Nutrition-dependent development of the Oral Microbiome in Early Pregnancy

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Abstract

Background: Most studies of the oral microbiome during pregnancy have focused on the second and third trimesters. Findings remain inconsistent- some report shifts in specific taxa, whereas others observe little change in diversity. To date, no large-scale longitudinal study has examined oral microbiome development across all three trimesters, leaving the early gestational dynamics largely unexplored.

Methods: We conducted a longitudinal analysis of the oral microbiome in 346 pregnant women from Israel and validated key findings in an independent cohort of 154 pregnant women from Russia. In Israel, Saliva samples were collected during the first (T1, 11-14 weeks), second (T2, 24-28 weeks), and third (T3, 32-38 weeks) trimesters; in Russia, samples were collected during the second and third trimesters at the same time points. Alongside sample collection, participants completed comprehensive dietary and health questionnaires to assess maternal factors that could influence microbial composition. Microbial profiles

were analyzed to test for (i) differential abundance across trimesters, (ii) the influence of maternal nutrition and lifestyle factors.
Results: Significant shifts in oral microbial composition were observed as early as the transition from T1 to T2. Alpha diversity decreased progressively from T1 (Shannon's diversity index: 3.261) to T2 (3.173) and T3 (3.109; Kruskal-Wallis test, $p = 0.0023$) in the Israeli cohort. Notable taxonomic changes included significant decreases in Verrucomicrobiota and Euryarchaeota from T1 to T2, with *Akkermansia muciniphila* showing a particular reduction. Conversely, Synergistota increased significantly during this transition. Gammaproteobacteria increased while Erysipelotrichia decreased between T1 and T2, suggesting an ecological shift from commensal, barrier-supporting taxa toward communities associated with dysbiosis and inflammation. Despite these systematic changes, individual microbial signatures- measured as pairwise distances between trimesters-remained relatively conserved across trimesters. Maternal factors that significantly modulated oral microbiome composition included adherence to gluten-free diets (the most robust association across all trimesters in Israeli women), smoking history, and conception method, with gluten-free diet showing consistent associations with taxa in the Lachnospiraceae and Ruminococcaceae families. **Conclusions:** This study provides the first large-scale evidence of significant oral microbiome changes beginning in early pregnancy, characterized by reduced diversity and a directional shift toward potentially pro-inflammatory communities. The strong associations with gluten consumption and smoking suggest that early pregnancy represents a critical window for microbiome modulation through dietary interventions. The alteration in the microbial composition highlights the oral microbiome as a sensitive marker of gestational physiology.

Keywords: oral microbiome, pregnancy, first trimester, Akkermansia muciniphila, gluten-free diet, smoking, Prevotella Copri

1 Introduction

The oral microbiome undergoes distinct compositional changes throughout pregnancy, influenced by hormonal, immunologic, and metabolic shifts[4, 25]. Although overall microbial diversity remains relatively stable, there is a trend towards decreased microbial richness and a shift in community structure, particularly in the second (T2) and third (T3) trimesters compared to non-pregnant or postpartum states[45, 5, 19].

Studies using 16S rRNA gene sequencing have shown significant increases in certain taxa in T2 and T3, such as the phylum Bacteroidota, and the genera *Veillonella* (notably *Veillonella dispar* and *Veillonella atypica*), and *Granulicatella*, with a concurrent decrease in *Streptococcus sanguinis* and *Selenomonas*[4, 8, 45]. T3 is associated with a further reduction in microbial richness and increased dominance of specific genera, suggesting a shift toward a more specialized, and potentially proinflammatory, community[41, 22].

These changes are correlated with increasing sex hormone levels, which can promote the growth of specific bacteria implicated in pregnancy gingivitis and periodontitis[35]. The oral microbiome tends to revert to a pre-pregnancy state within weeks postpartum[19]. Importantly, while the oral microbiome is more stable than the vaginal or gut microbiome, pregnancy-associated changes can increase susceptibility to oral disease and have been associated with adverse pregnancy outcomes[6]. Although the evolution of the oral microbiome in T2 and T3 has been extensively studied, to our knowledge, there are currently no large-scale studies looking at the evolution of the oral microbiome from the first trimester (T1) of pregnancy throughout, nor any clear evidence of a change in the oral microbiome in this period.

Diet is a central determinant of oral microbial ecology, shaping both community structure and function. Frequent intake of fermentable carbohydrates, particularly sugars, enriches acidogenic and aciduric taxa such as *Streptococcus mutans* and *Lactobacillus*, promoting dysbiosis and cariogenesis [30, 40]. Conversely, diets rich in fiber, plant polyphenols, and micronutrients such as vitamin D and calcium are associated with greater microbial diversity, resilience, and anti-inflammatory capacity [48, 18]. Nutritional deficiencies, including iron and folate, can also alter host immunity and indirectly affect microbial colonization [2]. Thus, dietary patterns interact with host physiology to create either a protective or disease-prone oral environment.

During pregnancy, nutritional demands increase to support maternal health and fetal development, and dietary habits often change due to nausea, cravings, or medical advice. These shifts can exacerbate pregnancy-related changes in the oral microbiome. For instance, increased snacking and carbohydrate intake during the first trimester (T1) may favor cariogenic bacteria, while micronutrient supplementation (e.g., folic acid, iron) may mitigate dysbiosis through improved host immunity [43, 31]. Restrictive diets such as gluten-free regimens, although necessary for individuals with celiac disease, can reduce dietary fiber and prebiotic intake, potentially leading to decreased microbial diversity and enrichment of opportunistic taxa in both the gut and oral cavity [42, 27]. Together, these findings suggest that nutrition is a key, though underexplored, modifier of pregnancy-associated oral microbiome changes.

In contrast to the oral microbiome, the gut microbiome shows distinct alterations already in the first trimester (T1). Increases in taxa such as *Akkermansia*, *Bifidobacterium*, and Bacillota support energy storage and metabolic adaptation to pregnancy [15, 23, 32]. Reduced diversity and early dysbiosis have been linked to gestational complications, including GDM, preeclampsia, and preterm birth [17, 26, 11]. Mechanistically, the gut microbiome influences maternal immune tolerance, metabolic homeostasis, and placental development, and interventions such as probiotics and high-fiber diets show potential to reduce risk [21, 47]. These findings highlight the importance of the microbiome early in pregnancy, yet the role of the oral microbiome in T1 remains underexplored.

To address the apparent discrepancy between the roles of the gut and oral microbiomes in T1, we analyzed the oral microbiome at the end of T1, T2, and T3 in 347 women, and compared that with questionnaires on nutrition and health status.

2 Methods

2.1 Study Population

To analyze the development of the oral microbiome during pregnancy, we recruited two geographically separated cohorts. The Israel, the cohort consisted of pregnant women sampled during T1, T2, and T3. Out of 467 oral microbiome samples, a total of 346 Israeli women (aged 18-40) were recruited: 235 in T1 (11-14 gestational weeks) at Clalit Health Services community clinics, Israel, during 2016-17; 144 in T2 (24-28 gestational weeks) at the Rabin Medical Center, Israel, during 2016-17; and 88 in T3 (32-38 gestational weeks) at the Rabin Medical Center, Israel. Exclusion criteria were only multiple gestation (see Table S1). Informed consent was obtained from all participants, in accordance with Helsinki ethics permit #0135-15-COM from the Clalit HMO Institutional Review Board and Helsinki ethics permit #0263-15-RMC from the Rabin Medical Center Institutional Review Board.

A second cohort of pregnant women, the Russian cohort, was recruited to validate our findings; this cohort was comprised of 138 pregnant women recruited in T2 (16-28 gestational weeks)

and 106 in T3 (34-37 gestational weeks) at the Federal Almazov North-West Medical Research Center in St. Petersburg, Russia, in 2018. In total, 154 pregnant women were recruited in this cohort. Exclusion criteria are the same as those described above. This cohort was approved by the local ethics committee of the Almazov National Medical Research Centre, Russia (protocol no. 119)[34].

2.2 Sample Collection

2.2.1 Israeli cohort

At recruitment, the height and weight of the study participants were recorded, and participants were interviewed by a dietitian for stress level, working hours, sleeping hours, smoking, education and 24-hour recall for food intake. We assumed that women with no reported gluten-based nutrition had a gluten-free diet. Participants then provided a saliva sample, according to Human Microbiome Project protocols[1], collected in 1.5 ml tubes and stored at -80°C until processing. Blood samples and stool samples were collected and stored at -80°C as part of a different study on the same cohort[33]. Similar procedures were repeated in the study population in T2 (24-28 gestational weeks) and T3 (32-38 gestational weeks) of pregnancy for the original recruits as well as additional participants recruited in T2 of pregnancy.

2.2.2 Russian cohort

At recruitment (T2; 16-28 weeks of gestation), the participants' pre-pregnancy height and weight were recorded, in addition to questionnaires such as smoking [34]. Participants then provided saliva and blood samples, as described above. Samples were collected again in T3 (34-37 gestational weeks).

2.3 Sample processing

DNA was extracted from all collected samples using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions and following a 2 min bead beating step (BioSpec, Bartlesville, OK, USA). DNA was purified using 4 μl, and then the variable V4 region was PCR-amplified using the 515F and 806R barcoded primers following the Earth Microbiome Project protocol[10]. Each PCR reaction contained 25 μl with $\sim$40 ng/μl of DNA, 2 μl 515F (forward, 10 μM) primer, 2 μl 806R (reverse, 10 μM) primer, and 25 μl PrimeSTAR Max PCR Readymix (Takara, Mountain View, CA, USA). PCR conditions were as follows: 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 20 s, followed by a final elongation at 72°C for 1 min. Amplicons were purified using AMPure magnetic beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the Picogreen dsDNA quantitation kit (ThermoFisher, Waltham, MA, USA). Equimolar amounts of DNA from individual samples were pooled and sequenced using the Illumina MiSeq platform at the Genomic Center at the Bar-Ilan University, Azrieli Faculty of Medicine.

2.4 Computational analysis and statistics

Sequencing data was run through the YaMAS pipeline (https://github.com/YarinBekor/YaMAS)[39]. Quality control measures were also applied in QIIME2 [7] to filter out low-quality reads and trim sequences. The filtered sequences were then processed to identify Amplicon Sequence Variants (ASVs), which were used to generate a taxa-table summarizing their abundance. Taxonomy

was assigned with a Naive Bayes classifier trained on the GreenGenes 13_8 99% ASVs reference database[12]. The resulting taxa-tables were jointly preprocessed using the MIPMLP pipeline[20], consisting of four steps: (1) merging similar features based on taxonomic classifications, (2) scaling the distribution, (3) standardizing features to z-scores, and (4) applying dimensionality reduction. Taxa were collapsed at the most resolved taxonomic level using mean and log normalization.

Statistical analyses were performed for the Israeli cohort using Python libraries integrated within the pipeline. Alpha diversity metrics, including Shannon diversity index and species richness, were calculated using scikit-bio [36]. Differences in alpha diversity across trimesters were assessed using the Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests for post-hoc comparisons. Changes in taxonomic abundances at the phylum level were evaluated using Mann-Whitney U tests. The GIMIC method [38] was employed to identify groups of taxa distinguishing consecutive trimesters (T1 vs. T2 and T2 vs. T3) and the taxonomic levels at which differences were significant. The miMic test [37] was used to detect taxa at different taxonomic levels that significantly differed between trimesters using Mann-Whitney U tests for binary targets followed by FDR correction. Cosine distances were computed on log-transformed and z-score normalized data to assess intra- and inter-individual microbiome variability, with significance tested via Mann-Whitney U tests. Associations between microbial taxa and maternal features (from questionnaires) were analyzed using the same normalization as done for computing the cosine distances and using Spearman's rank correlation for continuous variables and point-biserial correlation for binary variables, following correction for multiple testing using the False Discovery Rate (FDR) for all p-values per maternal feature in the trimester. To assess our findings, we repeated the analyses above on the Russian cohort using the same parameters.

3 Results

We analyzed the oral microbiome of 467 oral microbiome samples from 346 Israeli women at each trimester of pregnancy (Figure 1A). In addition to 16S rRNA gene sequencing, each participant completed dietary and health questionnaires and underwent clinical tests (Table S1 for an overview of all questionnaires and tests; Fig. S1 for feature distributions). We first identified phyla that exhibited noticeable shifts in mean abundance across trimesters (Fig S2) and then assessed their significance using the Mann–Whitney test and an FDR correction. From T1 to T2, Verrucomicrobiota and Euryarchaeota exhibited significant decreases ($p = 0.0003$ and $p = 0.0209$, respectively), while Synergistota and Cyanobacteria showed significant increases ($p = 0.0013$ and p = 0.0133, respectively). The decrease in Verrucomicrobiota and Euryarchaeota persisted toward T3 ($p = 0.0005$ for Verrucomicrobiota; $p = 0.0011$ for Euryarchaeota); however, no significant decrease in Verrucomicrobiota was observed between T2 and T3. In contrast, Synergistota abundance decreased from T2 to T3. Meanwhile, Cyanobacteria continued to increase significantly from T2 to T3 ($p = 0.0482$) (Fig. 1B, Table S2 for means and medians of phyla abundance across trimesters). Alpha diversity, as measured by Shannon diversity index, showed significant differences across pregnancy trimesters (Kruskal-Wallis test, $p = 0.0023$). Pairwise comparisons using the Mann-Whitney test revealed a significant decrease from T1 to T2 ($p = 0.0160$) and from T1 to T3 ($p = 0.0017$; Fig. 1C). Similar trends were observed for species richness and other diversity metrics (Fig. S2). The decrease in Shannon diversity index continued from T2 to T3, though not significantly, with median values of 3.261 in T1, 3.173 in T2, and 3.109 in T3 (Fig. 1C).

To further elucidate the evolution of the oral microbiome between trimesters, we applied the

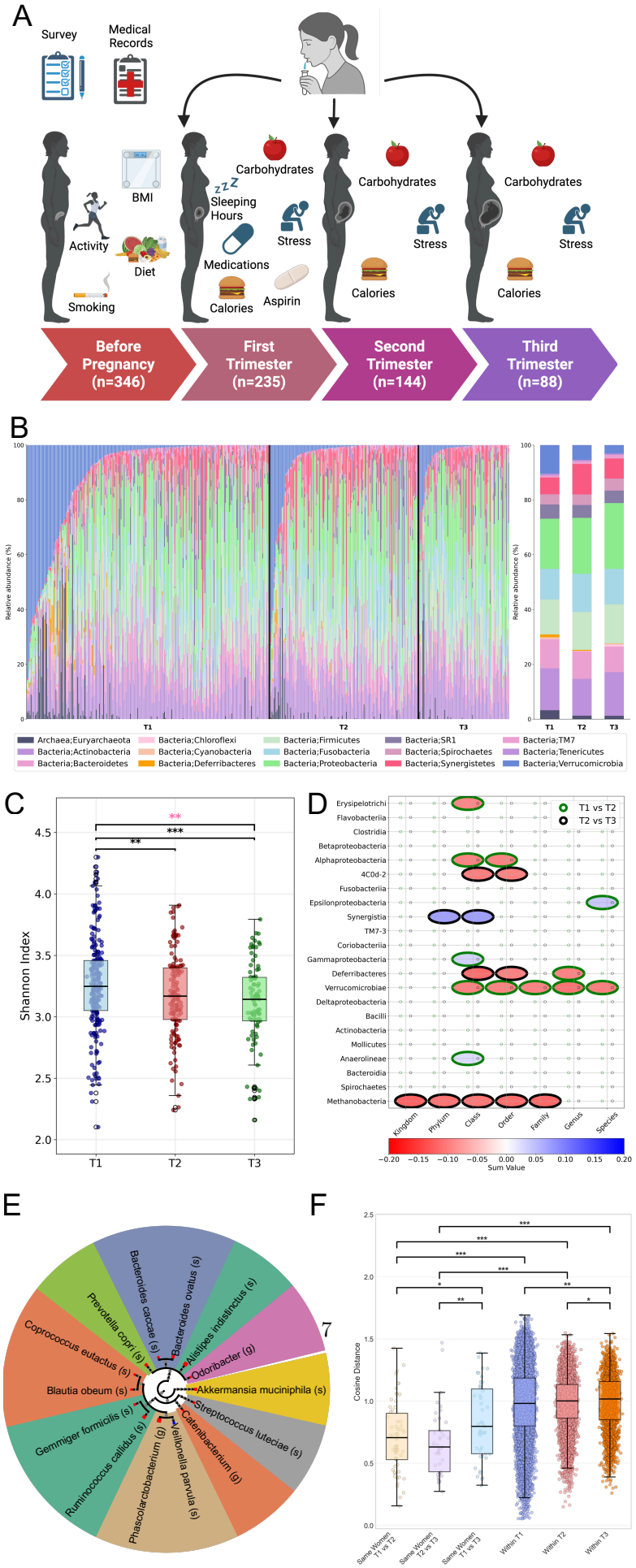
GIMIC comparison[38] (see methods and Fig. 1D). The GIMIC analysis revealed consistent dynamic shifts in microbial composition. Specifically, several lineages within the classes Alphaproteobacteria, Verrucomicrobiota, and Erysipelotrichia consistently decreased from T1 to T2, while Methanobacteria and Deferribacteres decreased from T2 to T3. The direction and magnitude of these shifts were stable across taxonomic levels, with Deferribacteres showing a consistent decline throughout pregnancy (Fig. 1D). In addition, a miMic test was performed between T1 and T2 and between T1 and T3 (Fig. 1E and Fig. S3)[37]. *Akkermansia muciniphila* of the Verrucomicrobiota class that consistently decreased in T2 (Fig. 1D), also showed a significant decrease at the species level (Fig. 1E). Other changes include an increase in Gammaproteobacteria in T2 and a decrease in Eryspelotrichia. A similar test between T2 and T3 yielded no significant differences.

To assess the variability in microbial composition over time within and between women, we calculated the cosine distances between samples (see methods). The typical distances between the microbiomes of the same women across consecutive trimesters (T1 to T2 and T2 to T3) were significantly lower than the distances between different women or between the same women from T1 to T3 ($p < 0.005$, Fig. 1F). Similar patterns were observed for other distance metrics (Supplementary Material, Fig. S4).

To further characterize the development of the oral microbiome across pregnancy, we examined associations between microbial taxa and maternal features, including nutrition, demographics, and medical history, within each trimester and between trimesters (T1 to T2 and T2 to T3). After normalizing the relative abundances (see methods), the normalized frequencies were compared to 15 maternal features (collected before pregnancy, and at each trimester, see Fig 1A and Table S1). Of these, only five variables demonstrated consistent and significant correlations overall with the oral microbiome after FDR correction, indicating that the majority of metadata features had little correlation with the oral community composition (Fig. 2). The most robust associations were observed for gluten-free diet, which correlated with oral microbiome composition in all three trimesters as well as with inter-trimester changes (T1 to T2 and T2 toT3). Tree-based association mapping (Fig. 2B) revealed that gluten-free diet was linked to multiple taxa, with the strongest effects involving members of the families Lachnospiraceae and Ruminococcaceae. Strikingly, the majority of these associations were positive, suggesting two possible mechanisms: either (i) microbes normally reduced by gluten-free diets leave ecological niches, allowing expansion of other taxa, or (ii) nutrients emphasized in gluten-free diets, such as alternative fibers and starches, selectively promote growth of these organisms.

Smoking history was also significantly associated with oral microbial composition. Most associations were in taxa within Clostridiales and Bacteroidales classes (Fig. 2A, 2C). These associations were present in multiple trimesters, suggesting that smoking history exerts a lasting effect on the maternal oral microbiome.

Conception mode (natural versus assisted) was likewise associated with oral microbial composition, overlapping with Clostridiales and Bacteroidales but uniquely extending to Actinomycetales, particularly in T2 (Fig. 2A, 3), suggesting that conception-related exposures may exert their influence most prominently during mid-gestation. In addition, Synergistia decreased from T1 to T2 across the general cohort, but women adhering to a gluten-free diet exhibited a significant positive correlation in T1 (FDR-adjusted $p = 0.017$). This divergence suggests that dietary factors may alter the timing or magnitude of microbial shifts during pregnancy. Together, these results highlight diet, smoking, and conception method as key host-related factors shaping the evolution of the oral microbiome across pregnancy, with diet exerting the most consistent and wide-reaching influence.


A
Survey
Medical Records
BMI
Activity
Diet
Smoking
Carbohydrates
Sleeping Hours
Stress
Medications
Calories
Aspirin
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Calories
Carbohydrates
Stress
Calories
Before Pregnancy (n=346)
First Trimester (n=235)
Second Trimester (n=144)
Third Trimester (n=88)
B
Relative abundance (%)
T1
T2
T3
Archaea;Euryarchaeota
Bacteria;Actinobacteria
Bacteria;Bacteroidetes
Bacteria;Chloroflexi
Bacteria;Cyanobacteria
Bacteria;Deferribacteres
Bacteria;Firmicutes
Bacteria;Fusobacteria
Bacteria;Proteobacteria
Bacteria;SR1
Bacteria;Spirochaetes
Bacteria;Synergistetes
Bacteria;TM7
Bacteria;Tenericutes
Bacteria;Verrucomicrobia
C
Shannon Index
T1
T2
T3
D
T1 vs T2
T2 vs T3
Erysipelotrichi
Flavobacteriia
Clostridia
Betaproteobacteria
Alphaproteobacteria
4C0d-2
Fusobacteriia
Epsilonproteobacteria
Synergistia
TM7-3
Coriobacteriia
Gammaproteobacteria
Deferribacteres
Verrucomicrobiae
Deltaproteobacteria
Bacilli
Actinobacteria
Mollicutes
Anaerolineae
Bacteroidia
Spirochaetes
Methanobacteria
Kingdom
Phylum
Class
Order
Family
Genus
Species
Sum Value
E
Bacteroides caccae (s)
Bacteroides ovatus (s)
Alistipes indistinctus (s)
Odoribacter (g)
Akkermansia muciniphila (s)
Streptococcus luteciae (s)
Catenibacterium (g)
Veillonella parvula (s)
Phascolarctobacterium (g)
Ruminococcus callidus (s)
Gemmiger formicilis (s)
Blautia obeum (s)
Coprococcus eutactus (s)
Prevotella copri (s)
7
F
Cosine Distance
Same Women T1 vs T2
Same Women T2 vs T3
Same Women T1 vs T3
Within T1
Within T2
Within T3

Figure 1: **Longitudinal changes in the oral microbiome between the different trimesters.** (A) Schematic overview of the study design and sampling strategy for the oral microbiome analysis of 346 pregnant women across trimesters (T1, T2, T3). (B) Stacked bar plots of microbial composition at the phylum level, with each color representing a different phylum and each bar corresponding to one sample (summing to 100% relative abundance per sample). The average distribution on the right highlights a decrease in Verrucomicrobiota during pregnancy (see Results). (C) Alpha diversity (Shannon diversity index) boxplots, with dots representing individual samples, showing medians and interquartile ranges. Statistical significance is indicated by asterisks (**$p < 0.01$, Mann-Whitney test, T1 > T2; ***$p < 0.001$, T1 > T3). A Kruskal-Wallis test confirmed overall group differences ($p < 0.01$, highlighted in pink). (D) GIMIC results depicting taxonomic shifts, with edge colors indicating comparisons: green for T1 vs. T2, black for T2 vs. T3. Bubbles summarize total differences across trimesters at each taxonomic level, with colors indicating enrichment (T1 vs. T2: red for T1, blue for T2; T2 vs. T3: red for T2, blue for T3), highlighting consistent shifts across taxonomic levels. (E) miMic test results identifying microbes with significant differences between T1 and T2, mapped onto a taxonomic tree. Dot colors indicate directionality (blue for increased in T2, red for decreased in T2), and dot size reflects the absolute Mann-Whitney test statistic. Background shading groups taxa by family. (F) Cosine distance boxplots comparing microbiome dissimilarity across scenarios, with colors distinguishing comparison types: same women across trimesters, different women within trimesters, and different women between trimesters. Higher values indicate greater dissimilarity, with significance marked by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Distances between samples from the same woman were consistently smaller than in other scenarios, with reduced variance in distances between different women in T2 and T3. See Fig. S3 for miMic results for T1 vs. T3; no results were found for T2 vs. T3, and Fig. S4 for additional distance metrics.

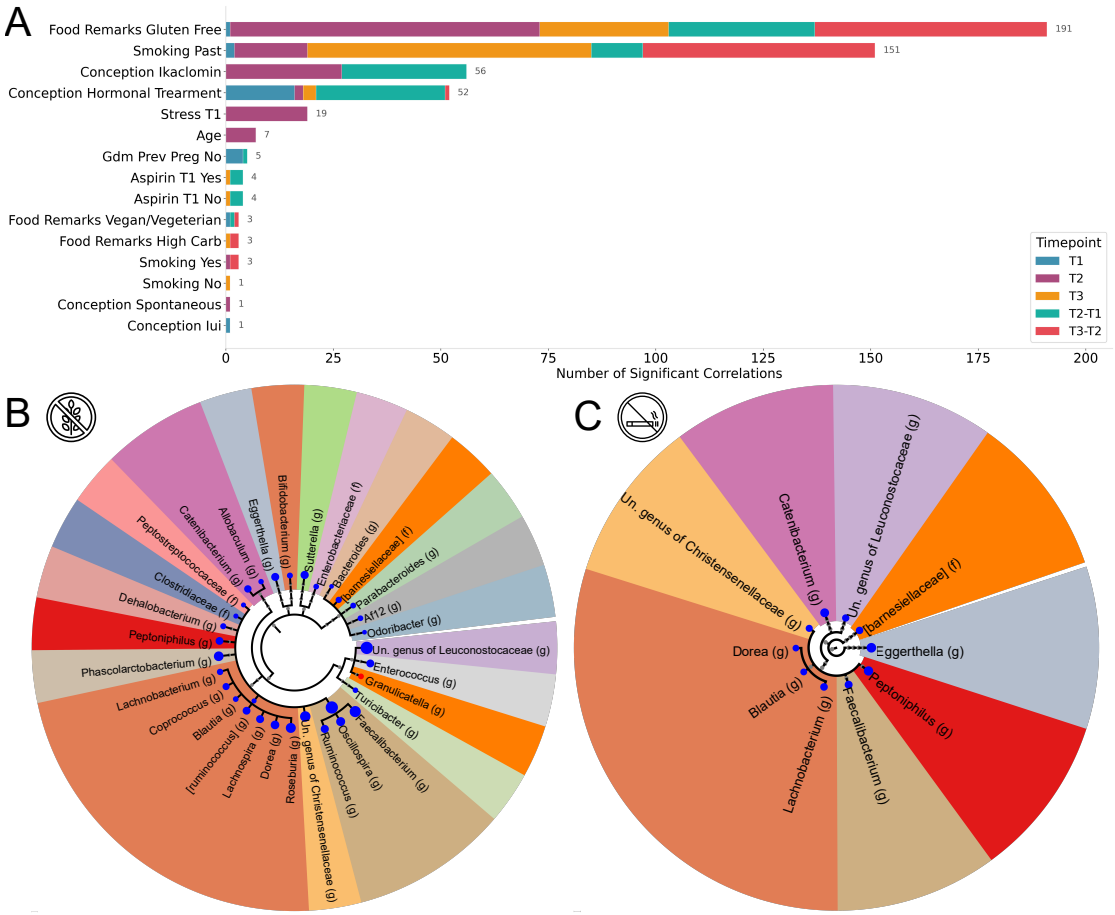

Figure 2: **Associations between Gluten-Free nutrition, conception, and the oral microbiome.**
(A) Bar plot showing the number of significant correlations (FDR-corrected, $p < 0.05$) between maternal features and microbial taxa in T1 (blue), T2 (purple), and T3 (orange), as well as differences between trimesters (T2–T1, light blue; T3–T2, red). The strongest associations were observed for adherence to a gluten-free diet, which was linked to microbial composition in all trimesters and changes between T1–T2 and T2–T3. Additional significant features included smoking in the past, among others. (B)(C) Circular taxonomic tree mapping microbes significantly associated with gluten-free nutrition, and smoking history, respectively, in oral microbiome T2. Following the same format as the miMic tree in Fig. 1E. Dot colors indicate directionality (blue for increased abundance, red for decreased abundance in women following a gluten-free diet, smoked in the past), dot size reflects the magnitude of the association, and background shading groups taxa by family. (B)(C) shared the same family colors. The trees were filtered to show only the significant genus and species taxa (See Fig. S7-S16 for additional full circular taxonomic trees for other features and trimesters, formatted similarly to the miMic tree).

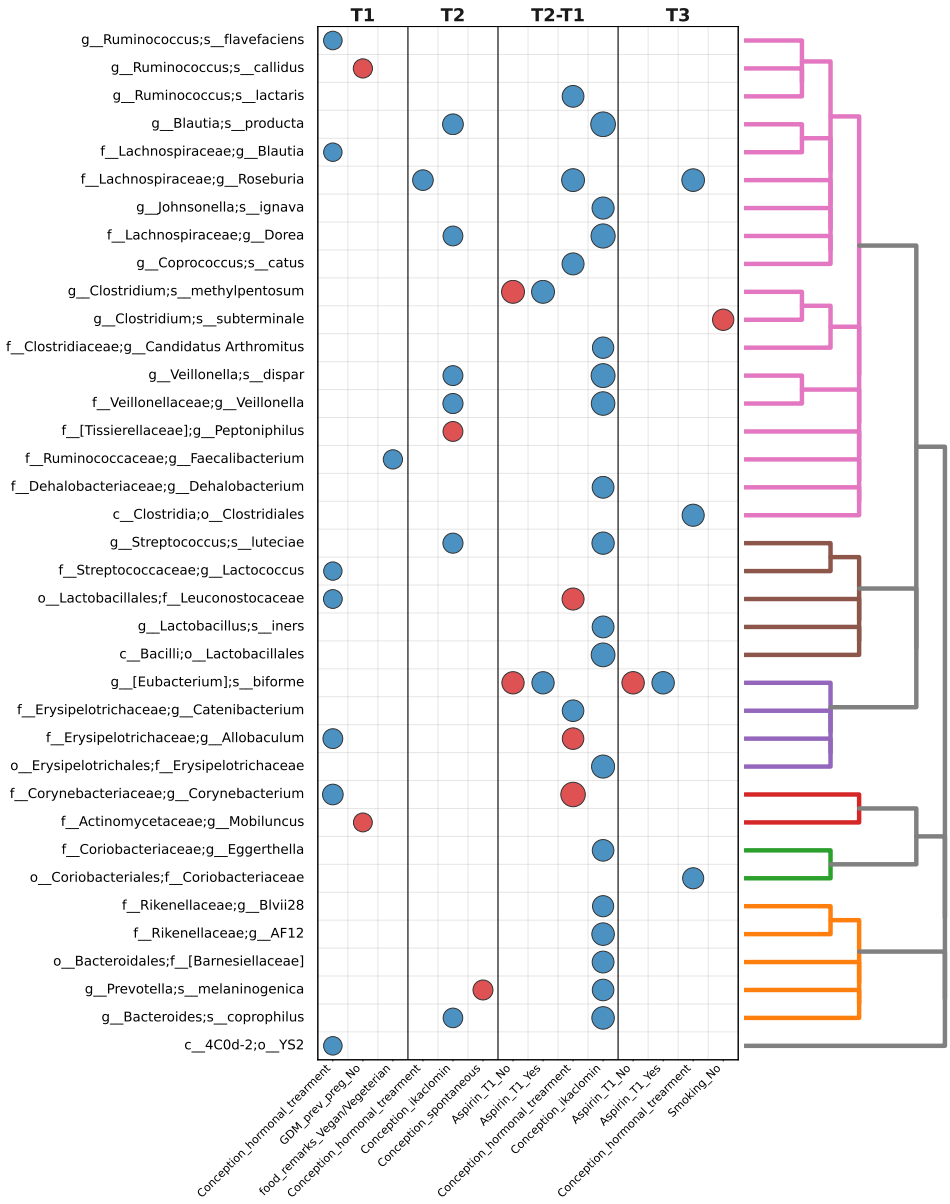

Figure 3: **Associations between maternal metadata and microbial taxa.** Heatmap of significant correlations (FDR-corrected, $p < 0.01$) between oral microbiome taxa and maternal metadata across pregnancy trimesters, excluding smoking and gluten-free nutrition (see Fig. 2). The left y-axis lists microbial taxa, and the right y-axis indicates their taxonomic linkage. The heatmap is divided into four panels: correlations within T1, within T2, differences between trimesters (T2–T1), and within T3. Differences between T3 and T2 were also analyzed but showed no significant associations after excluding smoking and gluten-free nutrition and applying FDR correction ($p < 0.01$). Columns represent maternal features (nutrition, demographics, and medical history) with significant associations (FDR-corrected, $p < 0.01$). Each dot denotes a significant association, with color indicating directionality (blue for positive, red for negative) and size reflecting the absolute value of the test statistic. Spearman's rank correlation was used for continuous metadata features, and biserial correlation was applied for binary features. See Fig. S6 for the complete heatmap of associations.

To validate our findings, we analyzed the oral microbiome in the Russian cohort (154 women) during T2 and T3, focusing on taxonomic shifts observed in the Israeli cohort. Consistent with the Israeli cohort, miMic test (see Methods) identified *Akkermansia muciniphila*, which continued to decrease significantly from T2 to T3 ($p = 0.0005$), reinforcing its decline throughout pregnancy. Similarly, *Veillonella parvula* maintained its increasing trend from T1 to T2 into T3 ($p = 0.004$), while *Phascolarctobacterium* showed a significant decrease in T3 ($p = 0.0009$). At the genus level, *Blautia* (containing *Blautia obeum*) and *Coprococcus* (containing *Coprococcus eutactus*), which were significant in the Israeli cohort, exhibited consistent decreases toward T3 (p = 7.502e-9 and p = 1.359e-5, respectively), mirroring their species-level trends. Additionally, *Prevotella copri* showed a bigger effect with a significant and progressive decline from T2 to T3 ($p = 1.031e-16$). The genus *Methanobrevibacter* also consistently decreased across all taxonomic levels toward T3 ($p = 4.53 - e5$) (Supplementary Material, Fig. S5). Alpha diversity, measured by Shannon diversity index, showed a reduction in T3 compared to T2 (median 2.98 and 2.93 in T2 and T3, respectively), though this decrease was not statistically significant ($p = 0.22$). Furthermore, in the Russian cohort, *Dialister* (Veillonellaceae family) in T2 was significantly associated with past smoking history (FDR-adjusted p = 0.01), aligning with smoking-related associations observed in the Israeli cohort. These results confirm the reproducibility of key microbial shifts and maternal factor associations across both cohorts, highlighting the robustness of the observed oral microbiome dynamics during pregnancy.

4 Discussion

To our knowledge, this study presents the first large-scale investigation of the evolution of the oral microbiome from T1 throughout pregnancy, addressing a significant gap in our understanding of microbial changes associated with early pregnancy. Our findings demonstrate significant changes in the structure of the oral microbial community during this critical period, characterized by a decrease in alpha diversity and distinct taxonomic changes, with notable associations with dietary factors and conception methods. Although earlier studies have documented oral microbiome alterations primarily in T2 and T3 [4, 8, 45], our results show that significant changes begin earlier in pregnancy. This pattern aligns with the well-established reduction in microbial richness observed in T3, suggesting a progressive shift toward a more specialized microbial community throughout pregnancy.

The significant decrease in Verrucomicrobiota, particularly *A. Muciniphila*, and the concurrent increase in Synergistota from T1 to T2 provides important insights into the microbial dynamics of early pregnancy. *A. Muciniphila*, a mucin-degrading bacterium best known for its role in gut health, is increasingly implicated in the maintenance of oral homeostasis[3]. Through its ability to strengthen mucosal barriers, modulate host immune responses, and produce anti-inflammatory metabolites such as short-chain fatty acids, *A. Muciniphila* may help counteract dysbiosis-driven oral diseases[13][9]. Experimental models show that supplementation with *A. Muciniphila* can attenuate inflammation and tissue damage induced by key oral pathogens, highlighting its potential role in protecting against periodontitis and peri-implantitis, and possibly in reducing risks linked to caries or oral mucositis[3]. In humans, *A. Muciniphila* has been detected in saliva, with prevalence varying by age and oral health status, underscoring its ecological relevance in the oral cavity. Together, these findings suggest that *A. Muciniphila* may act as a stabilizer of oral microbial communities during pregnancy. Its reduction in the oral cavity during early pregnancy may reflect systemic changes in host-microbe interactions driven by hormonal fluctuations. In contrast to the decline of *A. Muciniphila*, which is generally regarded as a stabilizing commensal, we observed that members of the Synergistota increased as pregnancy

progressed. Although typically low-abundance constituents of the oral microbiome, Synergistota have been consistently reported in subgingival plaque, periodontal pockets, and endodontic infections, often co-occurring with keystone periodontal pathogens such as *Porphyromonas gingivalis*and *Tannerella forsythia*[29]. Their enrichment has been associated with proteolytic metabolism, persistence in inflamed niches, and contribution to chronic biofilm dysbiosis. Thus, the simultaneous reduction of a protective commensal like *A. muciniphila* and the expansion of Synergistota may reflect a coordinated ecological remodeling of the oral microbiome during pregnancy, shifting from a barrier-supportive, anti-inflammatory profile toward one enriched in taxa linked to tissue destruction and inflammation[29].

The increase in Gammaproteobacteria and the decrease in Erysipelotrichia classes further support the concept of a directional shift in the structure of the microbial community between T1 and T2. Gammaproteobacteria are known members of the oral cavity, encompassing facultative anaerobes and opportunistic taxa that often expand under inflammatory or metabolically altered conditions[24]. Erysipelotrichia, in contrast, are more commonly associated with the gut microbiome, but they have also been detected in saliva, both in health and in association with metabolic conditions such as obesity[44]. Their presence in the oral cavity likely represents a low-abundance but responsive community whose dynamics may reflect broader host metabolic and immune status. In our cohort, the observed decline in Erysipelotrichia between T1 and T2, together with the rise in Gammaproteobacteria, suggests that early pregnancy creates an ecological environment that favors opportunistic oral taxa while reducing less-adapted groups. These findings are consistent with previous reports of increased opportunistic and pro-inflammatory taxa during pregnancy [14, 26], but in our data, these shifts emerged earlier, between T1 and T2.

Despite these systematic alterations, intra-individual profiles remained relatively conserved within individuals compared to between individuals. This suggests that while pregnancy exerts directional pressure on microbial community structure, individual microbial "signatures" persist. Importantly, the enrichment of Gammaproteobacteria and Synergistota together with the decline in *A. muciniphila* and Erysipelotrichia highlights an early ecological transition from commensal- and barrier-supporting taxa toward communities enriched in groups more often associated with dysbiosis, inflammation, and metabolic perturbations.

Building on the community-wide shifts observed between the trimesters, we next examined how maternal factors may modulate oral microbiome composition. Among the variables tested, the most striking associations were with diet, smoking history, and conception method. The strong association between a gluten-free (GF) diet and oral microbiome composition was among the most intriguing findings in our cohort. These associations persisted through T1 to T2 and T2 to T3 transitions. The predominantly positive correlations with taxa within Clostridiales and Bacteroidales suggest two non-exclusive mechanisms. First, eliminating gluten-rich staples may remove competing substrates, enabling expansion of taxa that exploit broader carbohydrate niches. Second, GF-focused substitutions (alternate starches and fibers) can reshape gut microbiome composition—this effect has been documented in human trials of low-gluten diets, which show shifts associated with fiber and carbohydrate intake. Smoking history also showed significant associations with oral microbial composition across trimesters, with the clearest effects evident in T2 and persisting into T3. This aligns with findings from cohort and review studies on smoking's impact, which highlight enrichment of periodontal-associated genera (e.g., *Prevotella*, *Porphyromonas*) and depletion of commensal *Proteobacteria* like *Neisseria*[16].

We also identified a robust association between conception method and the second-trimester oral microbiome, particularly involving Actinomycetales. Interestingly, this signal appeared to attenuate by T3, suggesting assisted reproductive technologies (ART) related effects may be strongest in early pregnancy. Possible mechanisms include hormonal stimulation during ART[46]

and links between oral dysbiosis and infertility-related conditions like polycystic ovary syndrome or endometriosis[28]. The conception method associations raise important questions about the long-term effects of ART on maternal microbial health. Future studies should investigate whether these changes persist throughout pregnancy and whether they correlate with pregnancy outcomes in ART-conceived pregnancies.

Importantly, these findings were validated in an independent cohort of 154 pregnant women from Russia, underscoring their generalizability across populations and geographic regions. In this cohort, we observed a continued decline in Akkermansia muciniphila from T2 to T3, reinforcing its role as a key taxon undergoing consistent reduction throughout pregnancy. Similarly, increases in *Veillonella parvula* and decreases in *Phascolarctobacterium* mirrored the temporal dynamics observed in the Israeli cohort. Additional taxa such as *Blautia*, *Coprococcus*, and *Prevotella copri* also demonstrated consistent trends, with significant decreases toward T3. The genus Methanobrevibacter showed a robust and progressive decline across trimesters, supporting a broader ecological restructuring. While the decrease in alpha diversity in T3 did not reach statistical significance in the Russian cohort, the overall direction of change and key taxonomic shifts were reproducible, affirming the robustness of our main findings. Notably, past smoking history in T2 was again associated with *Dialister* abundance, echoing the smoking-related associations found in the Israeli dataset. These results confirm that early and progressive changes in the oral microbiome during pregnancy are not only systematic but replicable across distinct populations, further supporting their relevance to maternal-fetal health.

4.1 Limitations and Considerations

Limitations of our study should be acknowledged. The observational design precludes causal inference, and oral health status (e.g., gingivitis, caries, salivary pH) was not systematically assessed, though these factors may influence microbiome composition. Sequencing was limited to 16S rRNA analysis, restricting taxonomic resolution and functional inference compared with metagenomics or metabolomics. Dietary data were self-reported, raising the possibility of recall bias, and sampling at fixed gestational weeks may not fully capture continuous microbial dynamics. Future work integrating functional omics, oral health assessments, and intervention studies will be critical to determine whether modulating the maternal oral microbiome can improve pregnancy outcomes and long-term offspring health.

5 Conclusion

This study provides the first large-scale longitudinal analysis of the oral microbiome across pregnancy, revealing early and progressive changes beginning in the first trimester. We demonstrate that pregnancy is associated with systematic shifts in microbial diversity and composition, including reductions in protective commensals such as *A. muciniphila* and increases in opportunistic taxa such as Synergistota and Gammaproteobacteria. These changes appear to reflect a directional ecological transition toward communities enriched in taxa linked to dysbiosis and inflammation. Importantly, maternal factors including diet, smoking history, and conception method were strongly associated with microbial profiles, highlighting the oral microbiome as a sensitive marker of gestational physiology. Validation in an independent cohort confirmed the reproducibility and generalizability of these findings across populations. Together, these results underscore the potential of the maternal oral microbiome as both a window into pregnancy-related physiological changes and a target for interventions aimed at improving maternal and fetal health.

6 Declarations

6.1 Ethics approval and consent to participate

Informed consent was obtained from all Israeli participants, in accordance with Helsinki ethics permit #0135-15-COM from the Clalit HMO Institutional Review Board and Helsinki ethics permit #0263-15-RMC from the Rabin Medical Center Institutional Review Board. Russian cohort was approved by the local ethics committee of the Almazov National Medical Research Centre, Russia (protocol no. 119)

6.2 Availability of data and material

All the datasets are available as taxa-tables with their respective tag in the GitHub repository- https://github.com/louzounlab/Development_of_oral_microbiome. All sequencing data were submitted to EBI (ERP143097).

6.3 Competing interests

We do not have competing interests.

6.4 Funding

No funding was received for this research.

Authors' contributions

S.F. performed the computational analysis and wrote the manuscript. T.S. contributed to writing the manuscript. P.P., A.S.T., E.A.V., A.D.A., E.A.P., T.M.P., and E.N.G. performed the Russian cohort validation study. Si.F., O.S., A.R., E.T., M.N.O., Y.P., M.H., B.S., and E.H. contributed to data collection and analysis. L.Y. and O.K. supervised this work and wrote the manuscript.

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