbiota in Immunity and Inflammation. *Cell* 157 (1), 121–141 doi: 10.1016/j.cell.2014.03.011

Biometry: The Principles and Practice of Statistics in Biological Research (1981).

Bodein, A., Chapleur, O., Droit, A., and Lê Cao, K.-A. (2019). A Generic Multivariate Framework for the Integration of Microbiome Longitudinal Studies With Other Data Types. *Front. Genet.* 0. doi: 10.3389/fgene.2019.00963

Borbet, T. C., Zhang, X., Müller, A., and Blaser, M. J. (2019). The Role of the Changing Human Microbiome in the Asthma Pandemic. *J. Allergy Clin. Immunol.* 144 (6), 1457–1466. doi: 10.1016/j.jaci.2019.10.022

Carrieri, A. P., Haiminen, N., Maudsley-Barton, S., Laura-Jayne, G., Murphy, B., Mayes, A. E., et al. (2021). Explainable AI Reveals Changes in Skin Microbiome Composition Linked to Phenotypic Differences. *Sci. Rep.* 11 (1), 1–18. doi: 10.1038/s41598-021-83922-6

Chen, L., Li, Z., Zeng, T., Zhang, Y.-H., Liu, D., Li, H., et al. (2020). Identifying Robust Microbiota Signatures and Interpretable Rules to Distinguish Cancer Subtypes. *Front. Mol. Biosci.* 7 (November), 604794. doi: 10.3389/fmolb.2020.604794

Dahan, E. (2021). *EasyMap: A Web Tool for Evaluating Clinical Variables Impact on Microbiome Changes (Version 1.0)* (Jerusalem: Github). Available at: https://github.com/yassourlab/EasyMap.

Davidson-Pilon, C. (2019). Lifelines: Survival Analysis in Python. *J. Open Source Software.* 4 (40), 1317. doi: 10.21105/joss.01317

De-Confounding Microbiome Association Studies n.d. Available at: https://www.natureasia.com/en/nature/hot-topics/detail/1788 (Accessed January 10, 2022).

Devkota, S. (2016). MICROBIOME. Prescription Drugs Obscure Microbiome Analyses. *Science* 351, 452–453. doi: 10.1126/science.aaf1353

Duvallet, C., Gibbons, S. M., Gurry, T., Irizarry, R. A., and Alm, E. J. (2017). Meta-Analysis of Gut Microbiome Studies Identifies Disease-Specific and Shared Responses. *Nat. Commun.* 8 (1), 1–10. doi: 10.1038/s41467-017-01973-8

Gou, W., Ling, C.-W., He, Y., Jiang, Z., Fu, Y., Xu, F., et al. (2021). Interpretable Machine Learning Framework Reveals Robust Gut Microbiome Features

Associated With Type 2 Diabetes. *Diabetes Care* 44 (2), 358. doi: 10.2337/dc20-1536

Huang, M., Liu, J., Liu, K., Chen, J., Wei, Z., Feng, Z., et al. (2021). Microbiome-Specific Statistical Modeling Identifies Interplay Between Gastrointestinal Microbiome and Neurobehavioral Outcomes in Patients With Autism: A Case Control Study. *Front. Psychiatry/Frontiers. Res. Foundation.* 0. doi: 10.3389/fpsyt.2021.682454

Jessica, G.-P., and Hanson, B. (2020). Tools for Analysis of the Microbiome. *Digest. Dis. Sci.* 65 (3), 674. doi: 10.1007/s10620-020-06091-y

Kanters, S. (2022). "Fixed- and Random-Effects Models," in *In Meta-Research* (New York, NY: Humana), 41–65.

Ma, Z. S. (2021). Cross-Scale Analyses of Animal and Human Gut Microbiome Assemblies From Metacommunity to Global Landscape. *mSystems* 6 (4), e0063321. doi: 10.1128/mSystems.00633-21

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., et al. (2021). Multivariable Association Discovery in Population-Scale Meta-Omics Studies. *PloS Comput. Biol.* 17 (11), e1009442. doi: 10.1371/journal.pcbi.1009442

Ma, S., Ren, B., Mallick, H., Moon, Y.S., Schwager, E., Maharjan, S., et al. (2021). A Statistical Model for Describing and Simulating Microbial Community Profiles. *PloS Comput. Biol.* 17 (9), e1008913. doi: 10.1371/journal.pcbi.1008913

Martin, V., Virkud, Y. V., Seay, H. L., Keet, C., Shreffler, W. G., and Yuan, Q. (2019). "A Prospective Assessment of Food Protein-Induced Allergic Proctocolitis from the GMAP Healthy Infant Cohort". *J. Allergy Clin. Immunol.* doi: 10.1016/j.jaci.2018.12.412

Ma, S., Shungin, D., Mallick, H., Schirmer, M., Nguyen, L. H., Kolde, R., et al. (2020). Population Structure Discovery in Meta-Analyzed Microbial Communities and Inflammatory Bowel Disease. *bioRxiv*. doi: 10.1101/2020.08.31.261214

Morgan, X. C., Tickle, T. L., Sokol, H., Gevers, D., Devaney, K. L., Ward, D. V., et al. (2012). Dysfunction of the Intestinal Microbiome in Inflammatory Bowel Disease and Treatment. *Genome Biol.* 13 (9), R79. doi: 10.1186/gb-2012-13-9-r79

Nagpal, R., Wang, S., Solberg Woods, L. C., Seshie, O., Chung, S. T., Shively, C. A., et al. (2018). Comparative Microbiome Signatures and Short-Chain Fatty Acids in Mouse, Rat, Non-Human Primate, and Human Feces. *Front. Microbiol.* 0. doi: 10.3389/fmicb.2018.02897

Nguyen, T. L., Vieira-Silva, S., Liston, A., and Raes, J. (2015). How Informative Is the Mouse for Human Gut Microbiota Research? *Dis. Models Mech.* 8 (1), 1–16. doi: 10.1242/dmm.017400

Niu, W., Yang, F., Fu, Z., Dong, Y., Zhang, Z., and Ju, J. (2021). The Role of Enteric Dysbacteriosis and Modulation of Gut Microbiota in the Treatment of Inflammatory Bowel Disease. *Microbial. Pathogene.* 165, 105381. doi: 10.1016/j.micpath.2021.105381

Oh, M., and Zhang, L. (2021). DeepGeni: Deep Generalized Interpretable Autoencoder Elucidates Gut Microbiota for Better Cancer Immunotherapy. *bioRxiv*. doi: 10.1101/2021.05.06.443032

Raimondi, S., Calvini, R., Candeliere, F., Leonardi, A., Ulrici, A., Rossi, M., et al. (2021). Multivariate Analysis in Microbiome Description: Correlation of Human Gut Protein Degraders, Metabolites, and Predicted Metabolic Functions. *Front. Microbiol.* 0. doi: 10.3389/fmicb.2021.723479

Ramette, A. (2007). Multivariate Analyses in Microbial Ecology. *FEMS Microbiol. Ecol.* 62 (2), 142–160. doi: 10.1111/j.1574-6941.2007.00375.x

Sorbara, M. T., and Pamer, E. G. (2022). Microbiome-Based Therapeutics. *Nat. Rev. Microbiol.*, 1–16. doi: 10.1038/s41579-021-00667-9

Srinivasjois, R. (2021). Fixed and Random-Effects Models for Meta-Analysis. In: S. Patole. (eds) *Principles. Pract. Syst. Rev. Meta-Analysis* Cham:Springer. doi: 10.1007/978-3-030-71921-0_7

TEDDY Study Group. (2008). The Environmental Determinants of Diabetes in the Young (TEDDY) Study. *Ann. New York. Acad. Sci.* 1150:1–13. doi: 10.1196/annals.1447.062

UK Biobank - UK Biobank (N.D). Available at: https://www.ukbiobank.ac.uk/ (Accessed January 10, 2022).

Vujkovic-Cvijin, I., Sklar, J., Jiang, L., Natarajan, L., Knight, R., and Belkaid, Y. (2020). Host Variables Confound Gut Microbiota Studies of Human Disease. *Nature* 587 (7834), 448–454. doi: 10.1038/s41586-020-2881-9

Xia, Y., and Sun, J. (2017). "Hypothesis Testing and Statistical Analysis of Microbiome." *Genes Dis.* 4 (3), 138–148. doi: 10.1016/j.gendis.2017.06.001

Zhang, Y., Thompson, K. N., Huttenhower, C., and Franzosa, E. A. (2021). Statistical Approaches for Differential Expression Analysis in Metatranscriptomics. *Bioinformatics* 37 (Suppl_1), i34–i41. doi: 10.1093/bioinformatics/btab327

Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D., et al. (2013). Characterization of Gut Microbiomes in Nonalcoholic Steatohepatitis (NASH) Patients: A Connection Between Endogenous Alcohol and NASH. *Hepatology* 57 (2), 601–609. doi: 10.1002/hep.26093

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.

frontiers | Frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 May 2022
doi: 10.3389/fcimb.2022.794170

Characteristics of the Intestinal Flora of TPOAb-Positive Women With Subclinical Hypothyroidism in the Second Trimester of Pregnancy: A Single-Center Prospective Cohort Study

Min Wu[1], Yuxi Yang[1], Yali Fan[2], Shan Guo[1], Tianhe Li[2], Muqing Gu[1], Tingting Zhang[1], Huimin Gao[1], Ruixia Liu[2] and Chenghong Yin[2]**

[1] Department of Internal Medicine, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing Maternal and Child Health Care Hospital, Beijing, China, [2] Department of Central Laboratory, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing Maternal and Child Health Care Hospital, Beijing, China

Pregnant women are at high risk of developing subclinical hypothyroidism (SCH), and anti-thyroid peroxidase antibody (TPOAb) positivity can further inhibit thyroxine synthesis. Emerging evidence indicates that intestinal flora can modulate metabolic and immune homeostasis. The characteristics of intestinal flora of TPOAb-positive women with SCH in their second trimester of pregnancy have not been reported. This single-center prospective observational cohort study investigated gut microbial composition and metabolic function using sequencing of the 16S rRNA gene in fecal samples from 75 TPOAb-positive women with SCH and 90 TPOAb-negative women with SCH during their second trimester of pregnancy. Women were treated with no levothyroxine (LT_4), low-dose LT_4 (≤50ug/d), or high-dose LT_4 (>50ug/d). Taxonomic analysis showed *Firmicutes* and *Bacteroidetes* were the dominant phyla, followed by *Actinobacteria* and *Proteobacteria*. *Faecalibacterium*, *Bacteroides*, *Prevotella 9*, *Bifidobacterium*, *Subdoligranulum*, *Lachnospira*, and *Megamonas* were the predominant genera. The intestinal flora of TPOAb-positive women with SCH who received no LT_4 was characterized by bacterial amplicon sequence variants (ASVs)/operational taxonomic units (OTUs) enriched in the genus *Subdoligranulum*. The intestinal flora of TPOAb-positive women with SCH who received low-dose or high-dose LT_4 were characterized by bacterial ASVs/OTUs depleted of the species *Ruminococcus* sp._or *Bacteroides massiliensis*, respectively. A total of 19 metabolic functions of intestinal flora, mainly involving lipid and amino acid metabolism, discriminated TPOAb-positive and TPOAb-

OPEN ACCESS

Edited by:
Maayan Levy, University of Pennsylvania, United States

Reviewed by:
Maria D'Accolti, University of Ferrara, Italy
Prince Parbie, National Institute of Infectious Diseases (NIID), Japan

***Correspondence:**
Chenghong Yin
yinchh@ccmu.edu.cn
Ruixia Liu
liuruixia@ccmu.edu.cn

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *13 October 2021*
Accepted: *21 April 2022*
Published: *19 May 2022*

Citation:
Wu M, Yang Y, Fan Y, Guo S, Li T, Gu M, Zhang T, Gao H, Liu R and Yin C (2022) Characteristics of the Intestinal Flora of TPOAb-Positive Women Aith Subclinical Hypothyroidism in the Second Trimester of Pregnancy: A Single-Center Prospective Cohort Study. Front. Cell. Infect. Microbiol. 12:794170. doi: 10.3389/fcimb.2022.794170

negative women with SCH. Our study suggests that there are differences in the composition and metabolic function of intestinal flora of TPOAb-positive and TPOAb-negative women with SCH treated with different doses of LT_4 in the second trimester of pregnancy. The findings provide insight into intestinal flora as novel targets for the treatment of TPOAb-positive women with SCH during pregnancy.

Keywords: subclinical hypothyroidism during pregnancy, anti-thyroid peroxidase antibody, intestinal flora, second trimester, levothyroxine

INTRODUCTION

Subclinical hypothyroidism (SCH) is diagnosed in patients with normal free thyroxine (FT_4) levels and mildly elevated thyroid-stimulating hormone (TSH) levels (Alexander et al., 2017). The prevalence of SCH in pregnancy ranges from 4 to 25%, and varies according to trimester-specific reference ranges for FT_4 and TSH (Springer et al., 2017; Dong and Stagnaro-Green, 2019; Fan et al., 2019). The enzyme thyroid peroxidase (TPO) is responsible for the oxidation and organization of iodine, and for the formation of FT_4 and free triiodothyronine (FT_3) (McLachlan and Rapoport, 1992). Although most women with SCH are asymptomatic, SCH during pregnancy may be associated with adverse outcomes, including miscarriage, preterm birth, pre-eclampsia and gestational diabetes, especially in women who are anti-thyroid peroxidase antibody (TPOAb) positive (van den Boogaard et al., 2011; Negro and Stagnaro-Green, 2014; Alexander et al., 2017; Arbib et al., 2017; Korevaar et al., 2019). The American Thyroid Association 2017 guidelines recommend assessing TSH levels in women at high risk of thyroid dysfunction, when they are seeking pregnancy or are newly pregnant. Levothyroxine (LT_4) may be administered to women who are TPOAb-positive with TSH levels higher than the pregnancy-specific reference range, or who are TPOAb-negative with TSH levels > 10.0 mIU/L (Lazarus et al., 2014; Alexander et al., 2017).

In humans, intestinal flora are important for maintaining the integrity of the intestinal mucosal, immune regulation, metabolism, and nutrition (Yang et al., 2016). Intestinal flora can affect the absorption of micronutrients linked to the synthesis and function of thyroid hormones (Virili and Centanni, 2015). Conversely, intestinal flora may interfere with the metabolism and storage of thyroid hormones (Virili and Centanni, 2015). Small intestinal bacterial overgrowth has been reported in patients with overt hypothyroidism; however, the identity of these pathogenic strains of bacteria remains to be elucidated (Lauritano et al., 2007).

To the authors' knowledge, there are no reports describing the characteristics of intestinal flora of TPOAb-positive women with SCH in their second trimester of pregnancy. In this single-center prospective observational cohort study, we aimed to identify altered gut microbial composition and metabolic function using sequencing of the 16S rRNA gene in fecal samples from 75 TPOAb-positive women with SCH and 90 TPOAb-negative women with SCH during their second trimester of pregnancy. Findings may identify intestinal flora as novel targets for the treatment of TPOAb-positive women with SCH during pregnancy.

MATERIALS AND METHODS

Study Population

This study was a nested prospective observational cohort study that was conducted at Beijing Obstetrics and Gynecology Hospital, Capital Medical University between June, 2020 and March, 2021. This study was approved by the Ethics Committee of the Beijing Obstetrics and Gynecology Hospital (No. 2018-KY-003-01, 2018-KY-003-02). All participants provided written informed consent. The study was registered in the Chinese Clinical Trial Registry (registration number ChiCTR2100047175) on June 10, 2021. All procedures were carried out in accordance with the Declaration of Helsinki.

Inclusion criteria were: (1) females with a singleton pregnancy; (2) recruitment at gestational age $6\text{-}13^{+6}$ weeks; (3) diagnosis of SCH based on thyroid function testing during the first trimester; and (4) provided informed consent.

Exclusion criteria were (1) abortion or loss to follow-up; (2) history of other severe systemic autoimmune disease; (3) history of severe heart, liver, kidney, lung and/or other organ dysfunction; (4) random adjustments to the daily dose of LT4; (5) failure to collect a fecal sample; (6) use of antibiotics or probiotics one month prior to collection of the fecal sample; (7) use of medications that affect thyroid function; (8) history of endemic goiter; or (9) history of mental illness.

Study Design

Pregnant women were screened to assess thyroid function in the first trimester, according to China's Guidelines for the Diagnosis and Treatment of Thyroid Diseases Pregnancy and Postpartum (Second Edition), 2019. Serum FT_4 (enzyme immunoassay), TSH3UL (enzyme immunoassay), and TPOAb levels were measured using an automatic chemiluminescence immunoanalyzer (CENTAUR XP, Siemens, USA). Women's clinical chemistry or hemoglobin were monitored with an automatic biochemical analyzer (CI16200, Abbott, USA) or blood cell analyzer (XN2000, Sysmex, Japanese), respectively.

TPOAb-positive women with SCH were identified based on TSH3UL>3.56mIU/L, FT4 11.80pmol/L-18.40pmol/L, and TPOAb>60.00U/ml. TPOAb-negative women with SCH were identified based on TSH3UL>3.56mIU/L, FT4 11.80pmol/L-18.40pmol/L, and TPOAb 0.00U/ml-60.00U/ml.

TPOAb-positive/negative women with SCH were stratified according to daily dose of LT_4 during pregnancy: no LT_4, low-dose LT_4 (≤50ug/d), or high-dose LT_4 (>50ug/d).

Fecal Sample Collection

Fecal samples were collected at 20-23^{+6} weeks of gestation using the PSP® Spin Stool DNA Plus Kit (SARSTEDT, Germany). Pregnant women collected their fecal samples in clean plastic bags after urination. Duplicate samples from the middle of the stool were placed into preservative in individual sterile tubes. Fecal samples were transported to the hospital on the day they were collected and stored at -80°C until analysis.

High Throughput 16S rRNA Amplicon Sequencing and Analysis

The hypervariable V3-V4 regions of the bacterial 16S rRNA genes were amplified using the following primers: 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). Total genomic DNA was extracted using the sodium dodecyl sulfate [SDS] and cetyltrimethyl ammonium bromide [CTAB] methods, according to the manufacturer (TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit)'s instructions. All PCR reactions were carried out under the following conditions: 15 μL of Phusion®High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and final elongation at 72°C for 5 min. PCR products were detected on 2% agarose gel electrophoresis. PCR products were mixed in equidensity ratios and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), according to the manufacturer's instructions, and index codes were added. Library quality was assessed on the Qubit®2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated.

Microbiome bioinformatics were performed with QIIME2 (2021.04) (Bolyen et al., 2019). Sequences were quality filtered, denoised, merged, and chimeras were removed using the DADA2 plugin (Callahan et al., 2016). Species annotation was performed using QIIME2. 16S annotation was performed using the Silva Database (Release138, http://www.arb-silva.de) (Quast et al., 2013). Alpha diversity indices (Chao1, Shannon, Simpson, Abundance-based Coverage Estimator [ACE]) were calculated with QIIME2 and displayed with R software (Version 3.6.2). Beta diversity was calculated using unweighted unifrac with QIIME2. Principal Coordinate Analysis (PCoA) was performed to visualize similarities and differences in data. A matrix of unweighted unifrac distances was transformed into a new set of orthogonal axes, where the maximum variation factor was demonstrated by the first principal coordinate (PCoA1), and the second maximum variation factor was demonstrated by the second principal coordinate (PCoA2). The two-dimensional PCoA results were displayed using the ade package and ggplot2 package in R (Version 3.6.2). The linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/; Segata et al., 2011) (LDA score threshold: 2 for functional prediction or 4 for microbiome taxa) was used for quantitative analysis of biomarkers. Functional profiling was performed with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (https://github.com/picrust/picrust2; Douglas et al., 2020) with a single script (PICRUSt2_pipeline. Py).

Statistical Analysis of Clinical Data

EpiData was used for data entry, with double data entry and validation. SPSS 26.0 software was used for statistical analysis of clinical data. Normally distributed continuous variables were reported as mean ± standard deviation, and were compared using the independent samples Student's t test. Non-normally distributed continuous variables were reported as medians and quartiles, and were compared using the Wilcoxon signed-rank test. Categorical variables were reported as frequency [n (%)]. The Wilcoxon signed-rank test was used to compare rank categorical variables. The chi-square test was used when there was no rank association between categorical variables. $P<0.05$ was considered a statistically significant difference.

RESULTS

Clinical Characteristics of Subjects

A total of 75 TPOAb-positive women with SCH and 90 TPOAb-negative women with SCH during pregnancy were included in this study. Compared to TPOAb-negative women with SCH, TPOAb-positive women with SCH were more likely to have a history of thyroid disease (41.3% vs. 14.4%, $P<0.0001$) and had significantly higher total cholesterol in the first trimester (4.34mmol/L vs. 4.06mmol/L, $P=0.022$). Other clinical characteristics were not significantly different between groups (**Table 1**).

After stratifying according to daily dose of LT_4 during pregnancy, 12 TPOAb-positive women with SCH and no LT_4 (A1), 24 TPOAb-positive women with SCH and low-dose LT_4 (A2), 39 TPOAb-positive women with SCH and high-dose LT_4 (A3), 30 TPOAb-negative women with SCH and no LT_4 (B1), 43 TPOAb-negative women with SCH and low-dose LT_4 (B2), and 17 TPOAb-negative women with SCH and high-dose LT_4 (B3). Microbiome bioinformatic information was compared in TPOAb-positive women with SCH who received no LT_4 (A1) and TPOAb-negative women with SCH who received no LT_4 (B1), TPOAb-positive women with SCH who received low-dose LT_4 (A2) and TPOAb-negative women with SCH who received low-dose LT_4 (B2), and TPOAb-positive women with SCH who received high-dose LT_4 (A3) and TPOAb-negative women with SCH who received high-dose LT_4 (B3).

TABLE 1 | Demographic and clinical characteristics of the study participants.

Characteristic	TPOAb-positive women with SCH (n = 75)	TPOAb-negative women with SCH (n = 90)	*P* value
General information			
Han ethnicity, *n* (%)	69 (92.0)	81 (90.0)	0.656
Education background (postgraduate and above), *n* (%)	16 (21.3)	21 (23.3)	0.846
Education background (undergraduate), *n* (%)	42 (56.0)	45 (50.0)	
Education background (college and below), *n* (%)	17 (22.7)	24 (26.7)	
Family income (over 4×10^5yuan/year), *n* (%)	22 (29.3)	25 (27.8)	0.925
Family income ($10^5$to 4×10^5yuan/year), *n* (%)	44 (58.7)	55 (61.1)	
Family income (less than 10^5 yuan/year), *n* (%)	9 (12.0)	10 (11.1)	
first pregnancy, *n* (%)	41 (54.7)	52 (57.8)	0.688
Thyroid disease history, *n* (%)	31 (41.3)	13 (14.4)	**0.000**
Natural pregnancy, *n* (%)	73 (97.3)	86 (95.6)	0.544
Smoking, *n* (%)	4 (5.3)	6 (6.7)	0.721
drinking, *n* (%)	5 (6.7)	4 (4.4)	0.531
Indicator in the first trimester			
Sickness, *n* (%)	25 (33.3)	41 (45.6)	0.111
Animals exposure, *n* (%)	11 (14.7)	16 (17.8)	0.591
Age (year), median (IQR)	33 (30-37)	33 (31-34)	0.623
BMI (kg/m^2), median (IQR)	21.6 (20.1-24.6)	21.7 (19.9-25.3)	0.961
SBP (mmHg), median (IQR)	112 (104-120)	110 (101-117)	0.153
DBP (mmHg), mean ± SD	67 ± 11	65 ± 9	0.193
ALT (U/L), median (IQR)	12.20 (9.70-20.60)	12.30 (9.78-17.43)	0.766
AST (U/L), median (IQR)	14.40 (13.00-17.30)	15.05 (12.68-16.93)	0.889
ALB (g/L), mean ± SD	43.96 ± 2.50	43.45 ± 1.91	0.188
GLU (mmol/L), median (IQR)	4.63 (4.49-4.93)	4.65 (4.45-4.84)	0.496
BUN (mmol/L), mean ± SD	3.11 ± 0.69	2.96 ± 0.56	0.116
UA (μmol/L), median (IQR)	214.30 (186.00-255.30)	213.70 (183.05-252.48)	0.973
CRE (μmol/L), mean ± SD	49.37 ± 6.76	48.70 ± 5.76	0.491
TC (mmol/L), median (IQR)	4.34 (3.84-4.85)	4.06 (3.74-4.54)	**0.022**
TG (mmol/L), median (IQR)	1.02 (0.70-1.40)	1.00 (0.78-1.44)	0.855
HDL-C (mmol/L), median (IQR)	1.54 (1.33-1.76)	1.42 (1.25-1.59)	0.093
LDL-C (mmol/L), median (IQR)	2.28 (1.93-2.79)	2.10 (1.89-2.46)	0.064
HGB (g/L), mean ± SD	130 ± 10	129 ± 10	0.572

IQR, interquartile range; TPOAb, thyroid peroxidase antibody; BMI, body mass index; SBP, systolicblood pressure; DBP, diastolic blood pressure; ALT, Alanine aminotransferase; AST, Aspartic acid aminotransferase; ALB, albumin; GLU, blood glucose; BUN, blood urea nitrogen; UA, uric acid; CRE, creatinine; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HGB, hemoglobin.
P<0.05 was considered a statistically significant difference.

Composition of Intestinal Flora

Among 165 fecal samples, the effective sequence number was 12,357,737. A total of 8,905,780 high-quality reads were identified after sequence denoising or clustering. The number of total/unique/common amplicon sequence variants/operating taxonomic units (ASVs/OTUs) are shown in **Supplementary Figure 1A**. The number of ASVs/OTUs classified to the family, genus and species levels are shown in **Supplementary Figure 1B**. Taxonomic analysis showed *Firmicutes* and *Bacteroidetes* were the dominant phyla, followed by *Actinobacteria* and *Proteobacteria* (**Figure 1A**). *Faecalibacterium*, *Bacteroides*, *Prevotella 9*, *Bifidobacterium*, *Subdoligranulum*, *Lachnospira* and *Megamonas* were the predominant genera (**Figure 1B**). There were no differences in the α-diversity indices between TPOAb-positive and TPOAb-negative women with SCH who received no LT_4, low-dose LT_4, or high-dose LT_4 (**Figure 2** and **Supplementary Table 1**).

The refraction curve of intestinal flora indicated the number of ASVs/OTUs analyzed was sufficient, and the distribution and abundance of species in each subgroup was high and adequate for data analysis (**Figure 3A**). On β-diversity, PCoA1 and PCoA2 explained 14.6% and 9.1% of the observed variation in the taxonomic profiles of intestinal flora across subgroups. The β-diversity of the intestinal flora in TPOAb-positive and TPOAb-negative women with SCH who received high-dose LT_4 was significantly different (PCoA1, *P*=0.047) (**Figures 3B–D** and **Supplementary Tables 2, 3**). There were no differences in the β-diversity of intestinal flora between TPOAb-positive and TPOAb-negative women with SCH who received no LT_4 or low-dose LT_4 (**Figures 3B–D**).

Intestinal Flora as Markers of SCH During Pregnancy

LEfSe analysis of differential species abundance was applied to identify intestinal flora that served as markers to distinguish

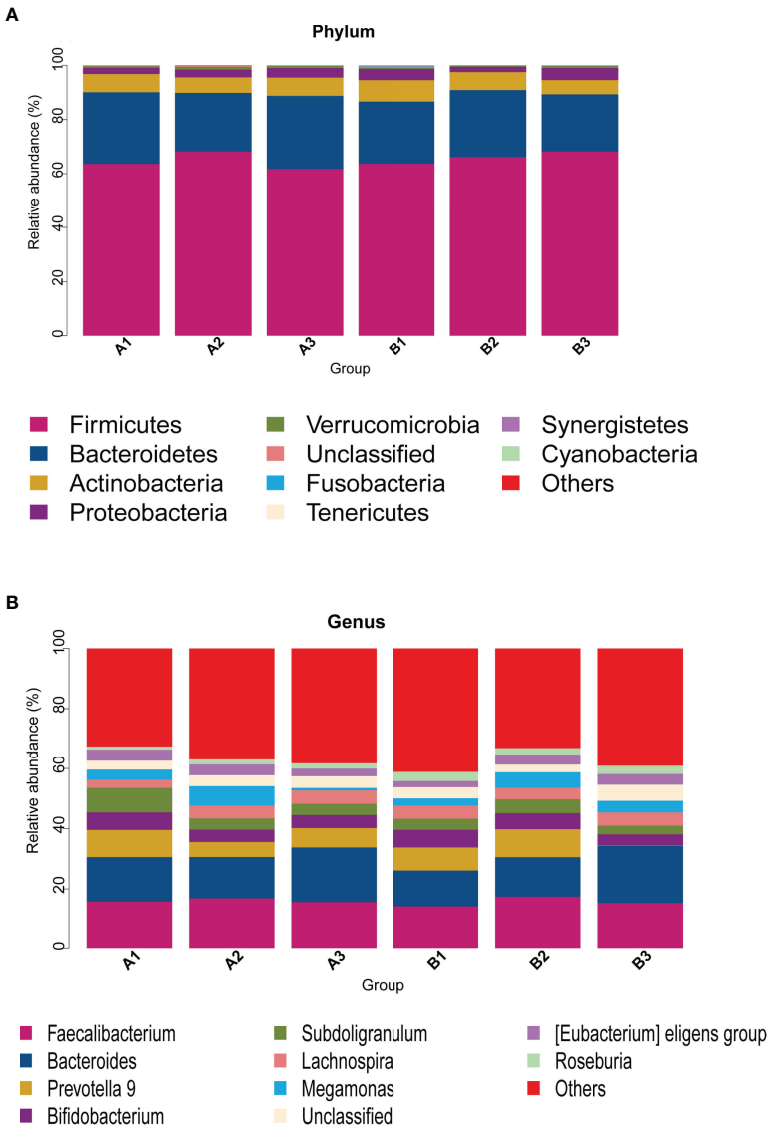


FIGURE 1 | **(A, B)** Taxonomic analysis at the phylum or genus level. A1, TPOAb-positive women with SCH and no LT_4; A2, TPOAb-positive women with SCH and low-dose LT_4; A3, TPOAb-positive women with SCH and high-dose LT_4; B1, TPOAb-negative women with SCH and no LT_4; B2, TPOAb-negative women with SCH and low-dose LT_4; B3, TPOAb-negative women with SCH and high-dose LT_4.

TPOAb-positive and TPOAb-negative women with SCH during pregnancy. The intestinal flora of TPOAb-positive women with SCH who received no LT_4 was characterized by bacterial ASVs/OTUs enriched in the genus *Subdoligranulum* (**Figures 4A, B** and **Supplementary Table 4**). The intestinal flora of TPOAb-positive women with SCH who received low-dose LT_4 was characterized by bacterial ASVs/OTUs depleted of the species *Ruminococcussp_N15_MGS_57* (**Figures 4C, D** and **Supplementary Table 4**). The intestinal flora of TPOAb-positive women with SCH who received high-dose LT_4 was characterized by bacterial ASVs/OTUs depleted of the species *Bacteroides massiliensis B84634_Timone84634_DSM17679_JCM13223* (**Figures 4E, F** and **Supplementary Table 4**). Three marker bacteria (genus *Subdoligranulum*, species *Ruminococcussp_N15_MGS_57* and *Bacteroides massiliensis B84634_Timone84634_DSM17679_JCM13223*) were uniformly distributed among the subgroups (**Supplementary Figures 2A, B** and **Supplementary Table 6**).

Functional Prediction

LEfSe analysis of functional abundance based on the KEGG pathway map was used to predict the metabolic functions of the intestinal flora that served as markers to distinguish TPOAb-positive and TPOAb-negative women with SCH during pregnancy. A total of 19 metabolic functions of intestinal flora discriminated TPOAb-positive and TPOAb-negative women

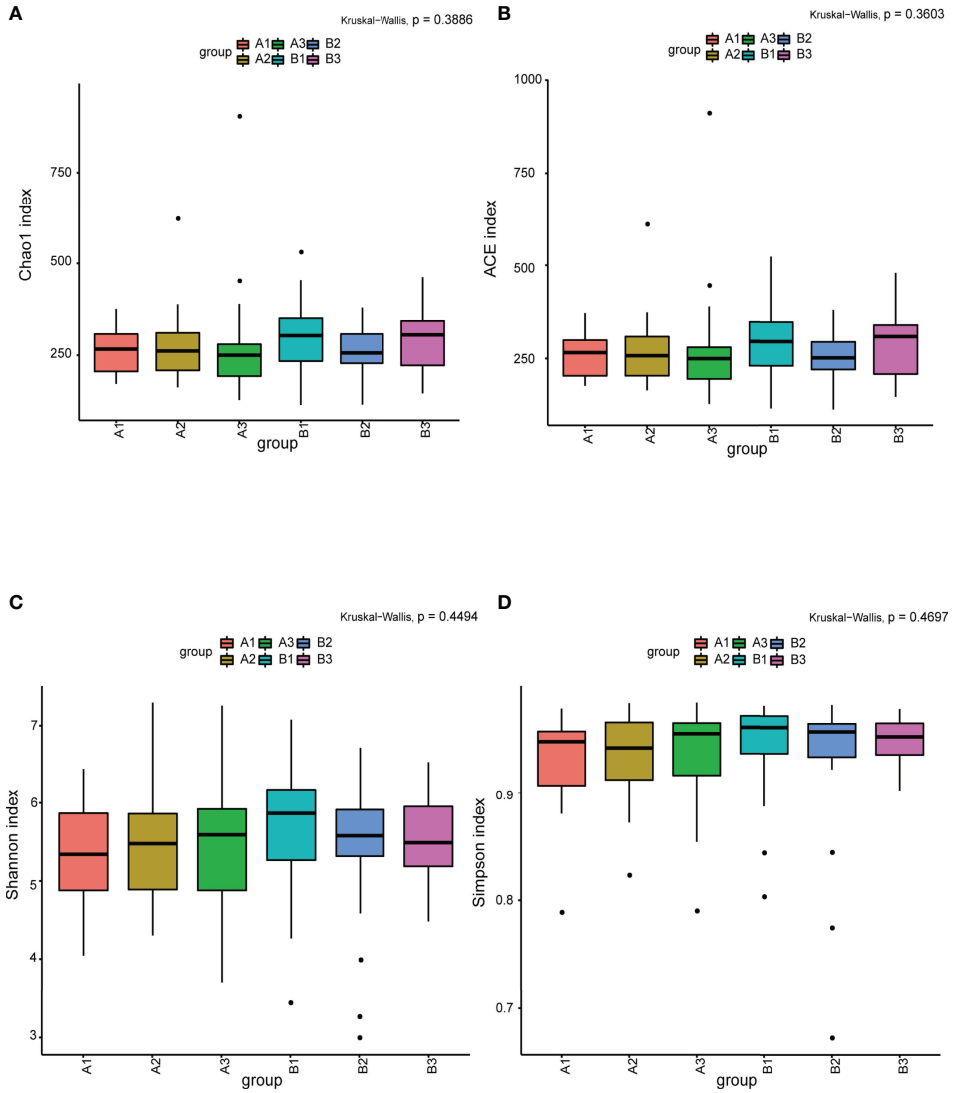


FIGURE 2 | **(A–D)** The α-diversity indices (Chao1, ACE, Shannon, Simpson) analysis. A1, TPOAb-positive women with SCH and no LT_4; A2, TPOAb-positive women with SCH and low-dose LT_4; A3, TPOAb-positive women with SCH and high-dose LT_4; B1, TPOAb-negative women with SCH and no LT_4; B2, TPOAb-negative women with SCH and low-dose LT_4; B3, TPOAb-negative women with SCH and high-dose LT_4.

with SCH. The intestinal flora of TPOAb-positive women with SCH who received no LT_4 was characterized by four enriched metabolic functions (including Histidine metabolism) and two depleted metabolic functions (including Arginine and Ornithine metabolism) (**Figure 5A** and **Supplementary Table 5**). The intestinal flora of TPOAb-positive women with SCH who received low-dose LT_4 was characterized by two enriched metabolic functions and five depleted metabolic functions (including Alanine, Aspartate and Glutamate metabolism) (**Figure 5B** and **Supplementary Table 5**). The intestinal flora of TPOAb-positive women with SCH who received high-dose LT_4 was characterized by three enriched metabolic functions and three depleted metabolic functions (including Linoleic acid metabolism) (**Figure 5C** and **Supplementary Table 5**).

DISCUSSION

In this single-center prospective cohort study, we described the composition and characterized the metabolic function of intestinal flora from TPOAb-positive women with SCH in the second trimester of pregnancy. To our knowledge, this is the first study to show that women diagnosed with TPOAb-positive SCH in the first trimester of pregnancy have distinct characteristics of intestinal flora in the second trimester.

In previous reports, non-pregnant female subjects with SCH were more likely to have a family history of thyroid disease than controls (Rafiq-Uddin et al., 2020). Similarly, in this study in pregnant females, TPOAb-positive women with SCH were more likely to have a history of thyroid disease or higher total

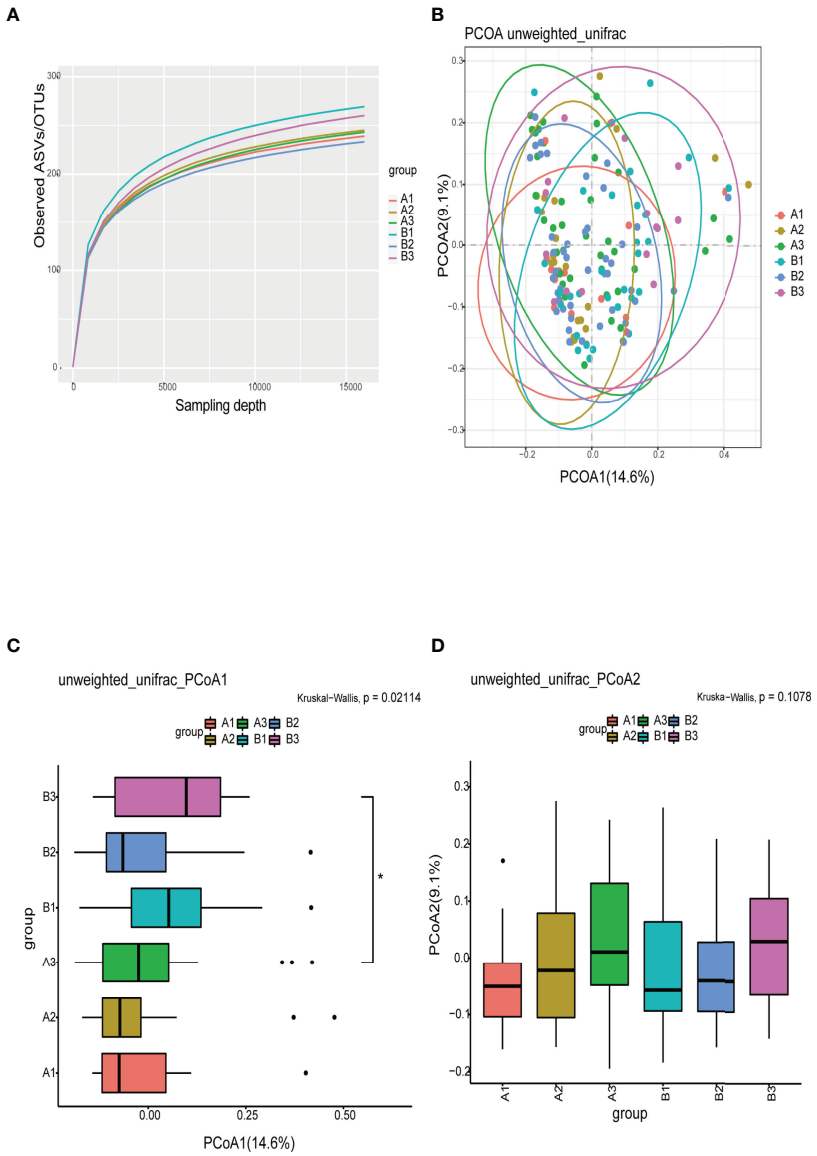


FIGURE 3 | **(A)** Refraction curves based on random extraction of sequencing data from fecal samples from the six subgroups. **(B–D)** β-diversity analysis conducted with the unweighted unifrac algorithm. Ellipses represent 95% confidence intervals for each subgroup in **Figure 3B**. *P<0.05. A1, TPOAb-positive women with SCH and no LT_4; A2, TPOAb-positive women with SCH and low-dose LT_4; A3, TPOAb-positive women with SCH and high-dose LT_4; B1, TPOAb-negative women with SCH and no LT_4; B2, TPOAb-negative women with SCH and low-dose LT_4; B3, TPOAb-negative women with SCH and high-dose LT_4.

cholesterol in the first trimester than TPOAb-negative women with SCH. However, total cholesterol of TPOAb-positive and TPOAb-negative women with SCH in the first trimester were within the normal range. Evidence suggests that dietary habits have an effect on gut microbiota composition (Qian et al., 2018). Thus, our study population was selected from Beijing, to minimize the influence of varying dietary habits across regions on intestinal flora. The baseline characteristics that may reflect the dietary habits of the women participating in this study, including ethnic, cultural and economic data, were not significantly different between groups.

The flower chart visually shows the number of common and unique ASVs/OTUs among the six subgroups of women included in this study. Taxonomic analysis revealed similar phyla and genera across the subgroups. α-diversity reflects microbial diversity in a sample; our boxplots showed no differences in the α-diversity indices across subgroups. β-diversity reflects the difference in taxonomic abundance profiles in different samples; We used unweighted unifrac algorithm and PCoA analysis to analyze β-diversity. Boxplots showed significant differences in species diversity at the PCoA1 axis in samples from TPOAb-positive and TPOAb-negative

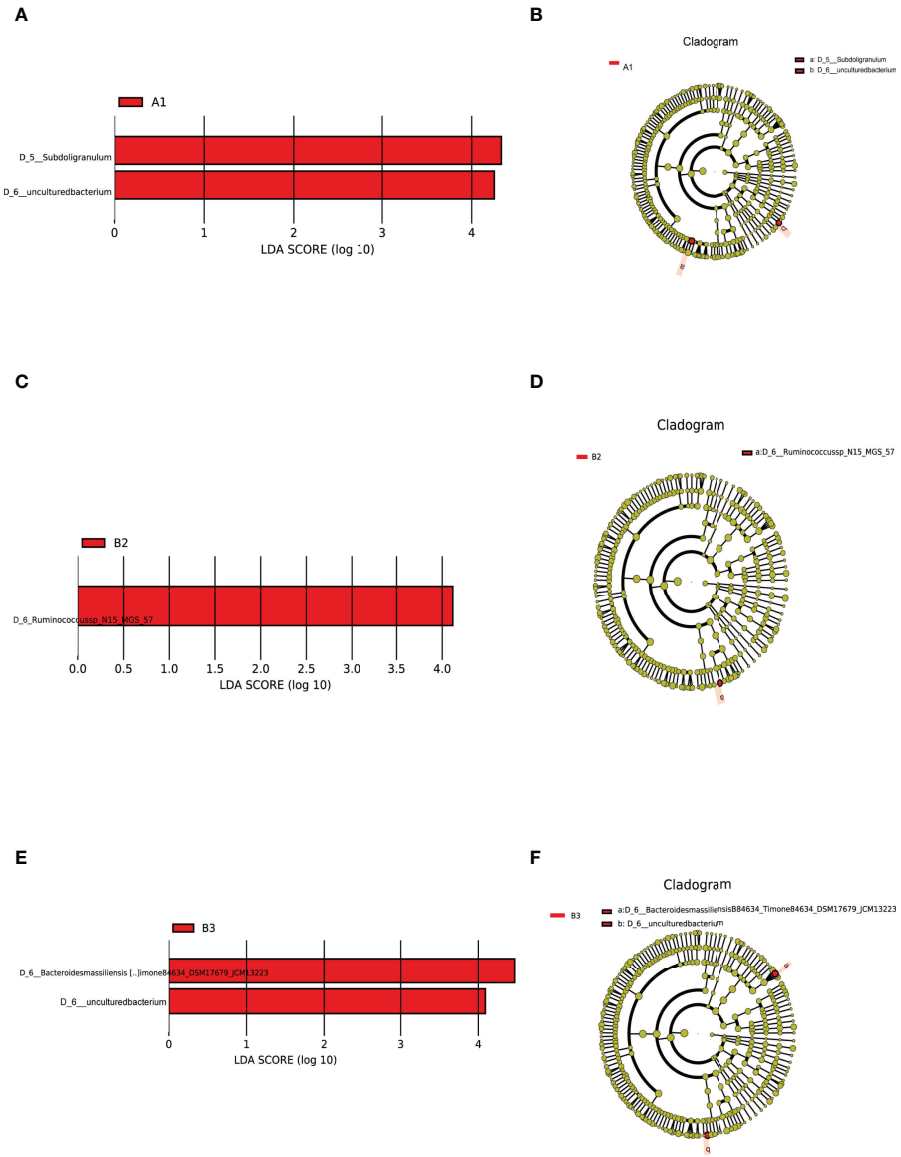


FIGURE 4 | Intestinal flora as markers in TPOAb-positive women with SCH. LEfSe analysis of differential species abundance. **(A, B)** TPOAb-positive vs. TPOAb-negative women with SCH and no LT_4; **(C, D)** TPOAb-positive vs. TPOAb-negative women with SCH and low-dose LT_4; **(E, F)** TPOAb-positive vs. TPOAb-negative women with SCH and high-dose LT_4. LDA value distribution histogram: red bars indicate higher abundance of intestinal flora. Cladograms: circles radiating from the inside to the outside represent taxonomic levels from phylum to species. A1, TPOAb-positive women with SCH and no LT_4; A2, TPOAb-positive women with SCH and low-dose LT_4; A3, TPOAb-positive women with SCH and high-dose LT_4; B1, TPOAb-negative women with SCH and no LT_4; B2, TPOAb-negative women with SCH and low-dose LT_4; B3, TPOAb-negative women with SCH and high-dose LT_4.

women with SCH who received high-dose LT_4. Interestingly, we found that median PCoA1 values of TPOAb-positive women with SCH who received no LT_4, low-dose LT_4, or high-dose LT_4 were smaller than those of TPOAb-negative women with SCH who received no LT_4, low-dose LT_4, or high-dose LT_4. In previous studies, immune-mediated diseases, such as type I diabetes (Gianchecchi and Fierabracci, 2017; Mullaney et al., 2018) and systemic lupus erythematosus (SLE) (Corrêa et al., 2017) were associated with a low intestinal microbial diversity (Rooks and Garrett, 2016; Vatanen et al., 2016; Kriss et al., 2018). Consistent with this, we demonstrated low β-diversity (PCoA1) for TPOAb-positive women with SCH.

Harmful or beneficial bacteria may reflect the severity of disease. Based on the results of this study, we can infer that the abundance of the genus *Subdoligranulum* was associated with promoting Histidine metabolism, and inhibiting Arginine and Ornithine metabolism. Thus, the genus *Subdoligranulum* could be harmful bacteria associated with disease progression in TPOAb-positive women with SCH in the second trimester of pregnancy. Conversely, some previous studies revealed that the

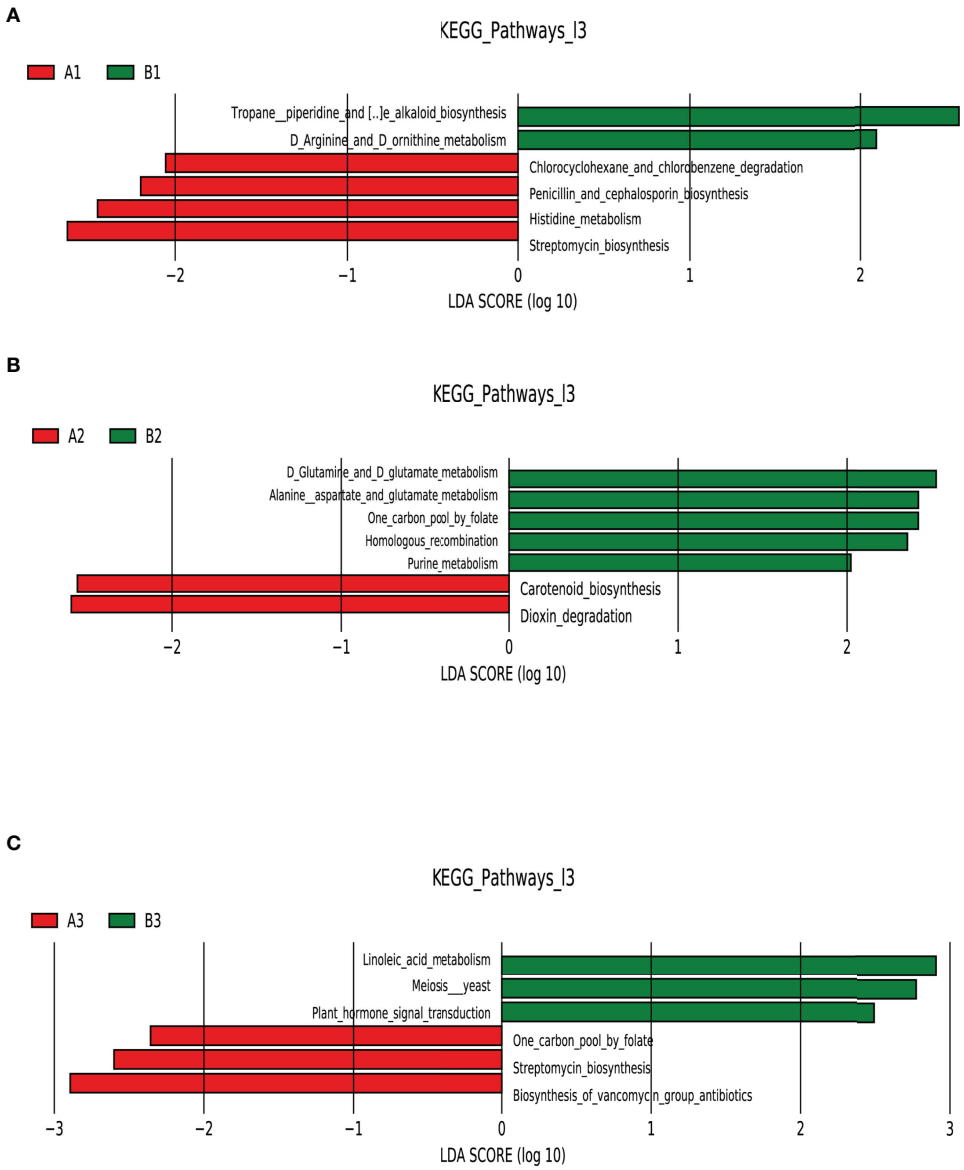


FIGURE 5 | Functional profiling performed with PICRUSt2. LEfSe analysis of differential functional abundance based on the KEGG pathway map. **(A)** TPOAb-positive vs. TPOAb-negative women with SCH and no LT_4; **(B)** TPOAb-positive vs. TPOAb-negative women with SCH and low-dose LT_4; **(C)** TPOAb-positive vs. TPOAb-negative women with SCH and high-dose LT_4. LDA value distribution histogram: the red and green bars represented metabolic functions with higher abundance. A1, TPOAb-positive women with SCH and no LT_4; A2, TPOAb-positive women with SCH and low-dose LT_4; A3, TPOAb-positive women with SCH and high-dose LT_4; B1, TPOAb-negative women with SCH and no LT_4; B2, TPOAb-negative women with SCH and low-dose LT_4; B3, TPOAb-negative women with SCH and high-dose LT_4.

genus *Subdoligranulum* had beneficial effects on obesity (Dao et al., 2016; Louis et al., 2016); Based on the results of this study, we can also infer that the abundance of the species *Ruminococcussp_N15_MGS_57* (associated with promoting Alanine, Aspartate and Glutamate metabolism) and *Bacteroides massiliensis B84634_Timone84634_DSM17679_JCM13223* (associated with promoting Linoleic acid metabolism) may be beneficial in SCH. Previous studies showed that the genus *Ruminococcus* was a prevalent butyrate-producing gut microbe (Beaud et al., 2005; Takahashi et al., 2016) that has a crucial role in the prevention of metabolic diseases (Arora and Bäckhed, 2016). The species *Bacteroides massiliensis* belongs to the genus *Bacteroides*, which is predominant in the human gut (Wexler and Goodman, 2017). In a previous report, the species *Bacteroides massiliensis* showed an inverse correlation with fecal SARS-CoV-2 load among patients hospitalized with COVID-19 (Zuo et al., 2020). To the authors' knowledge, our findings are the first to show that the species *Ruminococcussp_N15_MGS_57* and *Bacteroides massiliensis B84634_Timone84634_DSM17679_JCM13223* may have beneficial effects on SCH in the second

trimester of pregnancy. Future research should explore the causal relationship between these marker bacteria in the second trimester and TPOAb-positivity in SCH women.

Intestinal flora plays an important role in maintaining the metabolic and immunological balance of the host (Liang et al., 2018). Previous studies have confirmed that there may be a reciprocal interaction between gut microbiota and thyroid disorders (Zhou et al., 2014; Shen et al., 2019; Knezevic et al., 2020; Shin et al., 2020; Su et al., 2020). The mechanisms of intestinal flora in pregnant TPOAb-positive women with SCH remain to be elucidated. Consistent with previous studies that support a role for the molecular mechanisms of intestinal flora in the development of autoimmune diseases (Figura et al., 2019), we identified 19 metabolic functions that discriminate TPOAb-positive and TPOAb-negative women with SCH. The different metabolic functions were mainly involved in lipid and amino acid metabolism.

In conclusion, this single-center prospective cohort study described differences in the composition and metabolic function of intestinal flora between TPOAb-positive and TPOAb-negative women with SCH treated with different doses of LT_4 in the second trimester of pregnancy. Our data implied that in the second trimester of pregnancy, TPOAb-positive women with SCH possessed a gut microbiome abundant in harmful bacteria (genus *Subdoligranulum*) that regulated amino acid (Histidine, Arginine and Ornithine) metabolism but depleted in two species of beneficial bacteria (species *Ruminococcussp_N15_MGS_57* and *Bacteroides massiliensis B84634_Timone84634_DSM17679_JCM13223*) that promoted amino acid (Alanine, Aspartate and Glutamate) and lipid (Linoleic acid) metabolism, respectively. Although the dietary habits of the pregnant women were not recorded, and their contribution to the gut microbiota of our study population could not be determined, findings provide insights into intestinal flora as novel targets for the treatment of TPOAb-positive women with SCH during pregnancy. Large scale multicenter prospective cohort studies that include dynamic metagenomics and metabolomics analyses are required to verify our findings.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA751915.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Beijing Obstetrics and Gynecology Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CY, RL, and MW designed the study. MW, YY, YF, SG, MG, TZ, HG recruited the participants and collected the data and fecal samples. MW and TL performed the microbiological analyses. MW and YY analyzed the data. MW generated the figures and wrote the manuscript. CY and RL critically reviewed and edited the manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

FUNDING

This work was supported by The National Key Research and Development Program of China (No: 2016YFC1000101) and Supported by Beijing Municipal Science & Technology Commission (No. Z181100001718076). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank the volunteers for participating in the study and the medical staff for collecting clinical information, fecal samples, and blood samples. We thank Medjaden Inc. for scientific editing of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.794170/full#supplementary-material

REFERENCES

Alexander, E. K., Pearce, E. N., Brent, G. A., Brown, R. S., Chen, H., Dosiou, C., et al. (2017). 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. *Thyroid: Off. J. Am. Thyroid Assoc.* 27 (3), 315–389. doi: 10.1089/thy.2016.0457

Arbib, N., Hadar, E., Sneh-Arbib, O., Chen, R., Wiznitzer, A., and Gabbay-Benziv, R. (2017). First Trimester Thyroid Stimulating Hormone as an Independent Risk Factor for Adverse Pregnancy Outcome. *J. Maternal Fetal Neonatal Med.* 30 (18), 2174–2178. doi: 10.1080/14767058.2016.1242123

Arora, T., and Bäckhed, F. (2016). The Gut Microbiota and Metabolic Disease: Current Understanding and Future Perspectives. *J. Internal Med.* 280 (4), 339–349. doi: 10.1111/joim.12508

Beaud, D., Tailliez, P., and Anba-Mondoloni, J. (2005). Genetic Characterization of the Beta-Glucuronidase Enzyme From a Human Intestinal Bacterium, Ruminococcus Gnavus. *Microbiol. (Reading England)* 151 (Pt 7), 2323–2330. doi: 10.1099/mic.0.27712-0

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* 37 (8), 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-Resolution Sample Inference From Illumina Amplicon Data. *Nat. Methods* 13 (7), 581–583. doi: 10.1038/nmeth.3869

Corrêa, J. D., Calderaro, D. C., Ferreira, G. A., Mendonça, S. M., Fernandes, G. R., Xiao, E., et al. (2017). Subgingival Microbiota Dysbiosis in Systemic Lupus Erythematosus: Association With Periodontal Status. *Microbiome* 5 (1), 34. doi: 10.1186/s40168-017-0252-z

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., et al. (2016). Akkermansia Muciniphila and Improved Metabolic Health During a Dietary Intervention in Obesity: Relationship With Gut Microbiome Richness and Ecology. *Gut* 65 (3), 426–436. doi: 10.1136/gutjnl-2014-308778

Dong, A. C., and Stagnaro-Green, A. (2019). Differences in Diagnostic Criteria Mask the True Prevalence of Thyroid Disease in Pregnancy: A Systematic Review and Meta-Analysis. *Thyroid: Off. J. Am. Thyroid Assoc.* 29 (2), 278–289. doi: 10.1089/thy.2018.0475

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for Prediction of Metagenome Functions. *Nat. Biotechnol.* 38 (6), 685–688. doi: 10.1038/s41587-020-0548-6

Fan, J., Zhang, Y., Zhang, C., Barjaktarovic, M., Yang, X., Peeters, R. P., et al. (2019). Persistency of Thyroid Dysfunction From Early to Late Pregnancy. *Thyroid: Off. J. Am. Thyroid Assoc.* 29 (10), 1475–1484. doi: 10.1089/thy.2019.0115

Figura, N., Di Cairano, G., Moretti, E., Iacoponi, F., Santucci, A., Bernardini, G., et al. (2019). Helicobacter Pylori Infection and Autoimmune Thyroid Diseases: The Role of Virulent Strains. *Antibiotics (Basel Switzerland)* 9 (1), 12. doi: 10.3390/antibiotics9010012

Gianchecchi, E., and Fierabracci, A. (2017). On the Pathogenesis of Insulin-Dependent Diabetes Mellitus: The Role of Microbiota. *Immunol. Res.* 65 (1), 242–256. doi: 10.1007/s12026-016-8832-8

Knezevic, J., Starchl, C., Tmava Berisha, A., and Amrein, K. (2020). Thyroid-Gut-Axis: How Does the Microbiota Influence Thyroid Function? *Nutrients* 12 (6), 1769. doi: 10.3390/nu12061769

Korevaar, T., Derakhshan, A., Taylor, P. N., Meima, M., Chen, L., Bliddal, S., et al. (2019). Association of Thyroid Function Test Abnormalities and Thyroid Autoimmunity With Preterm Birth: A Systematic Review and Meta-Analysis. *JAMA* 322 (7), 632–641. doi: 10.1001/jama.2019.10931

Kriss, M., Hazleton, K. Z., Nusbacher, N. M., Martin, C. G., and Lozupone, C. A. (2018). Low Diversity Gut Microbiota Dysbiosis: Drivers, Functional Implications and Recovery. *Curr. Opin. Microbiol.* 44, 34–40. doi: 10.1016/j.mib.2018.07.003

Lauritano, E. C., Bilotta, A. L., Gabrielli, M., Scarpellini, E., Lupascu, A., Laginestra, A., et al. (2007). Association Between Hypothyroidism and Small Intestinal Bacterial Overgrowth. *J. Clin. Endocrinol. Metab.* 92 (11), 4180–4184. doi: 10.1210/jc.2007-0606

Lazarus, J., Brown, R. S., Daumerie, C., Hubalewska-Dydejczyk, A., Negro, R., and Vaidya, B. (2014). 2014 European Thyroid Association Guidelines for the Management of Subclinical Hypothyroidism in Pregnancy and in Children. *Eur. Thyroid J.* 3 (2), 76–94. doi: 10.1159/000362597

Liang, D., Leung, R. K., Guan, W., and Au, W. W. (2018). Involvement of Gut Microbiome in Human Health and Disease: Brief Overview, Knowledge Gaps and Research Opportunities. *Gut Pathog.* 10, 3. doi: 10.1186/s13099-018-0230-4

Louis, S., Tappu, R. M., Damms-Machado, A., Huson, D. H., and Bischoff, S. C. (2016). Characterization of the Gut Microbial Community of Obese Patients Following a Weight-Loss Intervention Using Whole Metagenome Shotgun Sequencing. *PloS One* 11 (2), e0149564. doi: 10.1371/journal.pone.0149564

McLachlan, S. M., and Rapoport, B. (1992). The Molecular Biology of Thyroid Peroxidase: Cloning, Expression and Role as Autoantigen in Autoimmune Thyroid Disease. *Endoc. Rev.* 13 (2), 192–206. doi: 10.1210/edrv-13-2-192

Mullaney, J. A., Stephens, J. E., Costello, M. E., Fong, C., Geeling, B. E., Gavin, P. G., et al. (2018). Correction to: Type 1 Diabetes Susceptibility Alleles are Associated With Distinct Alterations in the Gut Microbiota. *Microbiome* 6 (1), 51. doi: 10.1186/s40168-018-0438-z

Negro, R., and Stagnaro-Green, A. (2014). Diagnosis and Management of Subclinical Hypothyroidism in Pregnancy. *BMJ (Clinical Res. ed.)* 349, g4929. doi: 10.1136/bmj.g4929

Qian, L., Gao, R., Hong, L., Pan, C., Li, H., Huang, J., et al. (2018). Association Analysis of Dietary Habits With Gut Microbiota of a Native Chinese Community. *Exp. Ther. Med.* 16 (2), 856–866. doi: 10.3892/etm.2018.6249

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res.* 41 (Database issue), D590–D596. doi: 10.1093/nar/gks1219

Rafiq-Uddin, M., Kamrul-Hasan, A. B., Asaduzzaman, M., Aminul-Islam, A. K., Islam, M., Rauniyar, B. K., et al. (2020). Antithyroid Antibody Status in Non-Pregnant Adult Bangladeshi Patients With Subclinical Hypothyroidism. *Mymensingh Med. J. MMJ* 29 (1), 156–161.

Rooks, M. G., and Garrett, W. S. (2016). Gut Microbiota, Metabolites and Host Immunity. *Nat. Rev. Immunol.* 16 (6), 341–352. doi: 10.1038/nri.2016.42

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic Biomarker Discovery and Explanation. *Genome Biol.* 12 (6), R60. doi: 10.1186/gb-2011-12-6-r60

Shen, H., Han, J., Li, Y., Lu, C., Zhou, J., Li, Y., et al. (2019). Different Host-Specific Responses in Thyroid Function and Gut Microbiota Modulation Between Diet-Induced Obese and Normal Mice Given the Same Dose of Iodine. *Appl. Microbiol. Biotechnol.* 103 (8), 3537–3547. doi: 10.1007/s00253-019-09687-1

Shin, N. R., Bose, S., Wang, J. H., Nam, Y. D., Song, E. J., Lim, D. W., et al. (2020). Chemically or Surgically Induced Thyroid Dysfunction Altered Gut Microbiota in Rat Models. *FASEB J. Off. Publ. Fed. Am. Societies Exp. Biol.* 34 (6), 8686–8701. doi: 10.1096/fj.201903091RR

Springer, D., Jiskra, J., Limanova, Z., Zima, T., and Potlukova, E. (2017). Thyroid in Pregnancy: From Physiology to Screening. *Crit. Rev. Clin. Lab. Sci.* 54 (2), 102–116. doi: 10.1080/10408363.2016.1269309

Su, X., Zhao, Y., Li, Y., Ma, S., and Wang, Z. (2020). Gut Dysbiosis is Associated With Primary Hypothyroidism With Interaction on Gut-Thyroid Axis. *Clin. Sci. (London England: 1979)* 134 (12), 1521–1535. doi: 10.1042/CS20200475

Takahashi, K., Nishida, A., Fujimoto, T., Fujii, M., Shioya, M., Imaeda, H., et al. (2016). Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. *Digestion* 93 (1), 59–65. doi: 10.1159/000441768

van den Boogaard, E., Vissenberg, R., Land, J. A., van Wely, M., van der Post, J. A., Goddijn, M., et al. (2011). Significance of (Sub)Clinical Thyroid Dysfunction and Thyroid Autoimmunity Before Conception and in Early Pregnancy: A Systematic Review. *Hum. Reprod. Update* 17 (5), 605–619. doi: 10.1093/humupd/dmr024

Vatanen, T., Kostic, A. D., d'Hennezel, E., Siljander, H., Franzosa, E. A., Yassour, M., et al. (2016). Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* 165 (4), 842–853. doi: 10.1016/j.cell.2016.04.007

Virili, C., and Centanni, M. (2015). Does Microbiota Composition Affect Thyroid Homeostasis? *Endocrine* 49 (3), 583–587. doi: 10.1007/s12020-014-0509-2

Wexler, A. G., and Goodman, A. L. (2017). An Insider's Perspective: Bacteroides as a Window Into the Microbiome. *Nat. Microbiol.* 2, 17026. doi: 10.1038/nmicrobiol.2017.26

Yang, I., Corwin, E. J., Brennan, P. A., Jordan, S., Murphy, J. R., and Dunlop, A. (2016). The Infant Microbiome: Implications for Infant Health and Neurocognitive Development. *Nurs. Res.* 65 (1), 76–88. doi: 10.1097/NNR.0000000000000133

Zhou, L., Li, X., Ahmed, A., Wu, D., Liu, L., Qiu, J., et al. (2014). Gut Microbe Analysis Between Hyperthyroid and Healthy Individuals. *Curr. Microbiol.* 69 (5), 675–680. doi: 10.1007/s00284-014-0640-6

Zuo, T., Zhang, F., Lui, G., Yeoh, Y. K., Li, A., Zhan, H., et al. (2020). Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology* 159 (3), 944–955.e8. doi: 10.1053/j.gastro.2020.05.048

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cellular and Infection Microbiology

REVIEW
published: 08 June 2022
doi: 10.3389/fcimb.2022.873253

Infections and Pregnancy: Effects on Maternal and Child Health

*Manoj Kumar, Marwa Saadaoui and Souhaila Al Khodor**

Research Department, Sidra Medicine, Doha, Qatar

Pregnancy causes physiological and immunological adaptations that allow the mother and fetus to communicate with precision in order to promote a healthy pregnancy. At the same time, these adaptations may make pregnant women more susceptible to infections, resulting in a variety of pregnancy complications; those pathogens may also be vertically transmitted to the fetus, resulting in adverse pregnancy outcomes. Even though the placenta has developed a robust microbial defense to restrict vertical microbial transmission, certain microbial pathogens have evolved mechanisms to avoid the placental barrier and cause congenital diseases. Recent mechanistic studies have begun to uncover the striking role of the maternal microbiota in pregnancy outcomes. In this review, we discuss how microbial pathogens overcome the placental barrier to cause congenital diseases. A better understanding of the placental control of fetal infection should provide new insights into future translational research.

Keywords: preterm labor, miscarriage, TORCH, pregnancy complications, microbiome

OPEN ACCESS

Edited by:
Maayan Levy,
University of Pennsylvania,
United States

Reviewed by:
Piyali Chatterjee,
United States Department of Veterans Affairs, United States
Olivier Disson,
Institut Pasteur, France

***Correspondence:**
Souhaila Al Khodor
salkhodor@sidra.org

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *10 February 2022*
Accepted: *04 May 2022*
Published: *08 June 2022*

Citation:
Kumar M, Saadaoui M and Al Khodor S (2022) Infections and Pregnancy: Effects on Maternal and Child Health. Front. Cell. Infect. Microbiol. 12:873253. doi: 10.3389/fcimb.2022.873253

1 INTRODUCTION

Pregnancy is a critical "formative period" that has a significant impact on an individual's health trajectory from fetal life to adulthood (Lash, 2015). Pregnancy is governed by a series of interconnected physiological and cellular mechanisms that promote maternal homeostasis and maintain optimal maternal-fetal interface while boosting fetal growth (Ander et al., 2019). These mechanisms enable the woman's body to undergo, physiological and immunologic adaptations to host fetal antigens. From the mother's immune system perspective, the fetus is an allograft that contains foreign antigens from the father (Robinson and Klein, 2012). To protect the fetus from immune rejection, the maternal immune must strike a delicate balance between maintaining tolerance to the fetal allograft by inducing anti-inflammatory properties at the maternal-fetal interface and maintaining an elevated inflammatory response with rising levels of pro-inflammatory cytokines at mucosal surfaces such as the gut to protect against microbial challenges (Koren et al., 2012; Erlebacher, 2013; PrabhuDas et al., 2015; Nuriel-Ohayon et al., 2016; Marchant et al., 2017). Concurrently, the transition of the maternal immune system during pregnancy from more inflammatory states at the start of pregnancy to lower levels of inflammation in mid-pregnancy makes pregnant women more vulnerable to infections (Mor and Cardenas, 2010) and pregnancy complications. Although the exact etiology of pregnancy complications remains elusive, the complex interaction of microbial or other factors with host immune system is thought to be the underlying pathogenesis of pregnancy complications (Megli and Coyne, 2021).

The emerging findings from the various pregnancy cohorts (Piler et al., 2017; Pansieri et al., 2020), as well as many animal studies, demonstrated that pregnancy complications are heterogeneous and depend on a variety of factors, including intra- or extra-uterine infection, microbial dysbiosis, and aberrant immune system (Romero et al., 2014a; MacIntyre et al., 2015; Waken et al., 2017; Fettweis et al., 2019; Serrano et al., 2019; Jehan et al., 2020; Kumar et al., 2021a). During pregnancy, multiple immune signaling pathways and cytokines normally act as mediators to promote a healthy and successful pregnancy and to arbitrate defense against pathogens (Mor and Cardenas, 2010). However, the complexity of interaction between multiple host factors, including maternal infection or aberrant activation of the immune response during pregnancy, could lead to severe pregnancy complications and have a negative impact on pregnancy health or the developing fetus (Kumar et al., 2021a). Indeed, the emerging evidence indicates that these pregnancy complications may pose significant challenges to fetal growth and development during pregnancy, as well as susceptibility to a variety of diseases later in life (Rahman et al., 2012; Nimeri et al., 2013).

In this article, we review the complexity of the interaction between various host factors associated with different maternal infections and dynamic fluctuation of the maternal immune system in both inducing pregnancy complications and eliciting detrimental effects on the developing fetus.

2 MATERNAL INFECTIONS DURING PREGNANCY

Complications from various bacterial, viral, parasitic or fungal maternal infections can occur at any stage of pregnancy. Indeed, several studies suggest that pregnant women are more vulnerable to certain infections as a result of compensatory physiological and immunologic adaptations. The "TORCH" pathogens including *Toxoplasma gondii*, Other agents (syphilis, varicella-zoster, parvovirus B19), Rubella, Cytomegalovirus (CMV), and Herpes simplex virus, are known to cause various pregnancy complications such as congenital infections, abortion, and intrauterine fetal growth restrictions (Megli and Coyne, 2021). In addition to these most common infections linked to congenital defects, ZIKA infection, one of the newest TORCH pathogens, has recently sparked public concern, resulting in severe pregnancy complications ranging from fetal growth restriction to miscarriages in 2015-2017 (Coyne and Lazear, 2016). Most TORCH pathogens cause mild to moderate morbidity, but infections during pregnancy can have serious fetal consequences due to stimulation of systemic or local factors (**Table 1**). Emerging studies indicate that various microbial pathogens and neurotropic viruses can cross the placenta barrier, and an aberrant immune response to pathogens can cause various pregnancy complications (Platt et al., 2018), such as:

- Acute maternal infection during pregnancy: may cause maternal morbidity and/or mortality or a wide range of obstetric complications, including low birth weight, stillbirth, miscarriage, and preterm labor.
- Vertical transmission during pregnancy: which can result in congenital infection, intrauterine death, or permanent disability.
- Perinatal transmission during delivery: which can lead to severe neonatal diseases.

To better understand the pathophysiology and consequences of TORCH pathogens and other maternal infections during pregnancy, as well as their impact on pregnancy outcomes, we classified these pathogens into the following categories:

2.1 Bacterial Infections

Acute bacterial infections during pregnancy can increase pregnancy complications and even have a negative pregnancy outcome (**Table 1**). Bacterial infections, such as listeriosis, bacterial vaginosis, and sexually transmitted infections (STIs), can be caused by a single bacterial pathogen or by a microbial dysbiosis and can result in inflammasome signaling at the maternal-fetal interface and/or severe congenital anomalies in the developing fetus.

2.1.1 Listeriosis

Listeriosis is a foodborne bacterial infection caused by *Listeria monocytogenes* (Wang et al., 2021). Although this infection is uncommon in healthy people, pregnant women are particularly vulnerable to *L. monocytogenes* infection, possibly due to their altered immune status (Wang et al., 2021). Once transmitted through contaminated food, *L. monocytogenes* can cross the intestinal barrier to reach the placenta causing pregnancy complications such as preterm birth, stillbirth, congenital diseases, and sepsis (Mateus et al., 2013). A recent listeriosis outbreak in South Africa reported exceptionally high mortality rates among infected infants (>28%) and pregnant women (Thomas et al., 2020). Although the pathophysiology of *L. monocytogenes* placental transmission is still largely unknown, emerging studies show that the bacterium binds to E-cadherin on primary trophoblasts *via* the internalis protein InIA and InIB or InIP (Disson et al., 2008; Faralla et al., 2018), to survive in a hostile environment, suggesting that the bacterium uses trophoblast-specific virulence factors for placental colonization and fetal tissues infection (Bakardjiev et al., 2006). Concurrently, bacterial colonization in placental tissues leads to abscess development, innate immune cells recruitment, and aberrant IFN-γ secretion at the maternal-fetal interface (Charlier et al., 2020; Maudet et al., 2021) and subsequently stimulates inflammasome signaling and increases severity of neonatal outcomes. **A.**

2.1.2 Bacterial Vaginosis

Bacterial vaginosis (BV) is characterized by the loss of healthy vaginal microbiome composition and an increase in the abundance of pathogenic microbes (Isik et al., 2016). BV is the most common gynecological infection among women during reproductive age and pregnancy (Isik et al., 2016; Kumar et al., 2021a), resulting in serious pregnancy complications such as miscarriage and preterm birth (**Table 1**) (Leitich et al., 2003).

TABLE 1 | Pathogens associated with pregnancy complications and their pathological role in adverse pregnancy outcomes.

Pathogen	Transmission	Maternal symptoms	Immune response associated with infection	Pregnancy complications	Reference
Bacteria *Listeria monocytogenes*	Consumption of contaminated food	Fever, Flu-like symptoms, headache, vomiting	IFN-γ, IL-1β, IL-10	Vertical transmission, congenital disease, Miscarriage, stillbirths, fetal death	(Teixeira and Kaufmann, 1994; Thomas et al., 2020)
Brucella species	Consumption of contaminated food or contact with infected animal	Fever, join and muscle pain	IL-6, IL-8, MCP-1	Spontaneous abortions, preterm birth, chorioamnionitis	(Fernandez et al., 2016; Bosilkovski et al., 2020)
Chlamydia trachomatis	Sexual contact with infected person	Vaginal discharge, pelvic or abdominal pain	IL-1α, IL-6, IL-8, TNF-α, IFN-γ,	Premature rupture of membrane, Preterm, fetal eye infection	(Brunham and Rey-Ladino, 2005; Adachi et al., 2016)
Neisseria gonorrhoeae	Sexual contact with infected person	Vaginal discharge and bleeding, Painful urination, painful bowel movements	IL-1β, IL-6, IL-8, TNFα, MCP-1	Premature rupture of membrane, Preterm birth, low birth weight	(Lenz and Dillard, 2018; Vallely et al., 2021)
Treponema pallidum/Syphilis	Sexual contact with infected person	Fever, Swollen lymph nodes, headache and joint pains	IL-2, IFN-γ,TNFα	Vertical transmission, still birth, pregnancy loss, low birth weight	(Wicher and Wicher, 2001; Cerqueira et al., 2017)
Streptococci group B *S. pneumoniae*	Commensal Contaminated air	Normally no symptoms, but some women can have low grade fever, fast or slow heart rate and breathing rate, lethargy, Urinary tract infection	IL-1β, IL-8, IL-10, TNF-α	Vertical transmission (rare), Vertical transmission during delivery, preterm birth, neonatal sepsis	(Patras and Nizet, 2018; Flaherty et al., 2019; Phillips and Walsh, 2020)
Bacterial vaginosis *E. coli*	Commensal	Diarrhea, abdominal cramps, vomiting, fatigue, Urinary tract infection	IL-1β, IL-8, IL-10, TNF-α, IFN-γ	Preterm rupture of membranes, preterm birth, still birth	(Sacerdoti et al., 2018; Wilkie et al., 2019; Glaser et al., 2021; Megli and Coyne, 2022)
Gardnerella vaginalis	Sexual contact with infected person	Vaginal discharge, infection with fishy odor	IL-1β, IL-6, TNF-α,	Vertical transmission (no evidence), Preterm rupture of membranes, low birth weight, preterm birth	(Wong et al., 2018)
Trichomonas vaginalis *Ureaplasma urealyticum* *Mycoplasma hominis*	Sexual contact with infected person	Vaginal discharge, itching in the genitals	IL-1β, IL-6, IL-8	Premature rupture of membrane, Preterm birth, low birth weight	(Cauci and Culhane, 2007; Capoccia et al., 2013; Margarita et al., 2020)
Viruses Cytomegalovirus (cmv)	Ingestion of infected body fluids (blood, saliva, urine, breast milk, feces)	High fever, aching muscles, skin rash, sore throat	CXCL-10 (blood) TNF-α, IL-1β, IL-10, IL-12, IL-15, IL-17, CCL-2, CCL-4, CXCL-10 (amniotic fluid)	Vertical transmission, congenital disease, preterm birth, Fetal hearing loss, vision loss, intracranial calcifications	(Cannon et al., 2011; Scott et al., 2012; Liu et al., 2021)
Herpes simplex virus	Sexual or oral contact with infected person	Genital herpes, rash, cold sores on lips, gums	Anti-HHV-IgG, IgM	Vertical transmission during delivery, Spontaneous abortion, miscarriage, chorioretinitis, intracranial calcification in neonates	(Pinninti and Kimberlin, 2013; James et al., 2014)
Rubella	Contaminated respiratory droplets	Low-grade fever, headache, sore throat, conjunctivitis	Anti-rubella-IgG, IgM	Miscarriage, still birth, vertical transmission, fetal ocular disorder, auditory or speech disorder and autism	(Wilson et al., 2006; Arora et al., 2017; Yockey and Iwasaki, 2018)
HIV	Sexual or contaminated material	Weight loss, chronic diarrhea, night sweats, rash and increased susceptibility of infections	IL-1β, IL6, IL10, CD4+ ↑ IFNα ↓	Vertical transmission, congenital disease, neonatal high mortality and lifelong devastating effect, cardiovascular diseases and increased risk to infections	(Maartens et al., 2014; Johnson and Chakraborty, 2016; Moncunill et al., 2020)
Zika virus	Aedes species, sexual, blood borne	Fever, joint and muscle pain, rash	IL-6, IL-15, IL-17, IFN-γ, IFN-α, TNF-α (blood)	Pregnancy loss, still birth, congenital disease, neurological defects including intracerebral calcifications, enlarged ventricles and collapsing brain, echogenic bowel,	(Ornelas et al., 2017; Maucourant et al., 2019)

(Continued)

TABLE 1 | Continued

Pathogen	Transmission	Maternal symptoms	Immune response associated with infection	Pregnancy complications	Reference
SARS-CoV2 MERS	Respiratory or contact with infected material	Fever, cough, tiredness, loss of taste or smell	IL1, IL2, IL-7, IL10, TNF-α	Vertical transmission (no evidence), maternal mortality, preeclampsia, preterm birth	(Alfaraj et al., 2019; Kumar and Al Khodor, 2020; Saadaoui et al., 2021; Villar et al., 2021)
Hepatitis C virus	Ingestion of infected material	Cholestasis, itching, yellow eye or skin	CXCL-11, CXCL-12	Vertical transmission (rare), Vertical transmission during delivery, low birth weight, preterm birth, neonatal chronic liver disease	(Chudnovets et al., 2020)
Varicella-zoster virus	Contaminated respiratory droplets	Red rash, blisters, itching	IL-1α, IL-6, CXCL10, TGF-β	Vertical transmission (rare), Vertical transmission during delivery, Limb and gastrointestinal abnormalities	(Chudnovets et al., 2020; Nanthakumar et al., 2021)
Parvovirus B19 (Fifth disease)	Contaminated respiratory droplets	Mild fever, sore throat, red rash	IL-2, IL-12, IL-15, IFN-γ	Anemia, still birth, pregnancy loss	(Isa et al., 2007; Adams Waldorf and McAdams, 2013)
Influenza	Contaminated respiratory droplets	Fever with chills, cough, sore throat, runny or stuffy nose, body aches, headache	TNF-α, IL-1β, IL-6, IL-15, IFN-γ	Low birth weight	(Le Gars et al., 2016)
Enterovirus	Ingestion of infected material	Diarrhea, conjunctivitis or rash		Increased risk of type 1 diabetes in childhood	(Adams Waldorf and McAdams, 2013)
West Nile virus	Bite of infected mosquito Arbovirus (Culex species)	Fever, vomiting, neck stiffness, or seizures	IL-2, IL-4, TNF-α, IFN-γ	Meningitis/encephalitis, possible lissencephaly	(Stewart et al., 2013; Zidovec-Lepej et al., 2021)
Protozoa *Taxoplasma gondii*	Ingestion of contaminated food or oocysts	Usually cause no symptoms, but some infected people show symptoms, such as, Fever, aching muscles, tiredness, sore throat	IFN-γ, IL-12, IL-17 (blood) IL-4, IL-10, TGF-β (placenta)	Miscarriage, stillbirth, vertical transmission, congenital toxoplasmosis (blindness, deafness, intracranial calcifications)	(Abou-Bacar et al., 2004; Zhang et al., 2015)
Plasmodium falciparum Plasmodium vivax	Arthropod vector (*Anopletes* species)	Fever, shaking chills, headache, muscle aches, vomiting, diarrhea	IFN-γ, TNF-α, IL-10	Severe hypoglycemia, Fetus growth restriction, low birth weight, miscarriage, preterm, vertical transmission (rare)	(Artavanis-Tsakonas et al., 2003; Nasr et al., 2014; Romero et al., 2021; Chua et al., 2021) (Nasr et al., 2014; Briand et al., 2016; Cutts et al., 2020; Romero et al., 2021; Lee et al., 2021)
Fungi *Candida albicans Candida parapsilosis*	Normal vaginal flora, but during pregnancy *Candida* can cause infection due to microbial dysbiosis or vaginal hormonal fluctuation	Itching, burning, thick, white vaginal discharge	IL1β, IL8	Low birth weight, fetal candidiasis, premature rapture of membrane	(Maki et al., 2017; Ardizzoni et al., 2021)

Bacteria; Virus; Protozoa; Fungi.

Vaginal infections caused by group B *Streptococcus* (GBS), *Escherichia coli*, *Bacteroides* species, *C. trachomatis*, and *N. gonorrhoeae* can ascend to the genital tract and intraamniotic fluid causing chorioamnionitis (Galinsky et al., 2013; Jain et al., 2022). Infections caused by ascending genito-urinary tract pathogens are typically polymicrobial (Mendz et al., 2013) and often associated with microbial biofilm and antimicrobial cervical mucous plug to reach the intra-amniotic fluid or maternal-fetal interface and induce inflammation locally, which then endangers the fetus due to aberrant inflammation at the fetal membrane (Ayala et al., 2019). There is no clear evidence of how dysbiotic flora crosses the maternal barriers to reach the fetus, but *GBS* and *E. coli* are the most common pathogens found in the placenta and late-onset sepsis in neonates (Wilkie et al., 2019; Glaser et al., 2021). GBS and *E. coli* can both adhere to the fetal membrane *via* various virulence factors and stimulate neutrophils and macrophages to produce inflammatory cytokines and potentially develop extracellular traps to cause premature fetal membrane rupture (Armistead et al., 2020; Coleman et al., 2021; Deshayes de Cambronne et al., 2021).

2.1.3 Sexually Transmitted Infections

Changing the vaginal microenvironment during pregnancy may increase vaginal susceptibility to opportunistic STIs, which are frequently asymptomatic, but can cause severe pregnancy

complications if left untreated. Ascending transmission of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* can lead to pelvic inflammatory disease and endocarditis, as well as serious pregnancy complications like ectopic pregnancy, preterm birth, and low birth weight (Adachi et al., 2016; Heumann et al., 2017). Syphilis is another common STI (caused by *Treponema pallidum*). Although the pathophysiology of *T. pallidum* ascending transmission is unknown, it may be dependent on both the gestational age of the fetus and the maternal stage of infection (Kimball et al., 2020; Primus et al., 2020). Vertical transmission of this bacterium can cause excessive inflammation at the maternal-fetal interface resulting in mild to severe pregnancy complications such as low birth weight, preterm birth, congenital anomalies, and sometimes fetal loss (Primus et al., 2020; Megli and Coyne, 2021).

2.1.4 Maternal Microbiome

The maternal microbiome undergoes significant changes during the course of pregnancy and has been suggested to play an influencing role in the health of pregnant women and their neonates during pregnancy and beyond (Prince et al., 2015; Fettweis et al., 2019). The maternal microbiome consists of distinct microbial communities dominated by different bacterial taxa. For example, a vaginal microbial community dominated with *Lactobacillus* species are suggested to be associated with a healthy pregnancy, whereas the abundance of a complex vaginal microbial community of CST-IV including *Gardnerella*, *Prevotella, Chlamydia* and bacterial vaginosis (BV)-associated bacterium-I (BVAB-I) are associated with increased risk for adverse pregnancy outcomes and fetal infection (Ravel et al., 2011; Kumar et al., 2021a; Saadaoui et al., 2021). The gut and oral microbial communities, like the vaginal microbiome, undergo significant changes during pregnancy, including a significant decrease in alpha diversity and a significant enrichment in Actinobacteria and Proteobacteria species in the gut and oral environment (**Figure 1A**) (Offenbacher et al., 2006; Aagaard et al., 2012).

To ensure healthy pregnancy outcomes, this delicate balance between microbial communities and immune tolerance or immune response must be maintained (**Figure 1B**). Numerous studies have suggested that microbial dysbiosis is linked to a variety of pregnancy complications and fetal development (Seong et al., 2008; Han et al., 2010). For example, abnormal changes in the oral microbiota during pregnancy, such as a decrease in *Lactobacillus species* or an increase in the abundance of *Porphyromonas gingivalis*, may lead to further infections and the production of pro-inflammatory cytokines, which is thought to be a contributory factor to various pregnancy complications such as early labor, pregnancy loss, and low birth weight, among others (Aagaard et al., 2012; Koren et al., 2012; de Weerth et al., 2013; Romero et al., 2014b; DiGiulio et al., 2015; Goltsman et al., 2018). While the link between microbial dysbiosis and pregnancy complications is clear, the exact nature of these interactions is unknown. It is unclear whether dysbiosis impairs the maternal immune system or influences other mechanisms (Zhang et al., 2015; Kumar et al., 2020) to promote pregnancy complications and fetal development. These findings suggest that intra- or extra-uterine infection or vaginal dysbiosis induces an abnormal immune response in pregnant women and may be an important predictor marker for adverse outcomes of congenital infections.

2.2 Viral Infections

The human microbiome has a significant virome component, which includes a diverse collection of endogenous retroviruses, eukaryotic viruses, and bacteriophages (Wylie et al., 2012), and is increasingly recognized as an orchestrator of bacterial diversity and functionality (Mills et al., 2013; Barr, 2017). Although the majority of viruses are harmless, some pathogenic viruses can cross the maternal-fetal interface and influence placental functions, potentially causing fetal disease (**Table 1**).

2.2.1 Cytomegalovirus

Cytomegalovirus (CMV) is a DNA virus that belongs to the Herpesviridae family. CMV is the most common viral infection transmitted vertically *in utero*, causing a wide range of congenital disorders such as hearing and vision loss, intracranial calcifications, microcephaly, organ dysfunction, and intellectual disability (Liu et al., 2021). CMV is typically transmitted from person to person *via* infected bodily fluids such as blood, saliva, urine, and breast milk (Cannon et al., 2011). Once infected, the virus can live in bone marrow hematopoietic cells for the rest of one's life (Collins-McMillen et al., 2018). However, it is a primary infection during pregnancy, rather than a reactivation of a persistent infection, that causes adverse pregnancy outcomes (Boppana et al., 2001; Maidji et al., 2006). Although the exact pathophysiology of CMV is unknown, the severity of the infection and fetal consequences are dependent on gestational age at the time of maternal infection, implying that changes in maternal immune status and the maternal-fetal interface play an important role in CMV vertical transmission. According to new research, CMV may first infect placental pericytes before infecting the fetus (Aronoff et al., 2017). Additionally, CMV infected pregnant women have elevated level of cytokines including TNF-α, IL-1β, IL-10, IL-12, IL-15, IL-17, and CXCL10 which may cause various pregnancy complications or serious health problems to the baby, such as preterm birth or low birth weight, or hearing loss at birth or later in life, depending on the pregnancy (Scott et al., 2012).

2.2.2 Herpes Simplex Virus

Herpes simplex virus (HSV) infections are often asymptomatic or cause mild symptoms in adults; however, the changing maternal immune system from higher inflammatory status at the beginning of pregnancy to a lower level of inflammation in mid-pregnancy may predispose the pregnant women to different viral infections, including HSVs (Straface et al., 2012). Although the mechanism of its transplacental transmission is unknown, vertical transmission *via* direct contact with viral lesions in the genital tract during delivery is a more common route of neonatal infection (James et al., 2014). As a result, maternal HSV infection near the time of delivery increases the risk of vertical transmission, which can result in herpes simplex encephalitis, chorioretinitis, and intracranial calcification in neonates, with a

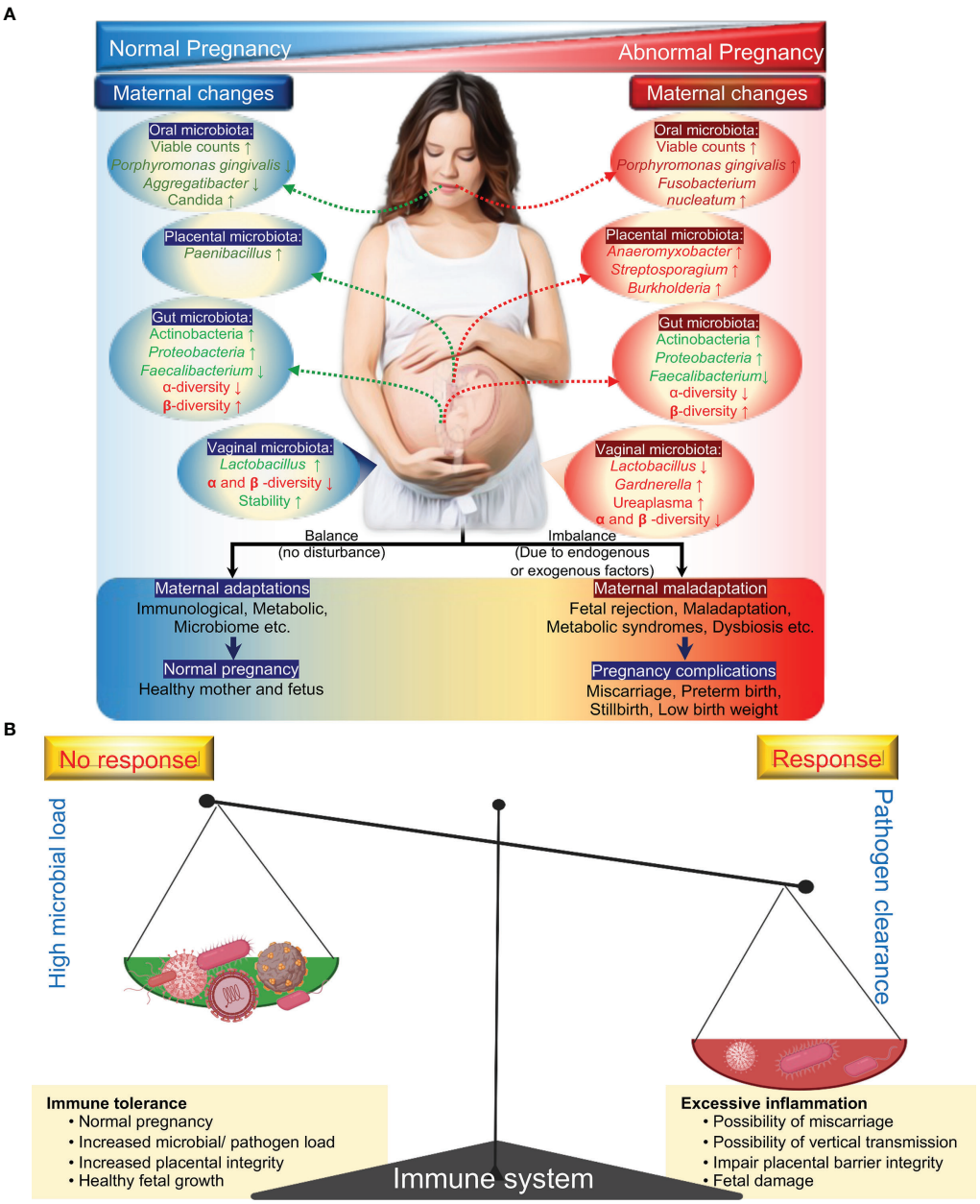


FIGURE 1 | General microbial dynamics during health pregnancy and complicated pregnancy. Known changes in the microbial composition: changes in a specific taxonomy (green) and changes in community diversity (red) **(A)**. Immune response during pregnancy: double-edged sword **(B)**. During pregnancy, the maternal immune system has to balance between sustaining the growth of the fetus and protecting both mother and fetus from pathogens.

50-80% mortality rate in untreated cases (Pinninti and Kimberlin, 2013).

2.2.3 Rubella Virus

Rubella virus is a contagious virus in the Togaviridae family. Rubella virus is primarily transmitted *via* respiratory droplets, and in healthy adults, the infection causes mild illness with a low-grade fever; however, pregnant women who acquire rubella infection are 85 percent more likely to have a miscarriage or stillbirth, and the virus can induce necrosis in the syncytiotrophoblasts allowing it to cross the placental barrier (Lambert et al., 2015; Arora et al., 2017). The neonatal infection can cause severe birth defects with devastating, lifelong consequences such as ocular disorder, auditory problems, cardiovascular defects, speech disorder, and autism (Lambert et al., 2015).

2.2.4 Human Immunodeficiency Virus

Despite the availability of effective anti-HIV therapies, approximately 38 million people are still infected with HIV;

among these 53% are women (Data, 2020). HIV can be transmitted through the placenta, perinatally (from direct contact to maternal vaginal fluids or blood during delivery), or postnatally (from breast milk or other sources) (Milligan and Overbaugh, 2014). As a result, congenital HIV transmission remains the leading cause of neonatal infections and the associated neonatal mortality or life-long devastation. Although it is unknown how HIV crosses the placental barrier, neonates born to HIV-infected women are always at a significantly high risk of vertical transmission (25 percent in the absence of antiretroviral therapy) (Bernstein and Wegman, 2018), which predispose them to serious health consequences, including developing acquired immunodeficiency syndrome (AIDS) and cardiovascular diseases (Maartens et al., 2014). Additionally, HIV infection is often associated with opportunistic infections, further increasing the risk of adverse pregnancy outcomes or vertical transmission (Johnson and Chakraborty, 2016).

2.2.5 Zika Virus

Zika virus (ZIKV) is an emerging arbovirus that is endemic in Africa, America, Asia, and Europe (Khaiboullina et al., 2018). ZIKV is primarily transmitted by the bite of an infected mosquito (Khaiboullina et al., 2018). Though ZIKV infection in adults causes mild symptoms with low-grade fever, headache, rash (Javanian et al., 2018), infection during pregnancy can cross the placenta and increase the risk of adverse pregnancy outcomes and postnatal developmental sequelae, such as miscarriage or stillbirth, or surviving infants show lifelong neurological defects such as enlarged ventricles, collapsing brains, and microcephaly. Emerging studies indicate that ZIKV can selectively infect decidual fibroblasts and macrophages, trophoblasts, hofbauer cells (fetal macrophages), and umbilical cord (Quicke et al., 2016; Tabata et al., 2016) and can significantly induce cytokine levels of IL-6, IL-15, IL-17, IFN-α, CXCL10 and IFN-γ at the maternal-fetal interface and in amniotic fluid, which may result in severe fetal neurological abnormalities (Ornelas et al., 2017; Maucourant et al., 2019). Accumulating evidence shows a link between ZIKV infection and congenital microcephaly (Tang et al., 2016; Gladwyn-Ng et al., 2018). ZIKV infection during gestation can trigger endoplasmic reticulum stress in the embryonic brain, which may perturb physiological unfolded protein response in the cerebral cortex and lead to microcephaly in the babies born from mothers infected with ZIKV (Mlakar et al., 2016; Gladwyn-Ng et al., 2018).

2.2.6 COVID-19

The most recent COVID-19 pandemic, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), infected over 308 million subjects, and killed 5.5 million people worldwide, highlighting the importance of focusing on women's health. SARS-CoV2 is primarily spread through close contact with an infected person, as well as through aerosols and respiratory droplets (Saadaoui et al., 2021) and can severely impact a variety of physiological and immunological processes, including pregnancy health and outcomes (Kumar and Al Khodor, 2020; Saadaoui et al., 2021). SARS-CoV-2 binds to host cells through the angiotensin-converting enzyme 2 (ACE2) receptor (Yan et al., 2020), which is expressed on the surface of various trophoblasts including, cytotrophoblast and syncytiotrophoblast cells at the maternal-fetal interface (Gengler et al., 2021). Although the virion genome has been observed in placental and vaginal samples (Dong et al., 2020), but the majority of recent reports show no evidence of vertical transmission (Saadaoui et al., 2021), suggesting that SARS-CoV2 cannot cross the placental barriers even in severely infected women. Despite the magnitude of the pandemic, pregnant women do not appear to vertically transfer the SARS-CoV2 to the fetus, but the inflammatory storm during SARS-CoV2 infection might indirectly induce pregnancy complications and even fetal developmental obstacles. For example, increasing levels of inflammatory cytokines during infection, such as IL-1, IL-2, IL-7, IL-10, and TNF-α in the maternal blood, at the maternal-fetal interface may lead to adverse pregnancy complications, including maternal mortality, preeclampsia, and preterm birth (Villar et al., 2021).

2.3 Parasites

Despite the fact that emerging knowledge and practices on prevention of mosquito-borne diseases have significantly reduced parasitic infections worldwide (Nguyen-Tien et al., 2021), some parasitic infections are still common during pregnancy due to the living conditions (Brummaier et al., 2019) or decreased host immunity. Due to reduced maternal immunity during pregnancy, parasitic infections are common among pregnant women living in low resource settings (Brummaier et al., 2019) and therefore can influence maternal and fetal health (**Table 1**).

2.3.1 Toxoplasmosis

Toxoplasmosis is caused by *Toxoplasma gondii* resulting in more than 200,000 cases of congenital toxoplasmosis worldwide each year (Bigna et al., 2020). *T. gondii* can be vertically transmitted during pregnancy to cause toxoplasmosis and can lead to a high risk of congenital diseases (Bigna et al., 2020). Although, vertical transmission of toxoplasmosis can occur only in 30-40% of patients, but *T. gondii* infection during pregnancy could lead to an aberrant immune response in blood to control the infection (Sasai and Yamamoto, 2019). Immune response toward the *T. gondii* infected cells leads to aberrant production of IFN-γ, IL-12, IL-17 which can result in miscarriage and stillbirth (Smith et al., 2021).

2.3.2 Malaria

Malaria parasites, mainly *Plasmodium falciparum* and *Plasmodium vivax*, are other pathogens associated with an elevated risk of pregnancy complications, including fetal growth restriction and preterm birth (Briand et al., 2016; Romero et al., 2021). Malaria parasite-infected erythrocytes during pregnancy can adhere to placental receptors and trigger placental inflammation and subsequent damage, causing harm to both mother and her infant (Chua et al., 2021). Emerging evidence suggests that malaria parasite-infected women have significantly higher systemic levels of pro-inflammatory cytokines and

chemokines, including TNF-α, IFN-γ, IL-10, which appear to be a key mediators of pregnancy complications (Nasr et al., 2014). IFN-γ response during pregnancy is a double-edged sword. It plays both protective and pathological roles during malaria infection (Nasr et al., 2014). IFN-γ response in malaria parasite-infected women is crucial for parasite clearance in both the liver and blood stages (Inoue et al., 2013), however high levels of IFN-γ may also exacerbate the disease severity, including cerebral malaria and other pregnancy complications such as embryotoxicity or abnormal placenta as shown in **Figure 3** (King and Lamb, 2015).

2.4 Fungal Infections

The vast majority of fungi are harmless, and serious fungal infections are uncommon during pregnancy; however, they may occur with higher frequency in pregnant women, which potentially can increase maternal complications, including prematurity or, in some cases, even fetal loss (Rasti et al., 2014).

2.4.1 Candidiasis

Candidiasis is the most common cause of infection worldwide and is caused by Candida, an opportunistic yeast (Manolakaki et al., 2010). Under normal conditions, most *Candida species* are commensals or endosymbionts, but some species, such as *Candida albicans* and *Candida parapsilosis*, can cause candidiasis (AN and Rafiq, 2021). Vaginal candidiasis is the most common gynecological infection during reproductive age and pregnancy. According to emerging studies, up to 40% of women have vaginal colonization with *Candida* spp. during pregnancy (DiGiulio, 2012), which can easily transmit to the maternal-fetal barrier and progress to intra-amniotic infection which may lead to severe pregnancy complications including low birth weight or fetal candidiasis (Siriratsivawong et al., 2014; Drummond and Lionakis, 2018).

3 PREGNANCY COMPLICATIONS ASSOCIATED WITH MATERNAL INFECTIONS

Although complications caused by maternal infections or extrinsic abnormalities can occur at any stage of pregnancy, the first trimester is critical for placental development and the formation of a selective barrier between maternal and fetal tissue (Burton et al., 2016). The placental barrier, which is made up of multiple layers of maternal and fetal tissues, serves as a strong barrier against human pathogens reaching the fetus (Burton et al., 2016). Syncytiotrophoblasts (SYNs) are multinucleated cells that form a strong barrier between maternal and fetal blood within the placenta (Ander et al., 2019). Despite the fact that SYNs are highly resistant to bacterial or viral infections and produce type III IFNs (Ander et al., 2019), some pathogens can still cross these barriers and reach the fetus (**Figure 2**). Although the mechanism(s) by which pathogens breach the strong barriers remains unknown, intrauterine infection and associated inflammation are significant contributors to pregnancy complications. Surprisingly, approximately 25% of preterm births are microbially induced, either through intrauterine infection or maternal extrauterine infection (Agrawal and Hirsch, 2012).

4 PREGNANCY COMPLICATION AS A RESULT OF ABERRANT IMMUNE RESPONSE

According to the findings of recent pathological and advanced metagenomic studies, which have been supplemented by cellular and experimental animal studies, a significant amount of pathogens can bypass the placental barrier integrity and modulate an abnormal immune response at the maternal-fetal interface or in the amniotic fluid (Megli and Coyne, 2021). Microbial pathogens commonly associated with periodontal disease or found in the lower genital tract can cross the placental barrier and react to amniotic fluid in women who had preterm labor, possibly *via* hematogenous dissemination *via* the transplacental passage or ascending microbial invasion into the amniotic fluid (chorioamnionitis) from the urinary tract (Cobb et al., 2017). Normally, microbial-induced pregnancy complications are mediated by an aberrant inflammatory process. Many studies have revealed an elevated level of proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α in cervicovaginal lavage or amniotic fluid of women experiencing pregnancy complications (Agrawal and Hirsch, 2012; Romero et al., 2014a; Gee et al., 2021). Interestingly, emerging evidence suggests that microbial infection or injection of microbial products such as PAMPs or recombinant inflammatory cytokines in pregnancy mice could lead to adverse pregnancy complications, including preterm birth or even fetal demise (Romero et al., 2014a). Microorganisms or their ligands such as LPS, CpG, Poly (I:C) are recognized by toll-like receptors (TLRs), to induce the production of chemokines (e.g., IL-8, and C-C motif legend 2 (CCL2), cytokines (e.g., IL-1β, and TNF-α), which act on the prostaglandins and proteases to induce the common pathway of parturition (**Figure 3**, **Table 2**) (Racicot et al., 2016; Yockey et al., 2018). Indeed, murine models revealed that microbial ligands or recombinant cytokines are likely to elicit miscarriage and preterm labor (Gonzalez et al., 2011), and can be used as a predictive biomarker of the onset of preterm labor (Romero et al., 2014a), emphasizing the role of microbial induced inflammation in pregnancy complications. These studies, when taken together, highlighted the role of microbial-induced inflammation in pregnancy complications and congenital disease.

5 FUTURE DIRECTIONS

Although technological advances over the past decade have made significant advances on multiple fronts, including a better understanding of molecular mechanisms, more precise diagnostics, and significantly improved therapeutic outcomes, the increasing incidences of pregnancy-related complications continue to pose

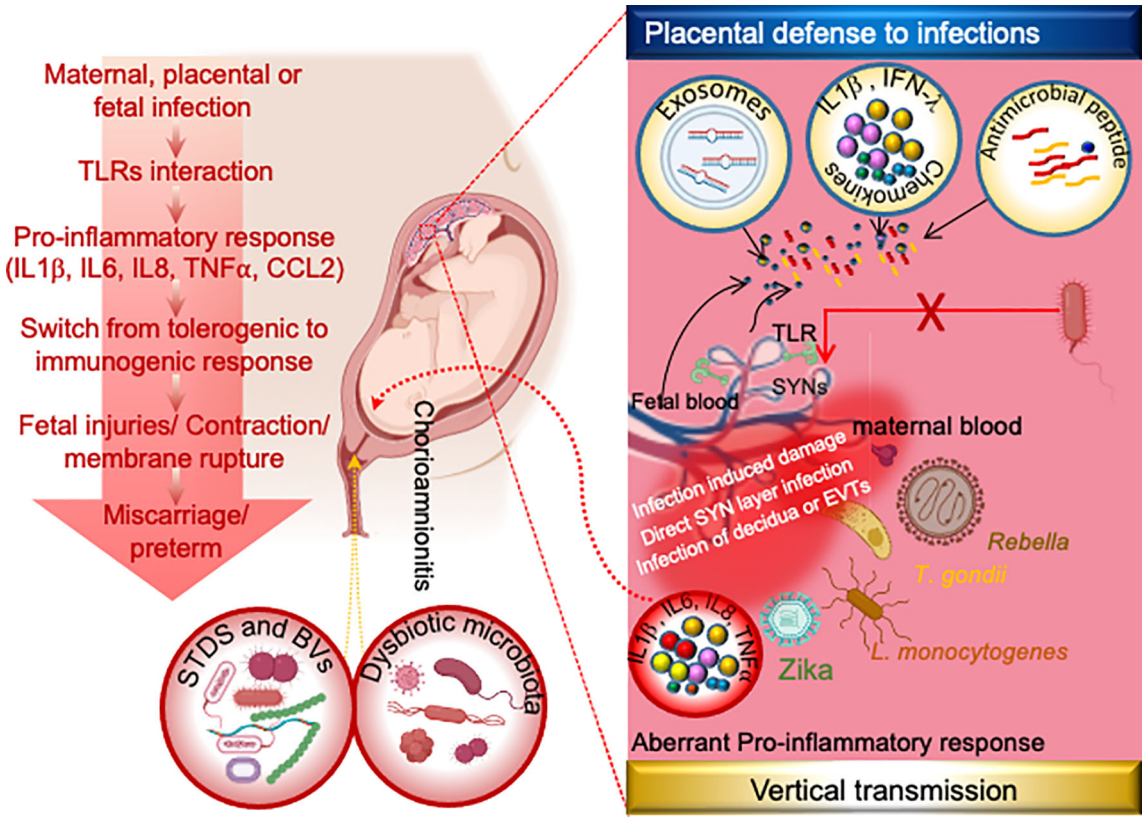


FIGURE 2 | Mechanism of placental physical and immune defense and possible mechanism of vertical transmission of TORCH and other pathogens during pregnancy. The human placenta has evolved several layers of defense including antimicrobial effectors such as exosomes, antimicrobial peptides, and/or innate immune response to infection by release of cytokines and/or highly integrated syncytiotrophoblast. Syncytiotrophoblast is the placental barrier between maternal and fetal blood that allows selective exchanges in nutrients and gases between the embryo and the mother but inhibits the microbial invasion. Although the exact mechanisms by which TORCH pathogens cross the placental barrier are still unclear. However emerging studies indicates these pathogens reach the fetus through infected maternal decidua, infected extracellular trophoblasts (EVTs) and/or through direct infection of the syncytium, while the vaginal pathogens gain access to the amniotic cavity *via* ascending transmission. Following the amniotic cavity infection, toll-like receptors (TLRs) at the fetal-maternal interface get activated and induce pro-inflammatory cytokines and chemokines, leading to further immune cells recruitment. Switching of maternal immune response from tolerogenic to inflammatory state leads to the premature activation of cervical ripening proteins and onset of labor. *Common STDs and BVs infections are C. trachomatis, N. gonorrhoeae, T. pallidum, GBS, E. coli etc. Dysbiotic microbiota infections are GBS, E. coli, C. trachomatis, Gardnerella, Prevotella etc. EVTs: Extravillous trophoblasts, GBS: Group B Streptococcus, SYNs:* syncytiotrophoblast.

daunting challenges in understanding their underlying pathogenesis, host-pathogen interaction at the maternal-fetal interface. As the incidence of maternal infections and associated pregnancy complications rises, a better understanding of the developmental events that result in host-pathogen interaction at the maternal-fetal interface and aberrant immune response is critical for the development of rational intervention strategies. With the help of advanced molecular techniques, the TORCH pathogens and their ability to cross the maternal-fetal barrier to cause congenital fetus disease, which was first proposed decades ago, have now been expanded to include emerging maternal infections and the effects of microbial dysbiosis.

Despite the progress made, there are still many unanswered and widely debated questions. For example, how the placental barrier remains uncompromised to multiple microbial pathogens that cause maternal systemic illness and bacteremia, such as methicillin-resistant *Staphylococcus aureus*, *E. coli*, *SARS-CoV2* virus, while other pathogens have mastered a variety of evasion mechanisms leading to serious maternal and fetal complications? Another controversial question is the whether the placenta harbors its own microbiome or not? (Aagaard et al., 2014), and how/when does the priming of the fetal immune system with the maternal microbiome occur? (Wampach et al., 2018; de Goffau et al., 2019). The intriguing question now is, what levels of proinflammatory cytokines are required systematically or locally at the maternal-fetal interface to modulate placental integrity and allow vertical transmission of pathogens? Finally, how does maternal dysbiotic microbiota influence the maternal-fetal interface or immune response to cause pregnancy complications? While emerging multi-omics have provided us with comprehensive information about the maternal microbiome (Jehan et al., 2020; Kumar et al., 2021a),

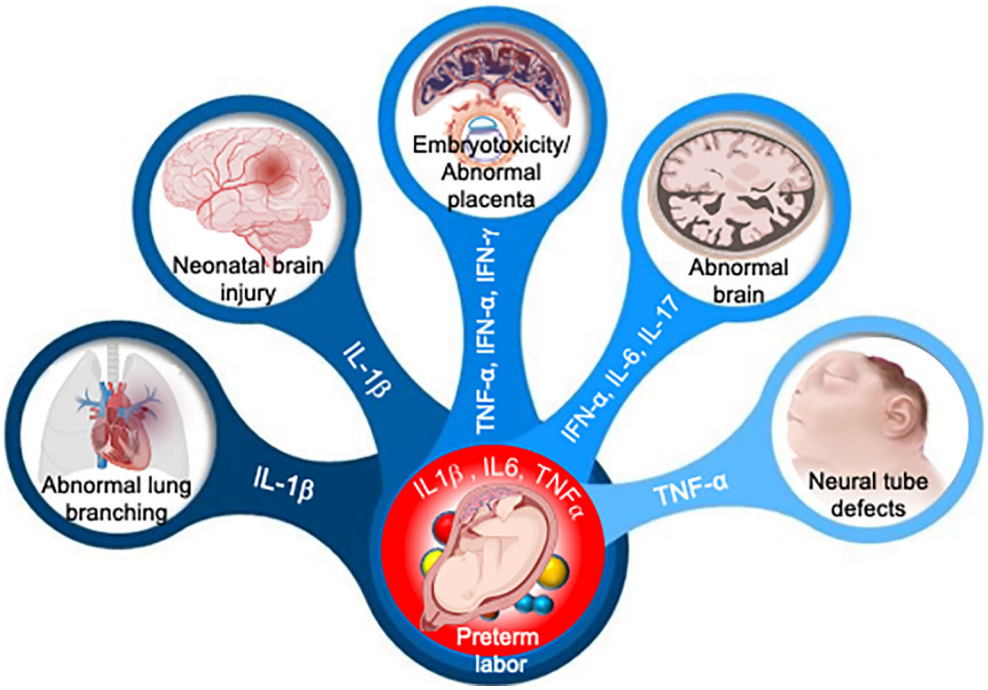


FIGURE 3 | Adverse pregnancy outcomes induced by aberrant cytokine response. Aberrant levels of IL-1β, IL-6, IL-17, TNF-α, IFN-α and IFN-γ in amniotic fluid can induce multiple organ development failure in fetus or induce premature activation of cervical ripening proteins and onset of preterm labor.

TABLE 2 | Roles of cytokines in human pregnancy complications.

Cytokines	Pathological roles in pregnancy	References
IFN-α	• Secreted as part of the immune response to modulate associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) • Contributes to the establishment and maintenance of successful pregnancy, mediating endometrial vascular remodeling and angiogenesis at the maternal-fetal interface • Overexpression correlates with viral infection or influence the placental development after ZIKV infection • Overexpression toxic to early embryo development	(Chesler and Reiss, 2002; Mogensen, 2009; Murphy et al., 2009; Yockey and Iwasaki, 2018; Ni and Lu, 2018)
IFN-γ	• Initiates endometrial vasculature remodeling and contributes to the normal health of the decidua • Secreted in the uterus during early pregnancy. • Overexpression prevents implementation and are toxic to the embryo • Induce the placental damage after Malaria or Toxoplasma infection	(Murphy et al., 2009)
IL-1β	• Sufficient to induce smooth muscle contraction in the uterus and preterm labor • Induces abnormal lung and neurological development	(Sadowsky et al., 2006; Hogmalm et al., 2014)
IL-2	• Overexpression modulates the pregnancy complication such as preeclampsia	(Hamai et al., 1997)
IL-6	• Mediates embryo implantation and placental development • Overexpression can mediate abnormal brain development	(Prins et al., 2012)
IL-10	• Plays a pivotal role in the maternal immune tolerance for survival of an allogeneic fetus.	(Murphy et al., 2005)
IL-15	• Convert decidual NK cells and macrophages to decidual phenotypes, including reduced cytotoxicity and secretion of angiogenic factors	(Ashkar et al., 2003; Chavan et al., 2016)
IL-17	• Modulates the production of other pro-inflammatory cytokines • Overexpression can mediate abnormal brain development	(Choi et al., 2016)
TNF-α	• Key cytokine to modulate responses against infection • TNF-α concentrations increase as gestation progresses albeit not excessively and may support the increased metabolic needs associated with pregnancy. • Important regulator of normal cell function, influencing vital biological processes including cell proliferation, apoptosis, and the production of other cytokines such as IL-6 • Overexpression can induce preterm labor and neural tube defects • Overexpression also toxic to early embryo development	(Baud and Karin, 2001; Waters et al., 2013; Spence et al., 2021)

their translational impact on women's health is still far from being achieved and requires more research.

Future research into the mechanism of host-pathogen interaction at the maternal-fetal interface, as well as how these interactions modulate immune responses and placental integrity, will have broader implications in understanding the mechanism of adverse pregnancy complications, such as miscarriage, preterm birth, and vertical transmission of pathogens. Additionally, it may lead to future therapeutic strategies to improve maternal health and prevent vertical transmission of pathogens. Advanced, cutting-edge statistical models, as well as high-throughput molecular multi-omics techniques, can be used to integrate various datasets for assessing their role in biological processes (Kumar et al., 2021b). It should be noted that numerous specific microbial therapies, such as bacteriophage or narrow-spectrum therapies that kill the specific pathogen without affecting other health microbes, are being developed and proving to be effective (Kumar et al., 2018; Brives and Pourraz, 2020). Studies are currently being conducted to determine whether these strategies will be effective for TORCH (Rodriguez-Melcon et al., 2018). Next-generation mRNA vaccines to control different maternal infections are being actively explored (Healy et al., 2019; Kumar and Al Khodor, 2021). These efforts can ultimately facilitate the design of targeted strategies to engineer the vaginal microbiota to lead to antibiotic-sparing strategies to modulate and restore a robust vaginal micro-environment, which may ultimately improve the reproductive health of women and their children.

AUTHOR CONTRIBUTIONS

MK, MS and SK Conceptualization, MK and SA. Writing—original draft preparation, MK and SA. Writing—review and editing, MK, MS and SA. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by Sidra Medicine, Qatar, grant number SDR400161, and the APC was funded by Research Department, Sidra Medicine, Qatar.

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The Placenta Harbors a Unique Microbiome. *Sci. Transl. Med.* 6 (237), 237ra65. doi: 10.1126/scitranslmed.3008599

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T. A., Coarfa, C., et al. (2012). A Metagenomic Approach to Characterization of the Vaginal Microbiome Signature in Pregnancy. *PloS One* 7 (6), e36466. doi: 10.1371/journal.pone.0036466

Abou-Bacar, A., Pfaff, A. W., Letscher-Bru, V., Filisetti, D., Rajapakse, R., Antoni, E., et al. (2004). Role of Gamma Interferon and T Cells in Congenital Toxoplasma Transmission. *Parasit. Immunol.* 26 (8-9), 315–318. doi: 10.1111/j.0141-9838.2004.00713.x

Adachi, K., Nielsen-Saines, K., and Klausner, J. D. (2016). Chlamydia Trachomatis Infection in Pregnancy: The Global Challenge of Preventing Adverse Pregnancy and Infant Outcomes in Sub-Saharan Africa and Asia. *BioMed. Res. Int.* 2016, 9315757. doi: 10.1155/2016/9315757

Adams Waldorf, K. M., and McAdams, R. M. (2013). Influence of Infection During Pregnancy on Fetal Development. *Reproduction* 146 (5), R151–R162. doi: 10.1530/REP-13-0232

Agrawal, V., and Hirsch, E. (2012). Intrauterine Infection and Preterm Labor. *Semin. Fetal Neonatal. Med.* 17 (1), 12–19. doi: 10.1016/j.siny.2011.09.001

Alfaraj, S. H., Al-Tawfiq, J. A., and Memish, Z. A. (2019). Middle East Respiratory Syndrome Coronavirus (MERS-CoV) Infection During Pregnancy: Report of Two Cases & Review of the Literature. *J. Microbiol. Immunol. Infect.* 52 (3), 501–503. doi: 10.1016/j.jmii.2018.04.005

Ander, S. E., Diamond, M. S., and Coyne, C. B. (2019). Immune Responses at the Maternal-Fetal Interface. *Sci. Immunol.* 4 (31): eaat6114. doi: 10.1126/sciimmunol.aat6114

AN, R., and Rafiq, N. B. (2021). *StatPearls*. (Treasure Island, FL: StatPearls Publishing).

Ardizzoni, A., Wheeler, R. T., and Pericolini, E. (2021). It Takes Two to Tango: How a Dysregulation of the Innate Immunity, Coupled With Candida Virulence, Triggers VVC Onset. *Front. Microbiol.* 12 (1449). doi: 10.3389/fmicb.2021.692491

Armistead, B., Herrero-Foncubierta, P., Coleman, M., Quach, P., Whidbey, C., Justicia, J., et al. (2020). Lipid Analogs Reveal Features Critical for Hemolysis and Diminish Granadaene Mediated Group B Streptococcus Infection. *Nat. Commun.* 11 (1), 1502. doi: 10.1038/s41467-020-15282-0

Aronoff, D. M., Correa, H., Rogers, L. M., Arav-Boger, R., and Alcendor, D. J. (2017). Placental Pericytes and Cytomegalovirus Infectivity: Implications for HCMV Placental Pathology and Congenital Disease. *Am. J. Reprod. Immunol.* 78 (3)10.1111/aji.12728. doi: 10.1111/aji.12728

Arora, N., Sadovsky, Y., Dermody, T. S., and Coyne, C. B. (2017). Microbial Vertical Transmission During Human Pregnancy. *Cell Host Microbe* 21 (5), 561–567. doi: 10.1016/j.chom.2017.04.007

Artavanis-Tsakonas, K., Tongren, J. E., and Riley, E. M. (2003). The War Between the Malaria Parasite and the Immune System: Immunity, Immunoregulation and Immunopathology. *Clin. Exp. Immunol.* 133 (2), 145–152. doi: 10.1046/j.1365-2249.2003.02174.x

Ashkar, A. A., Black, G. P., Wei, Q., He, H., Liang, L., Head, J. R., et al. (2003). Assessment of Requirements for IL-15 and IFN Regulatory Factors in Uterine NK Cell Differentiation and Function During Pregnancy. *J. Immunol.* 171 (6), 2937–2944. doi: 10.4049/jimmunol.171.6.2937

Ayala, O. D., Doster, R. S., Manning, S. D., O'Brien, C. M., Aronoff, D. M., Gaddy, J. A., et al. (2019). Raman Microspectroscopy Differentiates Perinatal Pathogens on Ex Vivo Infected Human Fetal Membrane Tissues. *J. Biophotonics.* 12 (9), e201800449. doi: 10.1002/jbio.201800449

Bakardjiev, A. I., Theriot, J. A., and Portnoy, D. A. (2006). Listeria Monocytogenes Traffics From Maternal Organs to the Placenta and Back. *PloS Pathog.* 2 (6), e66. doi: 10.1371/journal.ppat.0020066

Barr, J. J. (2017). A Bacteriophages Journey Through the Human Body. *Immunol. Rev.* 279 (1), 106–122. doi: 10.1111/imr.12565

Baud, V., and Karin, M. (2001). Signal Transduction by Tumor Necrosis Factor and its Relatives. *Trends Cell Biol.* 11 (9), 372–377. doi: 10.1016/S0962-8924(01)02064-5

Bernstein, H. B., and Wegman, A. D. (2018). HIV Infection: Antepartum Treatment and Management. *Clin. Obstet. Gynecol.* 61 (1), 122–136. doi: 10.1097/GRF.0000000000000330

Bigna, J. J., Tochie, J. N., Tounouga, D. N., Bekolo, A. O., Ymele, N. S., Youda, E. L., et al. (2020). Global, Regional, and Country Seroprevalence of Toxoplasma Gondii in Pregnant Women: A Systematic Review, Modelling and Meta-Analysis. *Sci. Rep.* 10 (1), 12102. doi: 10.1038/s41598-020-69078-9

Boppana, S. B., Rivera, L. B., Fowler, K. B., Mach, M., and Britt, W. J. (2001). Intrauterine Transmission of Cytomegalovirus to Infants of Women With Preconceptional Immunity. *N. Engl. J. Med.* 344 (18), 1366–1371. doi: 10.1056/NEJM200105033441804

Bosilkovski, M., Arapovic, J., and Keramat, F. (2020). Human Brucellosis in Pregnancy - An Overview. *Bosn. J. Basic Med. Sci.* 20 (4), 415–422. doi: 10.17305/bjbms.2019.4499

Briand, V., Saal, J., Ghafari, C., Huynh, B.-T., Fievet, N., Schmiegelow, C., et al. (2016). Fetal Growth Restriction Is Associated With Malaria in Pregnancy: A Prospective Longitudinal Study in Benin. *J. Infect. Dis.* 214 (3), 417–425. doi: 10.1093/infdis/jiw158

Brives, C., and Pourraz, J. (2020). Phage Therapy as a Potential Solution in the Fight Against AMR: Obstacles and Possible Futures. *Palgrave Commun.* 6 (1), 100. doi: 10.1057/s41599-020-0478-4

Brummaier, T., Syed Ahamed Kabeer, B., Lindow, S., Konje, J. C., Pukrittayaamee, S., Utzinger, J., et al. (2019). A Prospective Cohort for the Investigation of Alteration in Temporal Transcriptional and Microbiome Trajectories Preceding Preterm Birth: A Study Protocol. *BMJ Open* 9 (1), e023417. doi: 10.1136/bmjopen-2018-023417

Brunham, R. C., and Rey-Ladino, J. (2005). Immunology of Chlamydia Infection: Implications for a Chlamydia Trachomatis Vaccine. *Nat. Rev. Immunol.* 5 (2), 149–161. doi: 10.1038/nri1551

Burton, G. J., Fowden, A. L., and Thornburg, K. L. (2016). Placental Origins of Chronic Disease. *Physiol. Rev.* 96 (4), 1509–1565. doi: 10.1152/physrev.00029.2015

Cannon, M. J., Hyde, T. B., and Schmid, D. S. (2011). Review of Cytomegalovirus Shedding in Bodily Fluids and Relevance to Congenital Cytomegalovirus Infection. *Rev. Med. Virol.* 21 (4), 240–255. doi: 10.1002/rmv.695

Capoccia, R., Greub, G., and Baud, D. (2013). Ureaplasma Urealyticum, Mycoplasma Hominis and Adverse Pregnancy Outcomes. *Curr. Opin. Infect. Dis.* 26 (3), 231–240. doi: 10.1097/QCO.0b013e328360db58

Cauci, S., and Culhane, J. F. (2007). Modulation of Vaginal Immune Response Among Pregnant Women With Bacterial Vaginosis by Trichomonas Vaginalis, Chlamydia Trachomatis, Neisseria Gonorrhoeae, and Yeast. *Am. J. Obstet. Gynecol.* 196 (2), 133.e1–133.e7. doi: 10.1016/j.ajog.2006.08.033

Cerqueira, L. R. P., Monteiro, D. L. M., Taquette, S. R., Rodrigues, N. C. P., Trajano, A. J. B., Souza, F. M., et al. (2017). The Magnitude of Syphilis: From Prevalence to Vertical Transmission. *Rev. Inst. Med. Trop. Sao Paulo* 59, e78. doi: 10.1590/s1678-9946201759078

Charlier, C., Disson, O., and Lecuit, M. (2020). Maternal-Neonatal Listeriosis. *Virulence* 11 (1), 391–397. doi: 10.1080/21505594.2020.1759287

Chavan, A. R., Bhullar, B. A., and Wagner, G. P. (2016). What was the Ancestral Function of Decidual Stromal Cells? A Model for the Evolution of Eutherian Pregnancy. *Placenta* 40, 40–51. doi: 10.1016/j.placenta.2016.02.012

Chesler, D. A., and Reiss, C. S. (2002). The Role of IFN-Gamma in Immune Responses to Viral Infections of the Central Nervous System. *Cytokine Growth Factor Rev.* 13 (6), 441–454. doi: 10.1016/S1359-6101(02)00044-8

Choi, G. B., Yim, Y. S., Wong, H., Kim, S., Kim, H., Kim, S. V., et al. (2016). The Maternal Interleukin-17a Pathway in Mice Promotes Autism-Like Phenotypes in Offspring. *Science* 351 (6276), 933–939. doi: 10.1126/science.aad0314

Chua, C. L. L., Khoo, S. K. M., Ong, J. L. E., Ramireddi, G. K., Yeo, T. W., and Teo, A. (2021). Malaria in Pregnancy: From Placental Infection to Its Abnormal Development and Damage. *Front. Microbiol.* 12, 777343. doi: 10.3389/fmicb.2021.777343

Chudnovets, A., Liu, J., Narasimhan, H., Liu, Y., and Burd, I. (2020). Role of Inflammation in Virus Pathogenesis During Pregnancy. *J. Virol.* 95 (2), e01381–19. doi: 10.1128/JVI.01381-19

Cobb, C. M., Kelly, P. J., Williams, K. B., Babbar, S., Angolkar, M., and Derman, R. J. (2017). The Oral Microbiome and Adverse Pregnancy Outcomes. *Int. J. Womens Health* 9, 551–559. doi: 10.2147/IJWH.S142730

Coleman, M., Armistead, B., Orvis, A., Quach, P., Brokaw, A., Gendrin, C., et al. (2021). Hyaluronidase Impairs Neutrophil Function and Promotes Group B Streptococcus Invasion and Preterm Labor in Nonhuman Primates. *mBio* 12 (1): e03115–20. doi: 10.1128/mBio.03115-20

Collins-McMillen, D., Peppenelli, M., and Goodrum, F. (2018). Molecular Determinants and the Regulation of Human Cytomegalovirus Latency and Reactivation. *Viruses* 10 (8), 444. doi: 10.3390/v10080444

Coyne, C. B., and Lazear, H. M. (2016). Zika Virus - Reigniting the TORCH. *Nat. Rev. Microbiol.* 14 (11), 707–715. doi: 10.1038/nrmicro.2016.125

Cutts, J. C., Agius, P. A., Zaw, L., Powell, R., Moore, K., Draper, B., et al. (2020). Pregnancy-Specific Malarial Immunity and Risk of Malaria in Pregnancy and Adverse Birth Outcomes: A Systematic Review. *BMC Med.* 18 (1), 14. doi: 10.1186/s12916-019-1467-6

Data, U. (2020). *UNAIDS*. Available at: https://www.unaids.org/en/resources/fact-sheet

de Goffau, M. C., Lager, S., Sovio, U., Gaccioli, F., Cook, E., Peacock, S. J., et al. (2019). Human Placenta has No Microbiome But can Contain Potential Pathogens. *Nature* 572 (7769), 329–334. doi: 10.1038/s41586-019-1451-5

Deshayes de Cambronne, R., Fouet, A., Picart, A., Bourrel, A. S., Anjou, C., Bouvier, G., et al. (2021). CC17 Group B Streptococcus Exploits Integrins for Neonatal Meningitis Development. *J. Clin. Invest* 131 (5), e136737. doi: 10.1172/JCI136737

de Weerth, C., Fuentes, S., and de Vos, W. M. (2013). Crying in Infants: On the Possible Role of Intestinal Microbiota in the Development of Colic. *Gut Microbes* 4 (5), 416–421. doi: 10.4161/gmic.26041

DiGiulio, D. B. (2012). Diversity of Microbes in Amniotic Fluid. *Semin. Fetal Neonatal. Med.* 17 (1), 2–11. doi: 10.1016/j.siny.2011.10.001

DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., et al. (2015). Temporal and Spatial Variation of the Human Microbiota During Pregnancy. *Proc. Natl. Acad. Sci. U. S. A.* 112 (35), 11060–11065. doi: 10.1073/pnas.1502875112

Disson, O., Grayo, S., Huillet, E., Nikitas, G., Langa-Vives, F., Dussurget, O., et al. (2008). Conjugated Action of Two Species-Specific Invasion Proteins for Fetoplacental Listeriosis. *Nature* 455 (7216), 1114–1118. doi: 10.1038/nature07303

Dong, L., Tian, J., He, S., Zhu, C., Wang, J., Liu, C., et al. (2020). Possible Vertical Transmission of SARS-CoV-2 From an Infected Mother to Her Newborn. *JAMA* 323 (18), 1846–1848. doi: 10.1001/jama.2020.4621

Drummond, R. A., and Lionakis, M. S. (2018). Candidiasis of the Central Nervous System in Neonates and Children With Primary Immunodeficiencies. *Curr. Fungal Infect. Rep.* 12 (2), 92–97. doi: 10.1007/s12281-018-0316-y

Erlebacher, A. (2013). Immunology of the Maternal-Fetal Interface. *Annu. Rev. Immunol.* 31, 387–411. doi: 10.1146/annurev-immunol-032712-100003

Faralla, C., Bastounis, E. E., Ortega, F. E., Light, S. H., Rizzuto, G., Gao, L., et al. (2018). Listeria Monocytogenes InlP Interacts With Afadin and Facilitates Basement Membrane Crossing. *PloS Pathog.* 14 (5), e1007094. doi: 10.1371/journal.ppat.1007094

Fernandez, A. G., Ferrero, M. C., Hielpos, M. S., Fossati, C. A., and Baldi, P. C. (2016). Proinflammatory Response of Human Trophoblastic Cells to Brucella Abortus Infection and Upon Interactions With Infected Phagocytes. *Biol. Reprod.* 94 (2), 48. doi: 10.1095/biolreprod.115.131706

Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., et al. (2019). The Vaginal Microbiome and Preterm Birth. *Nat. Med.* 25 (6), 1012–1021. doi: 10.1038/s41591-019-0450-2

Flaherty, R. A., Borges, E. C., Sutton, J. A., Aronoff, D. M., Gaddy, J. A., Petroff, M. G., et al. (2019). Genetically Distinct Group B Streptococcus Strains Induce Varying Macrophage Cytokine Responses. *PloS One* 14 (9), e0222910. doi: 10.1371/journal.pone.0222910

Galinsky, R., Polglase, G. R., Hooper, S. B., Black, M. J., and Moss, T. J. (2013). The Consequences of Chorioamnionitis: Preterm Birth and Effects on Development. *J. Pregnancy* 2013, 412831. doi: 10.1155/2013/412831

Gee, S., Chandiramani, M., Seow, J., Pollock, E., Modestini, C., Das, A., et al. (2021). The Legacy of Maternal SARS-CoV-2 Infection on the Immunology of the Neonate. *Nat. Immunol.* 22 (12), 1490–1502. doi: 10.1038/s41590-021-01049-2

Gengler, C., Dubruc, E., Favre, G., Greub, G., de Leval, L., and Baud, D. (2021). SARS-CoV-2 ACE-Receptor Detection in the Placenta Throughout Pregnancy. *Clin. Microbiol. Infect.* 27 (3), 489–490. doi: 10.1016/j.cmi.2020.09.049

Gladwyn-Ng, I., Cordon-Barris, L., Alfano, C., Creppe, C., Couderc, T., Morelli, G., et al. (2018). Stress-Induced Unfolded Protein Response Contributes to Zika Virus-Associated Microcephaly. *Nat. Neurosci.* 21 (1), 63–71. doi: 10.1038/s41593-017-0038-4

Glaser, M. A., Hughes, L. M., Jnah, A., and Newberry, D. (2021). Neonatal Sepsis: A Review of Pathophysiology and Current Management Strategies. *Adv. Neonatal. Care* 21 (1), 49–60. doi: 10.1097/ANC.0000000000000769

Goltsman, D. S. A., Sun, C. L., Proctor, D. M., DiGiulio, D. B., Robaczewska, A., Thomas, B. C., et al. (2018). Metagenomic Analysis With Strain-Level Resolution Reveals Fine-Scale Variation in the Human Pregnancy Microbiome. *Genome Res.* 28 (10), 1467–1480. doi: 10.1101/gr.236000.118

Gonzalez, J. M., Franzke, C. W., Yang, F., Romero, R., and Girardi, G. (2011). Complement Activation Triggers Metalloproteinases Release Inducing Cervical Remodeling and Preterm Birth in Mice. *Am. J. Pathol.* 179 (2), 838–849. doi: 10.1016/j.ajpath.2011.04.024

Hamai, Y., Fujii, T., Yamashita, T., Nishina, H., Kozuma, S., Mikami, Y., et al. (1997). Evidence for an Elevation in Serum Interleukin-2 and Tumor Necrosis Factor-Alpha Levels Before the Clinical Manifestations of Preeclampsia. *Am. J. Reprod. Immunol.* 38 (2), 89–93. doi: 10.1111/j.1600-0897.1997.tb00281.x

Han, Y. W., Fardini, Y., Chen, C., Iacampo, K. G., Peraino, V. A., Shamonki, J. M., et al. (2010). Term Stillbirth Caused by Oral Fusobacterium Nucleatum. *Obstet. Gynecol.* 115 (2 Pt 2), 442–445. doi: 10.1097/AOG.0b013e3181cb9955

Healy, S. A., Fried, M., Richie, T., Bok, K., Little, M., August, A., et al. (2019). Malaria Vaccine Trials in Pregnant Women: An Imperative Without Precedent. *Vaccine* 37 (6), 763–770. doi: 10.1016/j.vaccine.2018.12.025

Heumann, C. L., Quilter, L. A., Eastment, M. C., Heffron, R., and Hawes, S. E. (2017). Adverse Birth Outcomes and Maternal Neisseria Gonorrhoeae Infection: A Population-Based Cohort Study in Washington State. *Sex Transm. Dis.* 44 (5), 266–271. doi: 10.1097/OLQ.0000000000000592

Hogmalm, A., Bry, M., Strandvik, B., and Bry, K. (2014). IL-1beta Expression in the Distal Lung Epithelium Disrupts Lung Morphogenesis and Epithelial Cell Differentiation in Fetal Mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 306 (1), L23–L34. doi: 10.1152/ajplung.00154.2013

Inoue, S., Niikura, M., Mineo, S., and Kobayashi, F. (2013). Roles of IFN-Gamma and Gammadelta T Cells in Protective Immunity Against Blood-Stage Malaria. *Front. Immunol.* 4, 258. doi: 10.3389/fimmu.2013.00258

Isa, A., Lundqvist, A., Lindblom, A., Tolfvenstam, T., and Broliden, K. (2007). Cytokine Responses in Acute and Persistent Human Parvovirus B19 Infection. *Clin. Exp. Immunol.* 147 (3), 419–425. doi: 10.1111/j.1365-2249.2006.03286.x

Isik, G., Demirezen, S., Donmez, H. G., and Beksac, M. S. (2016). Bacterial Vaginosis in Association With Spontaneous Abortion and Recurrent Pregnancy Losses. *J. Cytol.* 33 (3), 135–140. doi: 10.4103/0970-9371.188050

Jain, V. G., Willis, K. A., Jobe, A., and Ambalavanan, N. (2022). Chorioamnionitis and Neonatal Outcomes. *Pediatr. Res.* 91 (2), 289–296. doi: 10.1038/s41390-021-01633-0

James, S. H., Sheffield, J. S., and Kimberlin, D. W. (2014). Mother-To-Child Transmission of Herpes Simplex Virus. *J. Pediatr. Infect. Dis. Soc* 3 Suppl 1, S19–S23. doi: 10.1093/jpids/piu050

Javanian, M., Masrour-Roudsari, J., and Ebrahimpour, S. (2018). Clinical Diagnosis Challenges in Zika Virus Infection. *Caspian. J. Intern. Med.* 9 (4), 416–417. doi: 10.22088/cjim.9.4.416

Jehan, F., Sazawal, S., Baqui, A. H., Nisar, M. I., Dhingra, U., Khanam, R., et al. (2020). Multiomics Characterization of Preterm Birth in Low- and Middle-Income Countries. *JAMA Netw. Open* 3 (12), e2029655. doi: 10.1001/jamanetworkopen.2020.29655

Johnson, E. L., and Chakraborty, R. (2016). HIV-1 at the Placenta: Immune Correlates of Protection and Infection. *Curr. Opin. Infect. Dis.* 29 (3), 248–255. doi: 10.1097/QCO.0000000000000267

Khaiboullina, S., Uppal, T., Martynova, E., Rizvanov, A., Baranwal, M., and Verma, S. C. (2018). History of ZIKV Infections in India and Management of Disease Outbreaks. *Front. Microbiol.* 9, 2126. doi: 10.3389/fmicb.2018.02126

Kimball, A., Torrone, E., Miele, K., Bachmann, L., Thorpe, P., Weinstock, H., et al. (2020). Missed Opportunities for Prevention of Congenital Syphilis - United States, 2018. *MMWR Morb. Mortal Wkly. Rep.* 69 (22), 661–665. doi: 10.15585/mmwr.mm6922a1

King, T., and Lamb, T. (2015). Interferon-Gamma: The Jekyll and Hyde of Malaria. *PloS Pathog.* 11 (10), e1005118. doi: 10.1371/journal.ppat.1005118

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Backhed, H. K., et al. (2012). Host Remodeling of the Gut Microbiome and Metabolic Changes During Pregnancy. *Cell* 150 (3), 470–480. doi: 10.1016/j.cell.2012.07.008

Kumar, M., and Al Khodor, S. (2020). Pathophysiology and Treatment Strategies for COVID-19. *J. Transl. Med.* 18 (1), 353. doi: 10.1186/s12967-020-02520-8

Kumar, M., and Al Khodor, S. (2021). "Armed for the Future Coronavirus Pandemic": A Promising Use of the Multimeric SARS-CoV-2 Receptor Binding Domain Nanoparticle as a New Pan-Coronavirus Vaccine. *Signal Transduct Target Ther.* 6 (1), 305. doi: 10.1038/s41392-021-00721-1

Kumar, M., Mathur, T., Joshi, V., Upadhyay, D. J., Inoue, S. I., and Masuda, N. (2018). Effect of DS-2969b, a Novel GyrB Inhibitor, on Rat and Monkey Intestinal Microbiota. *Anaerobe* 51, 120–123. doi: 10.1016/j.anaerobe.2018.04.017

Kumar, M., Murugesan, S., Singh, P., Saadaoui, M., Elhag, D. A., Terranegra, A., et al. (2021a). Vaginal Microbiota and Cytokine Levels Predict Preterm Delivery in Asian Women. *Front. Cell Infect. Microbiol.* 11, 639665. doi: 10.3389/fcimb.2021.639665

Kumar, M., Saadaoui, M., Elhag, D. A., Murugesan, S., Al Abduljabbar, S., Fagier, Y., et al. (2021b). Omouma: A Prospective Mother and Child Cohort Aiming to Identify Early Biomarkers of Pregnancy Complications in Women Living in Qatar. *BMC Pregnancy Childbirth* 21 (1), 570. doi: 10.1186/s12884-021-04029-4

Kumar, M., Singh, P., Murugesan, S., Vetizou, M., McCulloch, J., Badger, J. H., et al. (2020). Microbiome as an Immunological Modifier. *Methods Mol. Biol.* 2055, 595–638. doi: 10.1007/978-1-4939-9773-2_27

Lambert, N., Strebel, P., Orenstein, W., Icenogle, J., and Poland, G. A. (2015). Rubella. *Lancet* 385 (9984), 2297–2307. doi: 10.1016/S0140-6736(14)60539-0

Lash, G. E. (2015). Molecular Cross-Talk at the Feto-Maternal Interface. *Cold Spring Harb. Perspect. Med.* 5 (12), a023010. doi: 10.1101/cshperspect.a023010

Lee, S., Choi, Y.-K., and Goo, Y.-K. (2021). Humoral and Cellular Immune Response to Plasmodium Vivax VIR Recombinant and Synthetic Antigens in Individuals Naturally Exposed to P. Vivax in the Republic of Korea. *Malaria J.* 20 (1), 288. doi: 10.1186/s12936-021-03810-2

Le Gars, M., Kay, A. W., Bayless, N. L., Aziz, N., Dekker, C. L., Swan, G. E., et al. (2016). Increased Proinflammatory Responses of Monocytes and Plasmacytoid Dendritic Cells to Influenza A Virus Infection During Pregnancy. *J. Infect. Dis.* 214 (11), 1666–1671. doi: 10.1093/infdis/jiw448

Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C., and Husslein, P. (2003). Bacterial Vaginosis as a Risk Factor for Preterm Delivery: A Meta-Analysis. *Am. J. Obstet. Gynecol.* 189 (1), 139–147. doi: 10.1067/mob.2003.339

Lenz, J. D., and Dillard, J. P. (2018). Pathogenesis of Neisseria Gonorrhoeae and the Host Defense in Ascending Infections of Human Fallopian Tube. *Front. Immunol.* 9, 2710. doi: 10.3389/fimmu.2018.02710

Liu, P. H., Hao, J. D., Li, W. Y., Tian, J., Zhao, J., Zeng, Y. M., et al. (2021). Congenital Cytomegalovirus Infection and the Risk of Hearing Loss in Childhood: A PRISMA-Compliant Meta-Analysis. *Med. (Baltimore)* 100 (36), e27057. doi: 10.1097/MD.0000000000027057

Maartens, G., Celum, C., and Lewin, S. R. (2014). HIV Infection: Epidemiology, Pathogenesis, Treatment, and Prevention. *Lancet* 384 (9939), 258–271. doi; 10.1016/S0140-6736(14)60164-1

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., et al. (2015). The Vaginal Microbiome During Pregnancy and the Postpartum Period in a European Population. *Sci. Rep.* 5, 8988. doi; 10.1038/srep08988

Maidji, E., McDonagh, S., Genbacev, O., Tabata, T., and Pereira, L. (2006). Maternal Antibodies Enhance or Prevent Cytomegalovirus Infection in the Placenta by Neonatal Fc Receptor-Mediated Transcytosis. *Am. J. Pathol.* 168 (4), 1210–1226. doi: 10.2353/ajpath.2006.050482

Maki, Y., Fujisaki, M., Sato, Y., and Sameshima, H. (2017). Candida Chorioamnionitis Leads to Preterm Birth and Adverse Fetal-Neonatal Outcome. *Infect. Dis. Obstet. Gynecol.* 2017, 9060138. doi: 10.1155/2017/9060138

Manolakaki, D., Velmahos, G., Kourkoumpetis, T., Chang, Y., Alam, H. B., De Moya, M. M., et al. (2010). Candida Infection and Colonization Among Trauma Patients. *Virulence* 1 (5), 367–375. doi: 10.4161/viru.1.5.12796

Marchant, A., Sadarangani, M., Garand, M., Dauby, N., Verhasselt, V., Pereira, L., et al. (2017). Maternal Immunisation: Collaborating With Mother Nature. *Lancet Infect. Dis.* 17 (7), e197–e208. doi: 10.1016/S1473-3099(17)30229-3

Margarita, V., Fiori, P. L., and Rappelli, P. (2020). Impact of Symbiosis Between Trichomonas Vaginalis and Mycoplasma Hominis on Vaginal Dysbiosis: A Mini Review. *Front. Cell Infect. Microbiol.* 10, 179. doi: 10.3389/fcimb.2020.00179

Mateus, T., Silva, J., Maia, R. L., and Teixeira, P. (2013). Listeriosis During Pregnancy: A Public Health Concern. *ISRN Obstet. Gynecol.* 2013, 851712. doi: 10.1155/2013/851712

Maucourant, C., Queiroz, G. A. N., Samri, A., Grassi, M. F. R., Yssel, H., and Vieillard, V. (2019). Zika Virus in the Eye of the Cytokine Storm. *Eur. Cytokine Netw.* 30 (3), 74–81. doi; 10.1684/ecn.2019.0433

Maudet, C., Levallois, S., Disson, O., and Lecuit, M. (2021). Innate Immune Responses to Listeria *In Vivo*. *Curr. Opin. Microbiol.* 59, 95–101. doi: 10.1016/j.mib.2020.11.006

Megli, C. J., and Coyne, C. B. (2021). Infections at the Maternal-Fetal Interface: An Overview of Pathogenesis and Defence. *Nat. Rev. Microbiol* 20 (2), 67–82. doi: 10.1038/s41579-021-00610-y

Megli, C. J., and Coyne, C. B. (2022). Infections at the Maternal–Fetal Interface: An Overview of Pathogenesis and Defence. *Nat. Rev. Microbiol.* 20 (2), 67–82. doi: 10.1038/s41579-021-00610-y

Mendz, G. L., Kaakoush, N. O., and Quinlivan, J. A. (2013). Bacterial Aetiological Agents of Intra-Amniotic Infections and Preterm Birth in Pregnant Women. *Front. Cell Infect. Microbiol.* 3, 58. doi: 10.3389/fcimb.2013.00058

Milligan, C., and Overbaugh, J. (2014). The Role of Cell-Associated Virus in Mother-to-Child HIV Transmission. *J. Infect. Dis.* 210 Suppl 3, S631–S640. doi: 10.1093/infdis/jiu344

Mills, S., Shanahan, F., Stanton, C., Hill, C., Coffey, A., and Ross, R. P. (2013). Movers and Shakers. *Gut Microbes* 4 (1), 4–16. doi: 10.4161/gmic.22371

Mlakar, J., Korva, M., Tul, N., Popovic, M., Poljsak-Prijatelj, M., Mraz, J., et al. (2016). Zika Virus Associated With Microcephaly. *N. Engl. J. Med.* 374 (10), 951–958. doi: 10.1056/NEJMoa1600651

Mogensen, T. H. (2009). Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clin. Microbiol. Rev.* 22 (2), 240–273. doi: 10.1128/CMR.00046-08

Moncunill, G., Dobano, C., Gonzalez, R., Smolen, K. K., Manaca, M. N., Balcells, R., et al. (2020). Association of Maternal Factors and HIV Infection With Innate Cytokine Responses of Delivering Mothers and Newborns in Mozambique. *Front. Microbiol.* 11, 1452. doi: 10.3389/fmicb.2020.01452

Mor, G., and Cardenas, I. (2010). The Immune System in Pregnancy: A Unique Complexity. *Am. J. Reprod. Immunol.* 63 (6), 425–433. doi; 10.1111/j.1600-0897.2010.00836.x

Murphy, S. P., Fast, L. D., Hanna, N. N., and Sharma, S. (2005). Uterine NK Cells Mediate Inflammation-Induced Fetal Demise in IL-10-Null Mice. *J. Immunol.* 175 (6), 4084–4090. doi: 10.4049/jimmunol.175.6.4084

Murphy, S. P., Tayade, C., Ashkar, A. A., Hatta, K., Zhang, J., and Croy, B. A. (2009). Interferon Gamma in Successful Pregnancies. *Biol. Reprod.* 80 (5), 848–859. doi: 10.1095/biolreprod.108.073353

Nanthakumar, M. P., Sood, A., Ahmed, M., and Gupta, J. (2021). Varicella Zoster in Pregnancy. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 258, 283–287. doi:10.1016/j.ejogrb.2021.01.009

Nasr, A., Allam, G., Hamid, O., and Al-Ghamdi, A. (2014). IFN-Gamma and TNF Associated With Severe Falciparum Malaria Infection in Saudi Pregnant Women. *Malaria J.* 13 (1), 314. doi; 10.1186/1475-2875-13-314

Nguyen-Tien, T., Pham, L. T., Vu, D. T., Tran, S. H., Vu, L. T., Bui, V. N., et al. (2021). Knowledge and Practice on Prevention of Mosquito-Borne Diseases in Livestock-Keeping and non-Livestock-Keeping Communities in Hanoi City, Vietnam: A Mixed-Method Study. *PloS One* 16 (2), e0246032. doi: 10.1371/journal.pone.0246032

Ni, L., and Lu, J. (2018). Interferon Gamma in Cancer Immunotherapy. *Cancer Med.* 7 (9), 4509–4516. doi: 10.1002/cam4.1700

Nimeri, N., Rahman, S., El Tinay, S., El Ansari, W., Tamano, E. A., Sellami, S., et al. (2013). The National Perinatal Mortality Rate in the State of Qatar During 2011; Trends Since 1990 and Comparative Analysis With Selected High-Income Countries: The PEARL Study Project. *J. Perinat. Med.* 41 (3), 323–330. doi: 10.1515/jpm-2012-0143

Nuriel-Ohayon, M., Neuman, H., and Koren, O. (2016). Microbial Changes During Pregnancy, Birth, and Infancy. *Front. Microbiol.* 7, 1031. doi; 10.3389/fmicb.2016.01031

Offenbacher, S., Boggess, K. A., Murtha, A. P., Jared, H. L., Lieff, S., McKaig, R. G., et al. (2006). Progressive Periodontal Disease and Risk of Very Preterm Delivery. *Obstet. Gynecol.* 107 (1), 29–36. doi: 10.1097/01.AOG.0000190212.87012.96

Ornelas, A. M., Pezzuto, P., Silveira, P. P., Melo, F. O., Ferreira, T. A., Oliveira-Szejnfeld, P. S., et al. (2017). Immune Activation in Amniotic Fluid From Zika Virus-Associated Microcephaly. *Ann. Neurol.* 81 (1), 152–156. doi: 10.1002/ana.24839

Pansieri, C., Pandolfini, C., Clavenna, A., Choonara, I., and Bonati, M. (2020). An Inventory of European Birth Cohorts. *Int. J. Environ. Res. Public Health* 17 (9), 3071. doi: 10.3390/ijerph17093071

Patras, K. A., and Nizet, V. (2018). Group B Streptococcal Maternal Colonization and Neonatal Disease: Molecular Mechanisms and Preventative Approaches. *Front. Pediatr.* 6, 27. doi: 10.3389/fped.2018.00027

Phillips, C., and Walsh, E. (2020). Group A Streptococcal Infection During Pregnancy and the Postpartum Period. *Nurs. Womens Health* 24 (1), 13–23. doi: 10.1016/j.nwh.2019.11.006

Piler, P., Kandrnal, V., Kukla, L., Andryskova, L., Svancara, J., Jarkovsky, J., et al. (2017). Cohort Profile: The European Longitudinal Study of Pregnancy and Childhood (ELSPAC) in the Czech Republic. *Int. J. Epidemiol.* 46 (5), 1379–137f. doi: 10.1093/ije/dyw091

Pinninti, S. G., and Kimberlin, D. W. (2013). Neonatal Herpes Simplex Virus Infections. *Pediatr. Clin. North Am.* 60 (2), 351–365. doi: 10.1016/j.pcl.2012.12.005

Platt, D. J., Smith, A. M., Arora, N., Diamond, M. S., Coyne, C. B., and Miner, J. J. (2018). Zika Virus-Related Neurotropic Flaviviruses Infect Human Placental Explants and Cause Fetal Demise in Mice. *Sci. Transl. Med.* 10 (426), eaao7090. doi: 10.1126/scitranslmed.aao7090

PrabhuDas, M., Bonney, E., Caron, K., Dey, S., Erlebacher, A., Fazleabas, A., et al. (2015). Immune Mechanisms at the Maternal-Fetal Interface: Perspectives and Challenges. *Nat. Immunol.* 16 (4), 328–334. doi: 10.1038/ni.3131

Primus, S., Rocha, S. C., Giacani, L., and Parveen, N. (2020). Identification and Functional Assessment of the First Placental Adhesin of Treponema Pallidum That May Play Critical Role in Congenital Syphilis. *Front. Microbiol.* 11, 621654. doi: 10.3389/fmicb.2020.621654

Prince, A. L., Chu, D. M., Seferovic, M. D., Antony, K. M., Ma, J., and Aagaard, K. M. (2015). The Perinatal Microbiome and Pregnancy: Moving Beyond the Vaginal Microbiome. *Cold Spring Harb. Perspect. Med.* 5 (6), a023051. doi: 10.1101/cshperspect.a023051

Prins, J. R., Gomez-Lopez, N., and Robertson, S. A. (2012). Interleukin-6 in Pregnancy and Gestational Disorders. *J. Reprod. Immunol.* 95 (1-2), 1–14. doi: 10.1016/j.jri.2012.05.004

Quicke, K. M., Bowen, J. R., Johnson, E. L., McDonald, C. E., Ma, H., O'Neal, J. T., et al. (2016). Zika Virus Infects Human Placental Macrophages. *Cell Host Microbe* 20 (1), 83–90. doi: 10.1016/j.chom.2016.05.015

Racicot, K., Kwon, J. Y., Aldo, P., Abrahams, V., El-Guindy, A., Romero, R., et al. (2016). Type I Interferon Regulates the Placental Inflammatory Response to Bacteria and is Targeted by Virus: Mechanism of Polymicrobial Infection-Induced Preterm Birth. *Am. J. Reprod. Immunol.* 75 (4), 451–460. doi: 10.1111/aji.12501

Rahman, S., Al Rifai, H., El Ansari, W., Nimeri, N., El Tinay, S., Salameh, K., et al. (2012). A PEARL Study Analysis of National Neonatal, Early Neonatal, Late Neonatal, and Corrected Neonatal Mortality Rates in the State of Qatar During 2011: A Comparison With World Health Statistics 2011 and Qatar's Historic Data Over a Period of 36 Years (1975-2011). *J. Clin. Neonatol.* 1 (4), 195–201. doi: 10.4103/2249-4847.105990

Rasti, S., Asadi, M. A., Taghriri, A., Behrashi, M., and Mousavie, G. (2014). Vaginal Candidiasis Complications on Pregnant Women. *Jundishapur J. Microbiol.* 7 (2), e10078. doi: 10.5812/jjm.10078

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., et al. (2011). Vaginal Microbiome of Reproductive-Age Women. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl 1, 4680–4687. doi: 10.1073/pnas.1002611107

Robinson, D. P., and Klein, S. L. (2012). Pregnancy and Pregnancy-Associated Hormones Alter Immune Responses and Disease Pathogenesis. *Horm. Behav.* 62 (3), 263–271. doi: 10.1016/j.yhbeh.2012.02.023

Rodriguez-Melcon, C., Capita, R., Garcia-Fernandez, C., and Alonso-Calleja, C. (2018). Effects of Bacteriophage P100 at Different Concentrations on the Structural Parameters of Listeria Monocytogenes Biofilms. *J. Food Prot.* 81 (12), 2040–2044. doi: 10.4315/0362-028X.JFP-18-177

Romero, R., Dey, S. K., and Fisher, S. J. (2014a). Preterm Labor: One Syndrome, Many Causes. *Science* 345 (6198), 760–765. doi; 10.1126/science.1251816

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., et al. (2014b). The Vaginal Microbiota of Pregnant Women Who Subsequently Have Spontaneous Preterm Labor and Delivery and Those With a Normal Delivery at Term. *Microbiome* 2, 18. doi: 10.1186/2049-2618-2-18

Romero, M., Leiba, E., Carrión-Nessi, F. S., Freitas-De Nobrega, D. C., Kaid-Bay, S., Gamardo, Á. F., et al. (2021). Malaria in Pregnancy Complications in Southern Venezuela. *Malaria J.* 20 (1), 186. doi: 10.1186/s12936-021-03728-9

Saadaoui, M., Kumar, M., and Al Khodor, S. (2021). COVID-19 Infection During Pregnancy: Risk of Vertical Transmission, Fetal, and Neonatal Outcomes. *J. Pers. Med.* 11 (6), 483. doi: 10.3390/jpm11060483

Sacerdoti, F., Scalise, M. L., Burdet, J., Amaral, M. M., Franchi, A. M., and Ibarra, C. (2018). Shiga Toxin-Producing Escherichia Coli Infections During Pregnancy. *Microorganisms* 6 (4), 111. doi: 10.3390/microorganisms6040111

Sadowsky, D. W., Adams, K. M., Gravett, M. G., Witkin, S. S., and Novy, M. J. (2006). Preterm Labor is Induced by Intraamniotic Infusions of Interleukin-1beta and Tumor Necrosis Factor-Alpha But Not by Interleukin-6 or Interleukin-8 in a Nonhuman Primate Model. *Am. J. Obstet. Gynecol.* 195 (6), 1578–1589. doi: 10.1016/j.ajog.2006.06.072

Sasai, M., and Yamamoto, M. (2019). Innate, Adaptive, and Cell-Autonomous Immunity Against Toxoplasma Gondii Infection. *Exp. Mol. Med.* 51 (12), 1–10. doi: 10.1038/s12276-019-0353-9

Scott, G. M., Chow, S. S., Craig, M. E., Pang, C. N., Hall, B., Wilkins, M. R., et al. (2012). Cytomegalovirus Infection During Pregnancy With Maternofetal Transmission Induces a Proinflammatory Cytokine Bias in Placenta and Amniotic Fluid. *J. Infect. Dis.* 205 (8), 1305–1310. doi: 10.1093/infdis/jis186

Seong, H. S., Lee, S. E., Kang, J. H., Romero, R., and Yoon, B. H. (2008). The Frequency of Microbial Invasion of the Amniotic Cavity and Histologic Chorioamnionitis in Women at Term With Intact Membranes in the Presence or Absence of Labor. *Am. J. Obstet. Gynecol.* 199 (4), 375 e1–375 e5. doi: 10.1016/j.ajog.2008.06.040

Serrano, M. G., Parikh, H. I., Brooks, J. P., Edwards, D. J., Arodz, T. J., Edupuganti, L., et al. (2019). Racioethnic Diversity in the Dynamics of the Vaginal Microbiome During Pregnancy. *Nat. Med.* 25 (6), 1001–1011. doi: 10.1038/s41591-019-0465-8

Siriratsivawong, R., Pavlis, M., Hymes, S. R., and Mintzer, J. P. (2014). Congenital Candidiasis: An Uncommon Skin Eruption Presenting at Birth. *Cutis* 93 (5), 229–232.

Smith, N. C., Goulart, C., Hayward, J. A., Kupz, A., Miller, C. M., and van Dooren, G. G. (2021). Control of Human Toxoplasmosis. *Int. J. Parasitol.* 51 (2-3), 95–121. doi: 10.1016/j.ijpara.2020.11.001

Spence, T., Allsopp, P. J., Yeates, A. J., Mulhern, M. S., Strain, J. J., and McSorley, E. M. (2021). Maternal Serum Cytokine Concentrations in Healthy Pregnancy and Preeclampsia. *J. Pregnancy* 2021, 6649608. doi: 10.1155/2021/6649608

Stewart, R. D., Bryant, S. N., and Sheffield, J. S. (2013). West Nile Virus Infection in Pregnancy. *Case Rep. Infect. Dis.* 2013, 351872. doi: 10.1155/2013/351872

Straface, G., Selmin, A., Zanardo, V., De Santis, M., Ercoli, A., and Scambia, G. (2012). Herpes Simplex Virus Infection in Pregnancy. *Infect. Dis. Obstet. Gynecol.* 2012, 385697. doi: 10.1155/2012/385697

Tabata, T., Petitt, M., Puerta-Guardo, H., Michlmayr, D., Wang, C., Fang-Hoover, J., et al. (2016). Zika Virus Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission. *Cell Host Microbe* 20 (2), 155–166. doi: 10.1016/j.chom.2016.07.002

Tang, H., Hammack, C., Ogden, S. C., Wen, Z., Qian, X., Li, Y., et al. (2016). Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. *Cell Stem Cell.* 18 (5), 587–590. doi: 10.1016/j.stem.2016.02.016

Teixeira, H. C., and Kaufmann, S. H. (1994). Role of NK1.1+ Cells in Experimental Listeriosis. NK1+ Cells are Early IFN-Gamma Producers But Impair Resistance to Listeria Monocytogenes Infection. *J. Immunol.* 152 (4), 1873–1882.

Thomas, J., Govender, N., McCarthy, K. M., Erasmus, L. K., Doyle, T. J., Allam, M., et al. (2020). Outbreak of Listeriosis in South Africa Associated With Processed Meat. *N Engl. J. Med.* 382 (7), 632–643. doi: 10.1056/NEJMoa1907462

Vallely, L. M., Egli-Gany, D., Wand, H., Pomat, W. S., Homer, C. S. E., Guy, R., et al. (2021). Adverse Pregnancy and Neonatal Outcomes Associated With Neisseria Gonorrhoeae: Systematic Review and Meta-Analysis. *Sex Transm. Infect.* 97 (2), 104–111. doi: 10.1136/sextrans-2020-054653

Villar, J., Ariff, S., Gunier, R. B., Thiruvengadam, R., Rauch, S., Kholin, A., et al. (2021). Maternal and Neonatal Morbidity and Mortality Among Pregnant Women With and Without COVID-19 Infection: The INTERCOVID Multinational Cohort Study. *JAMA Pediatr.* 175 (8), 817–826. doi: 10.1001/jamapediatrics.2021.1050

Waken, R. J., de Las Fuentes, L., and Rao, D. C. (2017). A Review of the Genetics of Hypertension With a Focus on Gene-Environment Interactions. *Curr. Hypertens. Rep.* 19 (3), 23. doi: 10.1007/s11906-017-0718-1

Wampach, L., Heintz-Buschart, A., Fritz, J. V., Ramiro-Garcia, J., Habier, J., Herold, M., et al. (2018). Birth Mode Is Associated With Earliest Strain-Conferred Gut Microbiome Functions and Immunostimulatory Potential. *Nat. Commun.* 9 (1), 5091. doi: 10.1038/s41467-018-07631-x

Wang, Z., Tao, X., Liu, S., Zhao, Y., and Yang, X. (2021). An Update Review on Listeria Infection in Pregnancy. *Infect. Drug Resist.* 14, 1967–1978. doi: 10.2147/IDR.S313675

Waters, J. P., Pober, J. S., and Bradley, J. R. (2013). Tumour Necrosis Factor in Infectious Disease. *J. Pathol.* 230 (2), 132–147. doi: 10.1002/path.4187

Wicher, V., and Wicher, K. (2001). Pathogenesis of Maternal-Fetal Syphilis Revisited. *Clin. Infect. Dis.* 33 (3), 354–363. doi: 10.1086/321904

Wilkie, G. L., Prabhu, M., Ona, S., Easter, S. R., Tuomala, R. E., Riley, L. E., et al. (2019). Microbiology and Antibiotic Resistance in Peripartum Bacteremia. *Obstet. Gynecol.* 133 (2), 269–275. doi: 10.1097/AOG.0000000000003055

Wilson, K. M., Di Camillo, C., Doughty, L., and Dax, E. M. (2006). Humoral Immune Response to Primary Rubella Virus Infection. *Clin. Vaccine Immunol.* 13 (3), 380–386. doi: 10.1128/CVI.13.3.380-386.2006

Wong, Y. P., Tan, G. C., Wong, K. K., Anushia, S., and Cheah, F. C. (2018). Gardnerella Vaginalis in Perinatology: An Overview of the Clinicopathological Correlation. *Malays J. Pathol.* 40 (3), 267–286.

Wylie, K. M., Weinstock, G. M., and Storch, G. A. (2012). Emerging View of the Human Virome. *Transl. Res.* 160 (4), 283–290. doi: 10.1016/j.trsl.2012.03.006

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural Basis for the Recognition of SARS-CoV-2 by Full-Length Human ACE2. *Science* 367 (6485), 1444–1448. doi: 10.1126/science.abb2762

Yockey, L. J., and Iwasaki, A. (2018a). Interferons and Proinflammatory Cytokines in Pregnancy and Fetal Development. *Immunity* 49 (3), 397–412. doi: 10.1016/j.immuni.2018.07.017

Yockey, L. J., Jurado, K. A., Arora, N., Millet, A., Rakib, T., Milano, K. M., et al. (2018b). Type I Interferons Instigate Fetal Demise After Zika Virus Infection. *Sci. Immunol.* 3 (19), eaao1680. doi: 10.1126/sciimmunol.aao1680

Zhang, D., Huang, Y., and Ye, D. (2015). Intestinal Dysbiosis: An Emerging Cause of Pregnancy Complications? *Med. Hypotheses* 84 (3), 223–226. doi: 10.1016/j.mehy.2014.12.029

Zhang, L., Zhao, M., Jiao, F., Xu, X., Liu, X., Jiang, Y., et al. (2015). Interferon Gamma is Involved in Apoptosis of Trophoblast Cells at the Maternal-Fetal Interface Following Toxoplasma Gondii Infection. *Int. J. Infect. Dis.* 30, 10–16. doi: 10.1016/j.ijid.2014.10.027

Zidovec-Lepej, S., Vilibic-Cavlek, T., Barbic, L., Ilic, M., Savic, V., Tabain, I., et al. (2021). Antiviral Cytokine Response in Neuroinvasive and Non-Neuroinvasive West Nile Virus Infection. *Viruses* 13 (2), 342. doi: 10.3390/v13020342

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership