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Women's multisite microbial modulation during pregnancy

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ABSTRACT

The composition of female microbiome varies with age, physiological and socio-behavior conditions. Also, changes in microbiome composition are observed as pregnancy progresses, especially in the vaginal site. Together with the physiological adaptations of gestation, changes in microbiome composition seem to be fundamental for proper fetal development. This study aimed at simultaneously evaluating the vaginal, gut, and oral microbiome of healthy pregnant women, and comparing it with those observed in healthy non-pregnant women of reproductive age. In a cross-sectional study, vaginal, oral and gut samples were collected from 42 pregnant and 18 non-pregnant women, and the microbiome composition was evaluated by *16S rRNA* sequencing, using Illumina platform. In the pregnant group, we observed a positive correlation between *Eubacterium* and *Akkermansia* in the gut samples; between *Eubacterium* and *Ruminococcus* in the vaginal samples; and between *Streptococcus* and *Gemella* in the oral samples. Notwithstanding, we observed a negative correlation between *Lactobacillus* and *Atopobium* and between *Lactobacillus* and *Gardnerella* in vaginal microbiome. *Prevotella* was the only genus found in all three sites studied; however, there was no signal of bacterial influence between sites during pregnancy. These results suggest that in addition to hormonal and immunological variations during healthy pregnancy, the female body also undergoes microbiome modulation in multiple sites in order to maintain an eubiotic status.

1. Introduction

Human body has a community of symbiotic microorganisms involved in several essential life processes [1].The microbiome exerts a critical influence on the host's metabolism and immune system modulation [2]. The host-microbiome relationship affects phenotypic plasticity and genetic expression, thus interfering on host health [3].

Female microbiome composition varies with age, hormone production, menstrual cycle, use of medicines, and sexual activity. Evidence shows that the microbiome pattern is important to reproductive and genital tract health [3]. Therefore, several physiological adaptations occur during pregnancy in order to enable adequate fetal development, while maintaining maternal health [4]. Several evidence suggest that changes in microbiome composition are observed as pregnancy progresses, especially in the vaginal site [5].

Vaginal microbiome of pregnant women is composed of a higher number of *Lactobacillus,* compared to non-pregnant women. This change seems to be beneficial, as it is well-known that these bacteria have an important role in vaginal immunity, and are responsible for producing lactic acidic, thereby decreasing local pH [6]. Overall, vaginal microbiome composition tends to remain more stable as pregnancy progresses; however, there is high intra-individual variability, in addition to enrichment of community groups, such as *Lactobacillus*, Actinomycetes and Bacteroidetes [7].

The gut microbiome modulates both local and systemic immunity, and affects the physiological adaptations occurring during pregnancy [8]. The composition of intestinal microbiome in the first trimester is similar to that observed in non-pregnant women. However, as pregnancy progresses, significant changes are observed, and diversity increases while richness decreases [9]. Recent studies have shown that a decreased diversity in gut microbiome is associated with a dysbiotic profile, inducing the production of inflammatory markers [8]. In turn, this altered pattern has been associated with different gestational pathologies [9,10].

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A similar microbial adaptation condition occurs in oral mucosa. The oral microbiome acts on the protection against pathogens and on metabolism, and this community is mainly composed of *Streptococcus, Neisseria* and *Prevotella* genera [11]. Dysbiosis in the oral tract due to chronic periodontitis during pregnancy is associated with many obstetric pathologies, such as preterm birth and preeclampsia [12].

In recent years, a growing number of studies have been published reporting the microbiome composition at different body sites in both pregnant and non-pregnant healthy women. However, most of these investigations assessed one single niche (vaginal, intestinal or oral); many of them evaluated solely non-pregnant women; and others compared the microbiome profile of healthy pregnant women with that observed in a group of patients with a specific obstetric disease. Considering these observations and the critical role of microbiome on reproductive process, we aimed at simultaneously evaluating vaginal, gut, and oral microbiome, and investigating whether there are changes in the microbiota during healthy pregnancy, in order to maintain an eubiotic profile.

2. Material and methods

2.1. Subjects

This study enrolled 42 women in the third trimester of pregnancy (28–36 weeks) at antenatal care clinics, and 18 non-pregnant women at the family planning clinic of the Obstetrics Department of the Federal University of São Paulo (Universidade Federal de São Paulo - UNIFESP-EPM), between 2014 and 2016. All women enrolled lived in the city of São Paulo.

Inclusion criteria for pregnant women included gestational age between 28 and 36 weeks at recruitment (based on menstrual dates confirmed through obstetric ultrasound), live fetus and singleton pregnancy. All pregnancies were monitored until the end, in order to ensure that there were no clinical intercurrences. The non-pregnant group consisted of healthy reproductive-aged (19–44 years old) women, who were instructed to appoint vaginal collection between the 10^{th} and 15^{th} day of menstrual cycle. Both pregnant and non-pregnant participants should be antibiotic-free for 3 months prior to sampling and were instructed to avoid sexual intercourse within three days prior to vaginal collection. All women enrolled were non-smokers and non-alcohol users.

The exclusion criteria for pregnant women included fetal death, multiple pregnancy, in vitro fertilization, uterine malformations, and placental abruption. Exclusion criteria for all participants were: infections, cancer and any other preexisting disease, such as autoimmune diseases, diabetes, hypertension, and use of vaginal or oral probiotics.

2.2. Sample collection

Vaginal, oral and fecal samples were collected from each woman enrolled in this study. Each participant was instructed to collect one stool sample at home using a dry sterile stool collector, keep it in a freezer (−20°C) and then transport it to the clinic in an ice-filled polystyrene container (previously supplied to the patient) in the medical appointment day. The vaginal sample was collected from the vaginal cervix by the attending physician on the same day the stool sample was delivered, and the secretion was placed in buffered medium (phosphate-buffered saline with 10% glycerol). The oral sample was collected on the same day using a sterile swab. After collection, samples were stored immediately in a freezer at - 80°C.

2.3. Microbiome sequencing

Bacterial DNA was obtained from vaginal and oral samples using the QIAamp DNA Blood Mini Kit (Qiagen). Bacterial DNA from stool samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). The amplification of V4 region (fecal samples [13]) or V3–V4 region (vaginal and oral samples [14,15]) of the *16S rRNA* gene was performed by 25 cycles, using the previously described primers and conditions. Negative controls with buffer from the DNA extraction kit were included in the PCR runs. The amplicons were pooled and loaded onto Illumina MiSeq clamshell style cartridge kit v2 at 500 cycles, for paired-end 250 sequencing at a final concentration of 12 pM. The library was clustered to a density of approximately 820 k/mm^2. The MiSeq platform was used for image analysis, base calling, and data quality assessment. The raw read files were demultiplexed and then analyzed using QIIME software, version 1.9 [16]. The chimeric sequences were identified and excluded using usearch61 [17]. Based on 99% similarity, the remaining sequences were compared against Silva's database version 128 and grouped into operational taxonomic units (OTUs) [18,19], which were subsequently filtered. The relative abundance rate of the bacteria was obtained in relation to the main phyla and genera found.

For each library, alpha and beta diversity indexes were calculated, and their analysis refers to the species variety and complexity in a community. For alpha diversity, Chao1 [20] was used to estimate richness by the total number of species found in a community. Shannon's diversity index was applied to assess the uncertainty degree in predicting the sum of the proportion of each species in relation to the total number of species in the community under analysis. Simpson's index reflects the probability that two individuals randomly chosen from the community belong to the same species, ranging from 0 to 1: the higher the index, the greater the probability that individuals belong to the same species, i.e., greater dominance and less diversity [21,22]. Then, using MicrobiomeAnalyst [23], a web-based tool, the main coordinate analysis (PCoA), based on weighted and unweighted UniFrac phylogenetic [24] distance matrices, was constructed to observe the differences in beta diversity between sites and groups [25]. Nucleic acid sequences are available at the Sequence Read Archive (SRA) under accession number SRP126329. The phylogenetic tree for *Prevotella* was constructed by filtering all sequences using Qiime 1.9 assigned to *Prevotella* genus; then, these sequences were aligned to '*Prevotella 16S* Ribossomal RNA' from all NCBI (www.ncbi.nih.gov.usa) databases using Muscle method by MEGA X [26]. The phylogenetic trees were carried out by using the general time reversible (GTR) model. Bootstrap values were calculated from 250 bootstrap replicates. The genetic distance for these samples was calculated in the same software [26,27]. For *Lactobacillus* species classified in vaginal samples, SPINGO software (Species-level Identification of metaGenOmic amplicons) was used, comparing the sequences obtained in the sequencing process with a known database, RDP (Ribosomal Database Project) [28], using default parameters. To this end, the software identifies unique sequences of specific species present in the *16S rRNA* gene to assign their classifications.

2.4. Statistical analyses

Kolmogorov-Smirnov or Shapiro-Wilk tests and Skewness and Kurtosis values were used to assess the distribution of quantitative variables. Student's *t*-test or Mann-Whitney test was applied for continuous variables. Spearman's correlation was used to correlate the results of bacterial genera within groups. All analyses were performed using standard software (GraphPad Prism, v7.0 for Windows). All tests were considered significant at $p < 0.05$. Phyla and bacterial abundance were presented as a percentage, following the order 'Non-pregnant group x pregnant group' in the results section.

2.5. Ethical approval

The study was approved by the Ethics Committee of Universidade Federal de São Paulo (UNIFESP-Process 842.143/2014 at October 21st, 2014) and written informed consent was obtained from all participants.

3. Results

3.1. Demographics

This study enrolled 60 subjects, comprising two groups: 18 non-pregnant women and 42 women in the third trimester of pregnancy. There was no statistically significant difference in age. As expected, the pregnant group had a higher body mass index (BMI). There was a significant difference between groups in ethnicity: half of the pregnant women had black and white ancestry (mixed race), while 72.22% of the non-pregnant women were white (Table S1). Logistic regression analysis showed that these variables (BMI and race) had no impact on the results.

3.2. Relative abundance (phyla and genera)

3.2.1. Vaginal samples

The relative abundance analysis showed predominance of Phylum Firmicutes in both groups, which was slightly increased in the pregnant group compared to the non-pregnant group (89.2% × 97.7%, $P = 0.10$) (Fig. S1a and Table S2).

Predominance of *Lactobacillus* was observed in both groups, with differences in the relative abundances (85.32% x 92.5%, $P = 0.30$) (Fig. 1a, Fig. S2a and Table S3), although not statically significant. The lowest *Lactobacillus* abundances and inter quartile ranges were observed in non-pregnant women (Table S3). Other genera, such as *Gardnerella, Prevotella, Sneathia* (Fig. 1b, c and 1d, respectively) showed lower relative abundances in the pregnant group compared to the non-pregnant group, but the differences did not achieve statistical significance. The increased abundance of *Lactobacillus* in vaginal samples during pregnancy was related to decreased *Atopobium* and *Gardnerella* abundances (Table S4 and Table S5). Statistical analyses revealed that the abundance of *Lactobacillus* showed a negative correlation with *Atopobium* ($r = -0.50$, $P < 0.01$) and *Gardnerella* ($r = -0.75$, $P < 0.01$), while the abundance of *Atopobium* showed a positive correlation with *Gardnerella* ($r = 0.61$, $P < 0.01$), *Sneathia* ($r = 0.55$, $P < 0.01$) and *Prevotella* ($r = 0.49$, $P < 0.01$) (Table S4 and Table S5).

The bacterial species in vaginal microbiota were also assessed qualitatively. In the classified sequences, there was a sharing of species in vaginal microbiota, such as *L. inners, L. jensenii, Gardnerella vaginallis, Prevotella timonensis* and *Atopobioum vaginae.* Most of the classified *Lactobacillus* observed in both pregnant and non-pregnant women were *L. iners* and *L. jensenii* species (Fig. S3). Species such as *Saccharofermentans acetigenes, Dialister micraerophilus, Mycoplasma hominis, Corynebacterium aurimucosum, Corynebacterium aurimucosum, Porphyromonas uenonis, Dialister succinatiphilus,* and *Lactobacus johnsonii*, were observed only in pregnant women.

3.2.2. Gut samples

Similar gut microbiome profiles were observed for non-pregnant and pregnant women, with a predominance of Firmicutes and Bacteroidetes in both groups. There are slight differences in the relative abundances of some Phylum across the groups (Fig. S1b and Table S2), but with no statistical significance.

The main genera found in both groups were *Bacteroides* and *Ruminococcus*. The pregnant group showed a tendency for increased abundance of *Akkermansia* and *Christensenellaceae*. In contrast, a trend

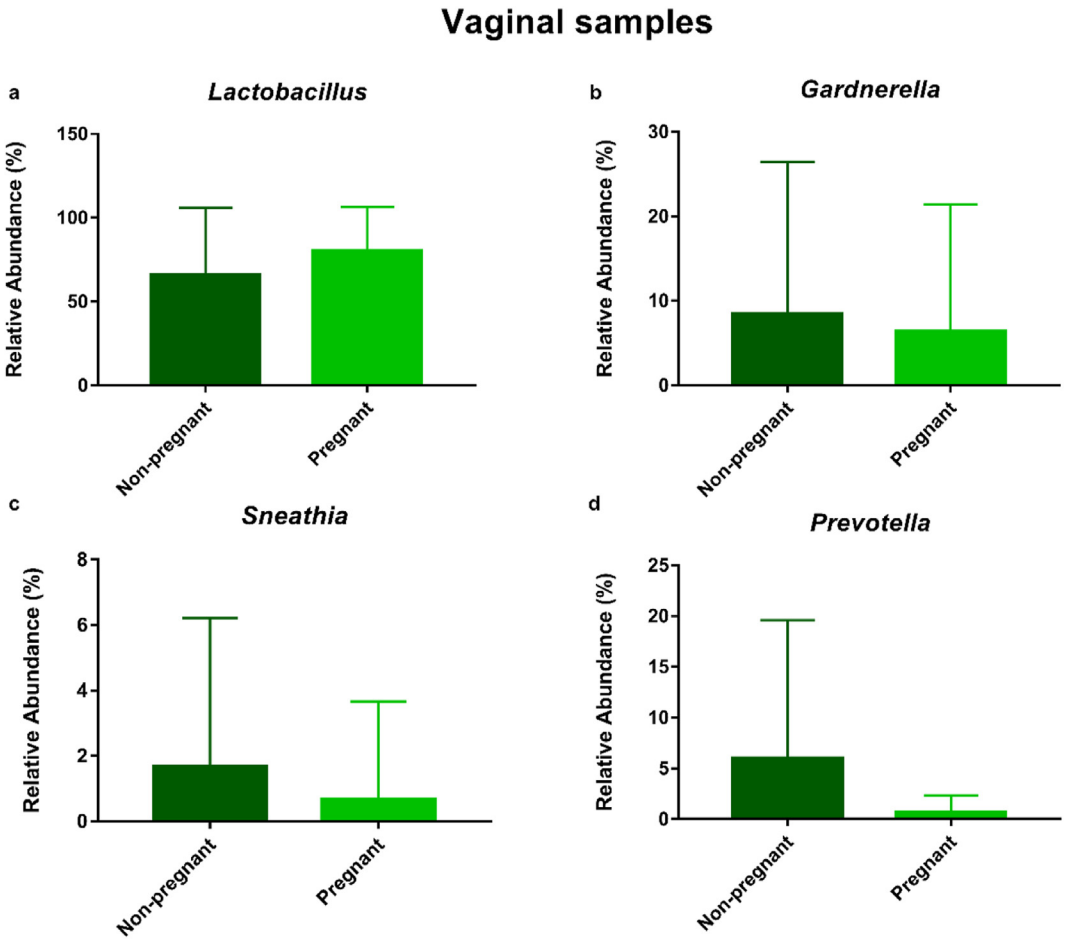

Fig. 1. Main bacterial genera variation between groups in vaginal site, (a) Lactobacillus; (b) Gardnerella; (c) Sneathia and (d) Prevotella (*$P < 0.05$).

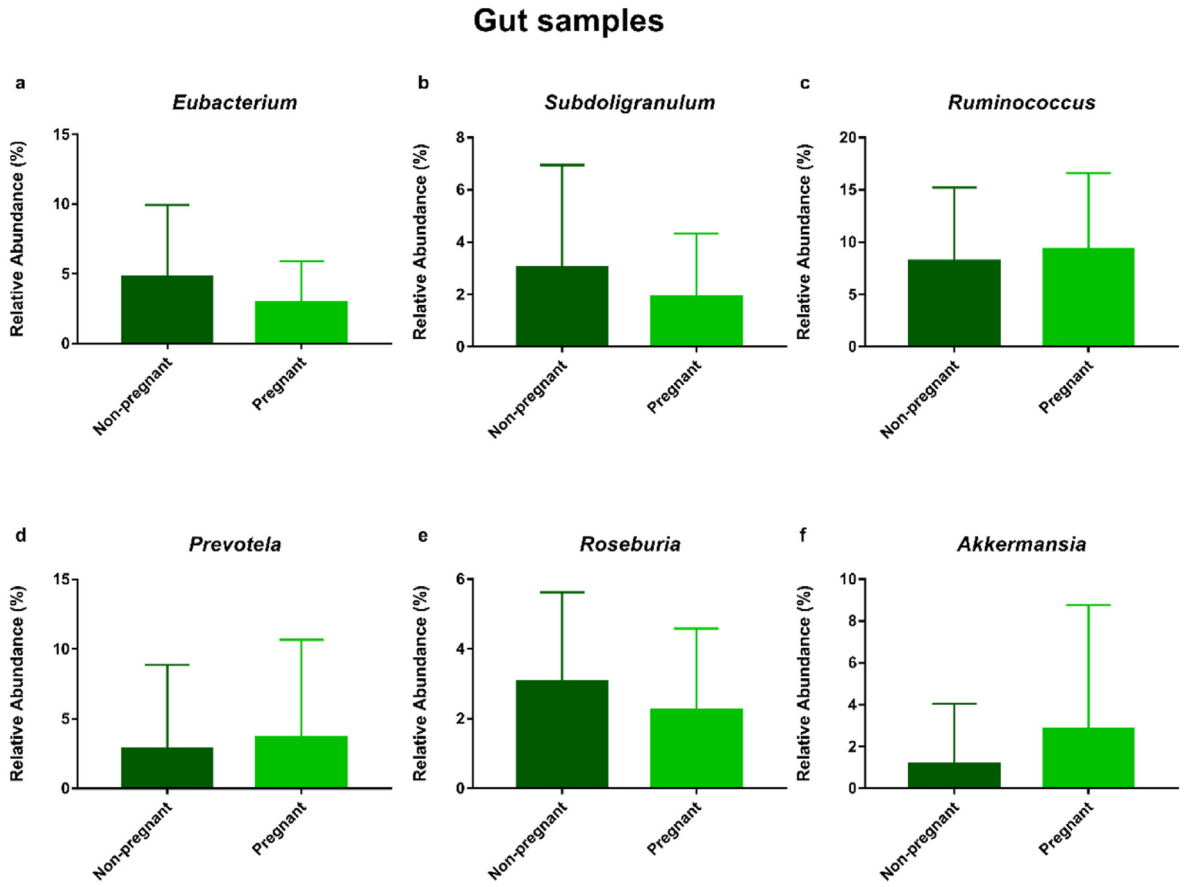

Fig. 2. Main bacterial genera variation between groups in gut site, (a) Eubacterium; (b) Subdoligranulum; (c) Ruminococcus; (d) Prevotella; (e) Roseburiaand (f) Akkermansia (*P < 0.05).

towards increased abundance of *Dialister*, *Eubacterium* and *Roseburia* was observed in the non-pregnant group (Fig. 2a and e, Fig. S2 and Table S3). Furthermore, a positive correlation of *Eubacterium* with *Akkermansia* ($r = 0.56$, $P < 0.01$) and *Eubacterium* with *Ruminococcus* ($r = 0.45$, $P = 0.01$) (Table S3) was noted in the gut microbiome of pregnant women (Table S6 and Table S7).

3.2.3. Oral samples

Significant differences were observed in the relative abundance of oral microbiome between the groups. Phylum Firmicutes was more abundant (37.4% × 60.0%, $P < 0.01$), whereas Proteobacteria was less abundant (36.6% × 17.15%, $P < 0.01$) in the pregnant group, compared to the non-pregnant group (Table S2). Additionally, Bacteroidetes and Fusobacteria Phyla were less abundant and Actinobacteria was more abundant in the pregnant group (Fig. S1 and Table S2). Regarding genera, a significant predominance of *Streptococcus* (12.9% × 26.0%, $P < 0.01$) and *Gemella* (2.0% × 4.2%, $P = 0.03$) was noted, as well as an increased abundance of *Granulicatella* (0.6% × 1.1%, $P = 0.05$) and *Prevotella* (1.8% × 2.5%, $P = 0.57$) in the pregnant group compared to the non-pregnant group (Fig. 3, Fig. S2 and Table S3). Genera *Haemophilus* (16.6% × 6.7%, $P < 0.01$), *Neisseria* (3.9% x 2.25, $P < 0.01$) and *Veillonella* (10.4% × 9.9%, $P = 0.80$) were less abundant in the pregnant compared to the non-pregnant group (Fig. 3, Fig. S2 and Table S3). In the oral microbiome, *Streptococcus* showed a positive correlation with *Gemella* ($r = 0.74$, $P < 0.01$) and a negative correlation with *Fusobacterium* ($r = -0.46$, $P < 0.01$) and *Prevotella* ($r = -0.60$, $P < 0.01$) in the pregnant group only (Table S8 and Table S9).

3.3. Alpha and beta diversity

Alpha diversity analysis of the vaginal samples showed a statistically significant increase in Chao 1 richness estimator in the pregnant group (1308 x 2108, $P = 0.04$) compared to the non-pregnant group (Fig. 4a). Furthermore, both Shannon and Simpson diversity indices were higher in the pregnant group (Shannon 2.67 x 2.89, $P = 0.15$ and Simpson 0.74 x 0.77, $P = 0.44$) (Fig. 4b and c). Increased Chao 1 value (3200 x 3230, $P = 0.89$) (Fig. 4d) and decreased Shannon (5.10 x 4.94, $P = 0.06$) (Fig. 4e) and Simpson (0.97 x 0.96, $P = 0.07$) (Fig. 4f) values were observed in the pregnant versus non-pregnant group, through the analysis of alpha diversity on gut samples. However, these differences were not statistically significant. The analysis of oral samples showed that Chao 1 richness estimator (2449 x 2130, $P = 0.34$) (Fig. 4g) and Shannon diversity index (4.41 x 4.42, $P = 0.98$) (Fig. 4h) were higher in the non-pregnant group, while the Simpson diversity index was higher in the pregnant group (0.93 x 0.94, $P = 0.59$) (Fig. 4i).

For beta diversity analysis, the PCoA based on weighted and unweighted UniFrac distance matrix was constructed. The analyses of the 3 sites in the same coordinates showed that each site clustered separately (Fig. 5a and b). There is a slightly proximity between bacterial communities in weighted plot seen in 2D screen, and there are no differences on clustering pattern of pregnant and non-pregnant groups for each site. We also analyzed the weighted and unweighted PCoA plot of each site individually (5c and 5d – Vaginal, 5e and 5f – Gut and 5g and 5h – Oral), where each spot represents one study participant. No statistically significant differences were observed between groups in vaginal and gut sample analyses. The vaginal microbiome community was not clustered according to the group, and the pregnant group seems to have a lower diversity than non-pregnant group, clustered within in

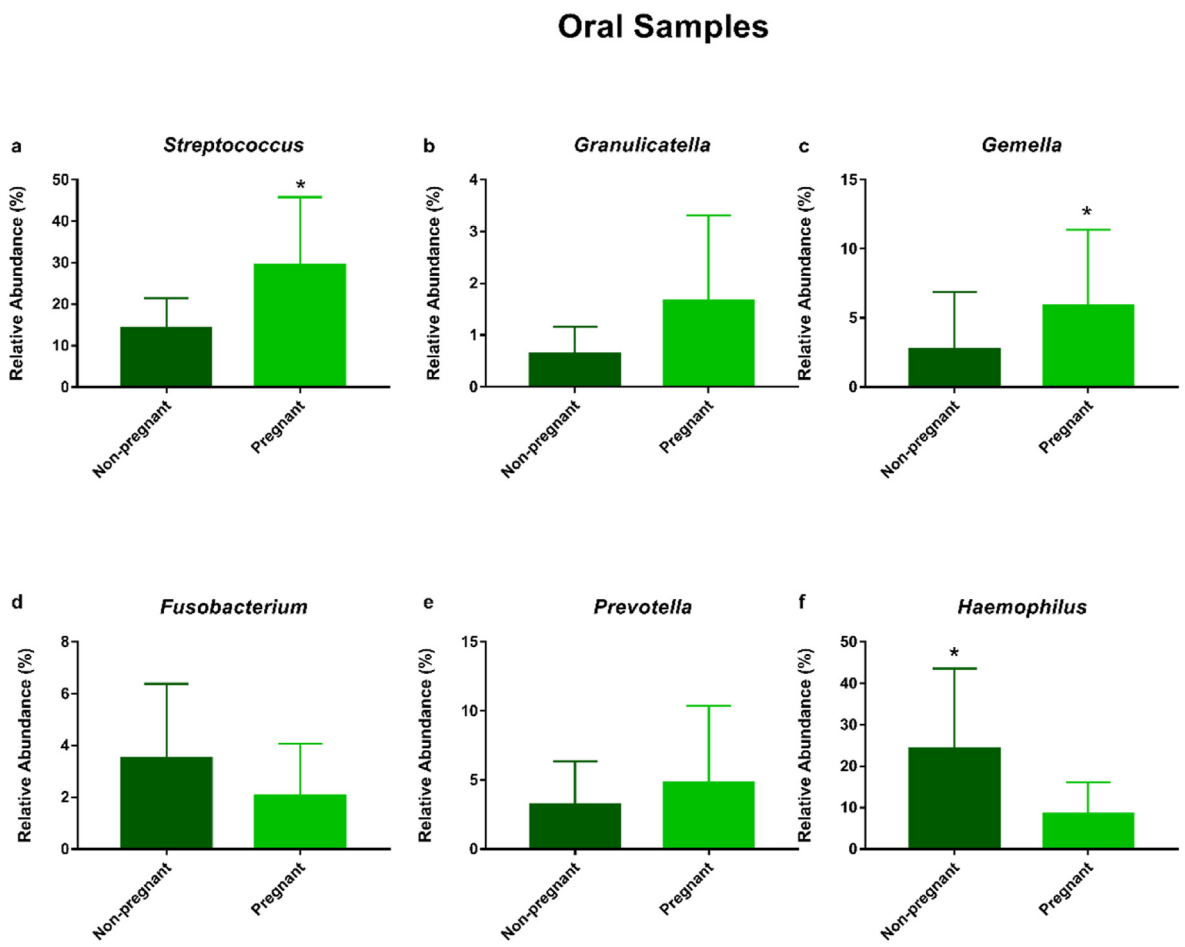

Fig. 3. Main bacterial genera variation between groups in oral site, (a) Streptococcus; (b) Granulicatella; (c) Gemella; (d) Fusobacterium; (e) Prevotella and (f) Haemophilus (*P < 0.05).

both weighted and unweighted plots (Fig. 5c and d). The gut microbial community showed a higher diversity in pregnant bacterial community, with the non-pregnant samples clustered within it in both weighted and unweighted plots (Fig. 5e and f). The oral microbial community showed a tendency to cluster in group in weighted plot, with statistically significant differences between groups ($P < 0.005$, F value = 1.31) (Fig. 5g and h).

4. Discussion

In addition to hormonal, metabolic and immunological changes observed in women's body during pregnancy [29], the microbiome is also known to have a major influence on gestation progress [5,9]. Although several studies have investigated the role of multiple sites microbiome in pregnancy, it remains unclear. Therefore, we simultaneously analyzed the composition of oral, vaginal and intestinal microbiome of healthy pregnant and non-pregnant women.

Vaginal microbiome composition changes throughout pregnancy, with an increase of *Lactobacillus* [5], related to estrogen production [3,30]. In turn, our results point out to a microbial modulation in vaginal site during pregnancy, since the *Lactobacillus* abundance was negatively correlated to *Atopobium* and *Gardnerella* abundances. This modulation was also observed between pathogenic genera, in which the presence of one or two pathogenic bacteria could stimulate the increase of others, since there was a positive correlation between *Atopobiun*, *Gardnerella*, *Sneathia* and *Prevotella*, all already described as potential dysbiotic members of the vaginal microbiome [5,31]. We can hypothesize that in women with increased *Gardnerella* and/or *Atopobium* abundance, the vaginal mucosal site is hostile to *Lactobacillus* colonization, and therefore a favorable environment for dysbiotic bacteria growth.

Physiological adaptations in the gut environment during pregnancy have shown to be related to changes in microbial composition [32]. Increased *Ruminococcus* and *Akkermansia* abundances were noted in the gut microbiome during pregnancy. *Ruminococcus* is a butyrate producer that increases the levels of short chain fatty acids (SCFA), thus increasing the presence of T regulatory cells (Treg), as previously suggested [31,32]. Increased *Akkermansia* in the gut microbiome of pregnant women is also related to eubiotic modulation, as this genus has been related to metabolic homeostasis changes in obese and diabetic patients after diet or bariatric surgery [33,34]. Our data showed a positive correlation between *Eubacterium* and *Akkermansia* and *Rummincoccus* in pregnant women. Thus, this might be a sign that these bacteria are involved in pregnancy eubiosis.

The oral microbiome in pregnant women showed a significantly increased abundance of *Streptococcus* and *Gemella,* with a concomitant decrease in *Fusobacterium* and *Prevotella*. Several studies have shown that *Streptococcus* has a crucial role in the oral microbiome, supporting the dental plaque microbiome formation [35]. *Fusobacterium* is a dysbiotic bacterial genus, associated with periodontal disease, as well as the attachment and invasion of epithelial cells [12]. Recent investigations suggest that the oral microbiome has a great influence on placental microbiome composition [36]. However, it remains unclear how the oral and placental microbiome interact, and the relation between the bacterial communities of both sites. A recent study in non-pregnant and pregnant women suggested a shaping in the supragingival

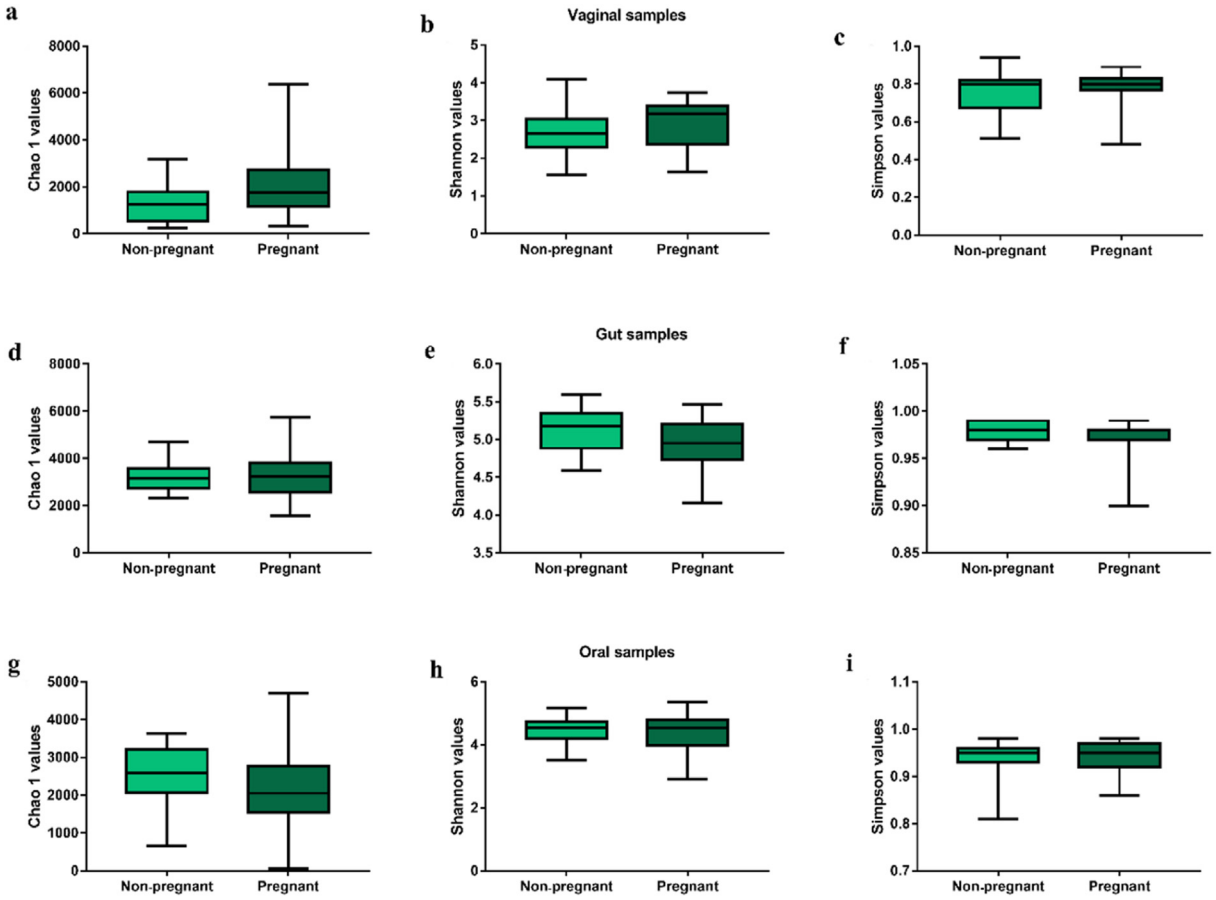

Fig. 4. Alpha diversity indices between groups in: Vaginal samples - (a) Chao 1, (b) Shannon and (c) Simpson; Gut samples - (d) Chao 1, (e) Shannon and (f) Simpson and Oral samples - (g) Chao 1, (h) Shannon and (i) Simpson.

microbiome associated with pregnancy [37]. Our results are in accordance with the literature, reinforcing the importance of oral microbial modulation during pregnancy, maintaining an eubiotic environment, and protecting the host.

Interestingly, using a cutoff of 1% as a filter for the microbial abundance of each studied site, *Prevotella* was the only bacterial genus found in all three microbiomes. Its abundance was higher in oral and gut sites and lower in the vaginal site. In pregnant women, there was a closer phylogenetic relationship between *Prevotella* OTUs from gut, oral, and to a lesser extent from vaginal sites (Fig. S1 and S2). Several reports describe the importance of *Prevotella* for human microbiome in eubiotic [38,39] and dysbiotic [40,41] conditions. Despite this genetic plasticity [42], and the presence of *Prevotella* in the three studied sites, we failed to establish any relation between microbiome's niches. This proposition is corroborated by UniFrac analyses, since the dominant bacteria of each site determine the microbial community.

To date, this is the first study presenting the microbiome profile of three different sites in samples collected simultaneously from healthy pregnant and non-pregnant women. Wang et al. (2018) recently described the microbial composition of these sites in gestational diabetes mellitus (GDM), reinforcing the GDM-related dysbiosis status [43]. DiGiulio et al. (2015) characterized the microbial variation during pregnancy in four body sites: vagina, distal gut (stool), saliva, and tooth/gum, describing microbial stability during pregnancy at all body sites [6]. Goltsman et al. (2018) assessed the microbiome composition from different sites of preterm and term pregnant women and found that obstetric complications were correlated to changes in the profile of gut and oral microbiome. However, the study did not enroll non-pregnant women to compare the results [44].

Our results show that, despite the presence of *Prevotella* in all three studied sites, there was no bacterial influence between sites during pregnancy. The homeostasis observed in the microbiome of the sites during pregnancy may have some role in systemic control.

We can point out that there are differences between the groups regarding race and BMI; however, logistic regression analysis showed that these variables did not affect the results. We also recognize that the small sample size of non-pregnant women is a limitation for this study. The inclusion of more participants could increase the strength of data on microbial abundance variation and bacteria genera, and lead to significant results, especially in the gut microbiome, where we found higher bacterial diversity compared to the other sites.

5. Conclusions

Our results suggest that there was no bacterial influence between sites during pregnancy; however, there is microbiome modulation in multiple sites in order to maintain an eubiotic status during pregnancy. Therefore, the characterization of microbiome profile and its modulation as pregnancy progresses will contribute to the improvement of female reproductive health.

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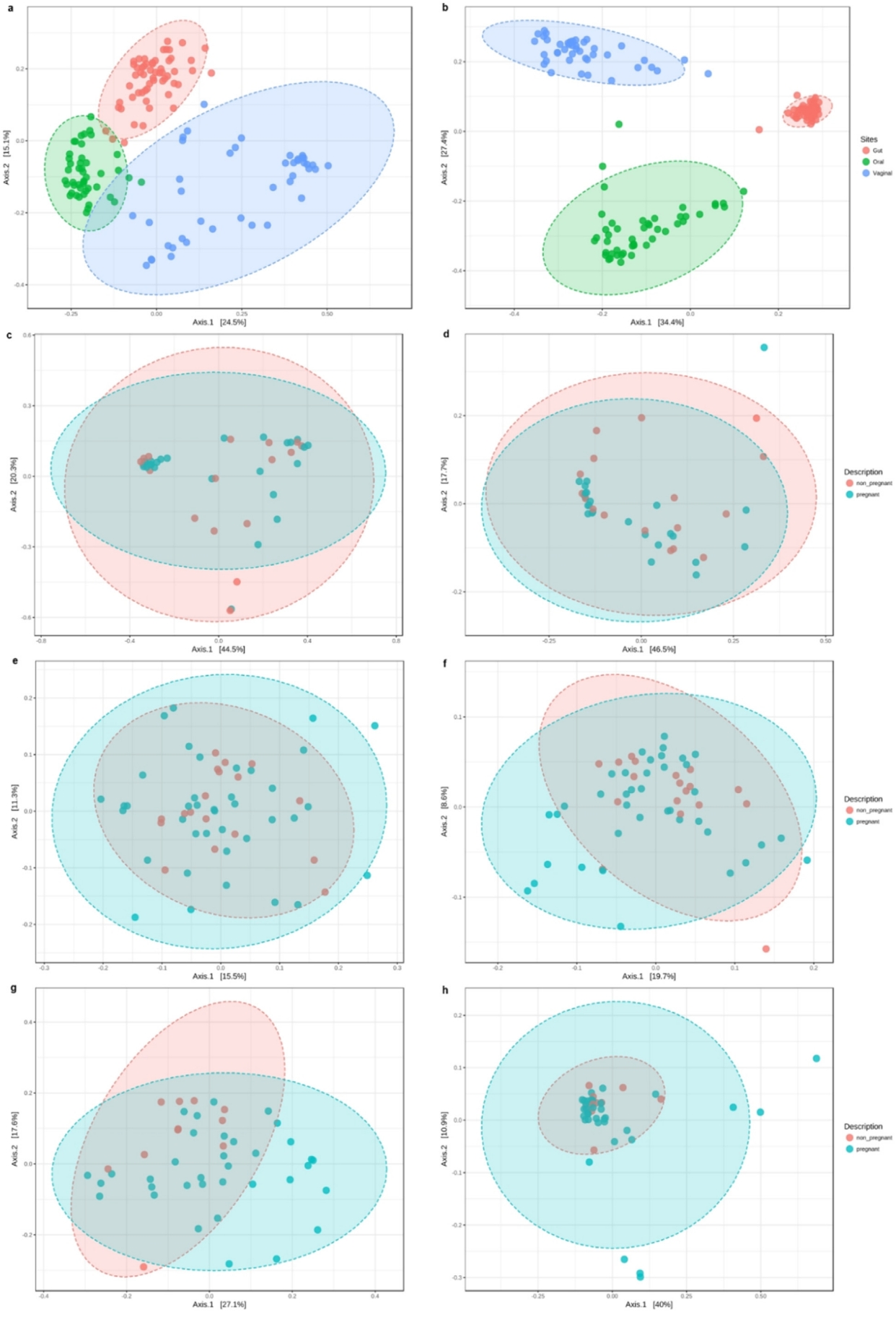

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Fig. 5. Main coordinate analysis (PCoA) from: All sites - (a) weighted and (b) unweighted; Vaginal - (c) weighted and (d) unweighted; Gut - (e) weighted and (f) unweighted and Oral (g) weighted and (h) unweighted (g and h: $P < 0.005$; F value = 1.31).

Authors statement

LGS: Formal analysis; investigation; roles/writing- original draft.

RCV: Formal analysis; investigation; roles/writing- original draft.

SD: Conceptualization; funding acquisition; project administration writing - review & editing.

MP: Formal analysis; investigation.

SS; MUN: Resources; investigation.

CRT: Conceptualization; methodology; supervision; writing - review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104230.

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