UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXV CICLO DEL DOTTORATO DI RICERCA IN

SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

Characterization of chorionic villus microbiome in the first trimester and of the amniotic fluid microbiome in the second trimester of pregnancy: relationship with the vaginal, rectal and oral maternal microbiome

Settore scientifico-disciplinare: MED/07

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ABSTRACT

The presence of a microbiome in healthy uterus has long been a matter of debate.

Until a few years ago, the placenta was thought to be a sterile tissue, therefore, the amniotic cavity and the fetus were also supposed to be sterile. Many studies were conducted to detect the presence of bacterial DNA in uterus, but most studies involved women with obstetric pathologies. Additionally, culture techniques were utilized, while it is now known that bacteria that grow in culture represent only a small part of all pathogens: most of them do not grow in culture or are suppressed in culture by other fast-growing bacteria. The concept of a sterile uterus in healthy women has changed thanks to the advent of new sequencing techniques based on metagenomics and 16S rRNA gene amplicon sequencing. A multitude of recent studies exploiting high-throughput sequencing technologies has challenged this paradigm, proposing that the placenta harbors a unique microbiome, neither the fetus, therefore, nor the amniotic fluid are sterile, and that acquisition of microbes begins *in utero*. Bacterial DNA similar to human oral and vaginal flora in placental tissue has been detected, and the association between alterations of the vaginal and oral microbiome and adverse pregnancy outcomes has been shown. Conversely**,** several studies have assessed that the human uterus and placenta have not a distinct microbiome, suggesting that the findings of bacteria in the intrauterine environment were due to contamination by reagents and sample processing. The introduction of methods for removing the set of contaminants from sequencing reagents, called “kitome”, minimizes contamination in microbiome workflows to the point that it is barely detectable. Therefore, studies that adopted this methodology found bacterial genetic signatures that were distinguishable from respective controls of contamination, confirming the hypothesis of a distinct microbial community in the placenta. Current knowledge of the placental microbiome is based on the results obtained from placentas sampled at the time of delivery, and it is not possible to know in which period of gestation the colonization took place. In this context, the aim of this study is to characterize,

using the Next-Generation Sequencing technique, fetus-placental bacteriome in the early stages of pregnancy and to compare it with that of other maternal districts (rectal, vaginal, oral). In addition, the immune profile of the fetus-placental complex and vaginal environment was investigated. In this study, 60 women, afferent to IRCCS Burlo Garofolo for the execution of villocentesis or amniocentesis, were enrolled. A total of 240 biological samples was analyzed, including chorionic villi (CVS, n= 23) and amniotic fluid samples (AF, n=37), and the matched samples including vaginal swabs, rectal swabs, and saliva samples. From the microbiome analysis, 12 (32%) AF samples and 10 (44%) CVS samples tested positive for the presence of bacterial DNA. The identified bacteria in the positive CVS and AF samples belonged to commensal and opportunistic pathogens of the reproductive tract and of the oral cavity (*Lactobacillus* and *Streptococcus*). Our results showed that CVS samples harbor a greater microbial heterogeneity, in particular regarding the possibly derived oral species, suggesting that the placenta could be colonized also from the oral route. When looking at a possible predisposing microbiome of maternal body districts to the colonization of CVS and AF, we found a decrease of probiotic *Streptococcus salivarius (S. salivarius)* in saliva samples matched to CVS and AF tested positive for the presence of bacterial DNA. To note, this probiotic species is able to inhibit immune activation by oral dysbiosis and periodontal disease pathogens. In vaginal samples, the most evident result was the decrease of *Lactobacillus crisptaus (L. crispatus)* in the samples matched to the CVS/AF samples that tested positive for the presence of bacteria compared to the samples matched to the negative CVS/AF samples. As shown by previous studies, *L. crispatus* shows a potential role to inhibit dysbiotic vaginal microbiome and infectious inflammation. Moreover, the decrease of this bacterium in the vaginal environment during pregnancy has been associated with a higher risk of infection and preterm delivery. Lastly, to compare the immune profile of fetus-placental complex and that of vaginal environment, the concentration of 27 soluble immune proteins, including Th1/pro‐inflammatory and anti‐inflammatory cytokines,

chemokines, and trophic factors, was measured in the AF/CVS samples and vaginal swabs. In particular, markers of intraamniotic inflammation, such as IL-8 and G-CSF, were increased in presence of bacterial DNA in the AF samples. Conversely, an immune hyporesponsiveness in the vaginal swabs matched to the positive AF samples was observed, suggesting, in according to previous studies, that the mother, placenta, and fetus all possess unique innate immune systems. To conclude, this study, confirms, for the first time, the presence of bacterial DNA in fetus-placental complex in the early stages of pregnancy, supporting the hypothesis of an *in utero* microbiome. The results from our pilot study show that the placenta can be colonized not only from the urogenital route but also from the oral route, suggesting hematogenous access. To date, the hematogenous source is supported by experimental evidence only on animal models. In addition, we speculate that the immune hyporesponsiveness in the vaginal milieu could contribute to the bacterial DNA translocation in the amniotic fluid where, in absence of an ongoing infection, the up-regulation of inflammatory cytokines was revealed. Further studies are needed to understand the variations in placental microbiome-induced metabolic pathways and their role in pregnancy outcomes.

RIASSUNTO

La presenza di un microbioma nell'utero sano è un argomento molto discusso. Per lungo tempo si è ritenuto che la placenta, pur permettendo il passaggio di ossigeno e nutrienti provenienti dalla madre e destinati al feto, fungesse da barriera per le infezioni. Questo ha portato per decenni a ritenere la placenta un organo sterile, così come, di conseguenza, la cavità amniotica e il feto, purché in assenza di patologia. Per molti anni gli studi effettuati hanno utilizzato tecniche di coltura o d'indagine tradizionale con risultati limitati nelle capacità identificative dei microrganismi, anche e, soprattutto, a causa delle basse quantità di microrganismi presenti nel tessuto da analizzare. I recenti progressi della biologia molecolare e l'utilizzo di nuove tecnologie di sequenziamento del genoma, hanno migliorato la nostra conoscenza dei microrganismi, in particolare di quelli presenti in quantità molto piccole rispetto all'intera comunità microbica. Il concetto di "utero sterile" nelle donne sane è cambiato grazie all'introduzione della tecnica di sequenziamento di nuova generazione (Next-Generation Sequencing) dell'amplicone del gene batterico 16S rRNA. Recenti studi condotti su tessuto uterino e placentale hanno dimostrato che sia l'utero che la placenta ospitano un "proprio microbioma", e sono state osservate somiglianze tra il microbioma intrauterino e quello dell'ambiente orale e vaginale. Inoltre, è stato dimostrato che alterazioni del microbioma vaginale e orale sono associate a parto pre-termine e allo sviluppo di patologie durante la gravidanza.

Al contrario, ci sono diversi studi che hanno dimostrato che l'utero umano e la placenta non hanno un microbioma distinto, ma le sequenze batteriche rilevate nell'ambiente intrauterino derivavano da contaminanti contenuti nei reagenti di sequenziamento o contaminazioni avvenute durante le procedure di analisi del campione. L'introduzione di metodi per la rimozione del set di contaminanti dei reagenti, chiamato "kitome", riduce al minimo la contaminazione al punto che è appena rilevabile. Pertanto, gli studi che hanno adottato questa metodologia hanno trovato sequenze genetiche batteriche distinguibili dai rispettivi

controlli di contaminazione, confermando l'ipotesi di una distinta comunità microbica nell'ambiente intrauterino. Le attuali conoscenze del microbioma placentare si basano sui risultati ottenuti dalle placente campionate al momento del parto (a termine o pre-termine), e non è possibile sapere in quale periodo di gestazione sia avvenuta la colonizzazione. Pertanto, lo scopo di questo studio è di caratterizzare, utilizzando la tecnica del 16S rRNA Next-Generation Sequencing, il batterioma feto-placentare nelle prime fasi della gravidanza e di confrontarlo con quello di altri distretti materni (rettale, vaginale, orale). Inoltre, è stato studiato il profilo immunitario del complesso feto-placentare e dell'ambiente vaginale. In questo studio sono state arruolate 60 donne, afferenti al Burlo Garofolo per l'esecuzione di villocentesi o amniocentesi. È stato analizzato un totale di 240 campioni biologici, composti da campioni di villi coriali (CVS, n= 23), e campioni di liquido amniotico (AF, n=37), e i corrispondenti tamponi vaginali, tamponi rettali e campioni di saliva. Dall'analisi del microbioma, sono risultati positivi per la presenza di DNA batterico 12 (32%) campioni AF e 10 (44%) campioni CVS. I batteri identificati nei campioni positivi di CVS e di AF appartenevano a patogeni commensali e opportunisti del tratto riproduttivo e del cavo orale (*Lactobacillus* e *Streptococcus*). I nostri risultati hanno mostrato che i campioni di CVS ospitavano una maggiore eterogeneità microbica, in particolare per quanto riguarda le specie appartenenti al microbioma orale. Questa evidenza suggerisce che l'origine della colonizzazione batterica placentale non è solo l'ambiente urogenitale, ma batteri dalla cavità orale, probabilmente, attraverso il torrente sanguigno, possono raggiungere l'ambiente intrauterino. Nell'evidenziare un possibile microbioma predisponente alla colonizzazione di CVS e AF negli altri distretti materni, è stata osservata una diminuzione del probiotico *S. salivarius* nei campioni di saliva abbinati ai CVS e AF risultati positivi per la presenza di batteri. È noto che questa specie probiotica è in grado di inibire la risposta infiammatoria indotta da disbiosi orale e dalla presenza di patogeni associati a malattie parodontali. Nei campioni vaginali, il risultato più evidente è stato la diminuzione di abbondanza relativa di

Lactobacillus crisptaus (*L. crispatus*) nei tamponi vaginali abbinati ai campioni CVS/AF risultati positivi per la presenza di batteri. Come dimostrato da studi precedenti, *L. crispatus* mostra un potenziale ruolo nell'inibire la disbiosi vaginale, e la diminuzione di questo batterio nell'ambiente vaginale durante la gravidanza è stata associata a un rischio maggiore di infezione e parto pre-termine. Per confrontare il profilo immunitario del complesso feto-placentare con quello dell'ambiente vaginale, è stata misurata la concentrazione di 27 proteine immunitarie solubili, comprese citochine Th1/proinfiammatorie e Th2/antinfiammatorie, chemochine e fattori trofici, nei campioni CVS/AF e nei tamponi vaginali. É stato osservato un significativo aumento di concentrazione di due fattori pro-infiammatori, IL-8 e G-CSF, in presenza di DNA batterico nei campioni di liquido amniotico. Al contrario, è stata osservata un'iporeattività immunitaria nei tamponi vaginali abbinati ai campioni di liquido amniotico risultati positivi alla presenza di batteri, suggerendo, come dimostrato da studi precedenti, una diversa risposta immunitaria nei due ambienti. In conclusione, questo studio conferma, per la prima volta, la presenza di DNA batterico nel complesso feto-placentare nelle prime fasi della gravidanza, supportando l'ipotesi di un microbioma in utero. I risultati del nostro studio pilota dimostrano che la placenta può essere colonizzata non solo dalla via urogenitale ma anche dalla via orale, suggerendo un accesso ematogeno. Ad oggi, l'accesso ematogeno è supportato solo da evidenze sperimentali su modelli animali. Inoltre, ipotizziamo che l'iporeattività immunitaria osservata nell'ambiente vaginale possa contribuire alla traslocazione del DNA batterico nel liquido amniotico dove, in assenza di un'infezione in corso, è stata rivelata la sovra-regolazione di due citochine infiammatorie. Comunque, sono necessari ulteriori studi per comprendere i cambiamenti dei processi metabolici indotti dal microbioma feto-placentale e il loro ruolo nell'esito della gravidanza.

INDEX

1. INTRODUCTION

The human microbiome is the set of microorganisms that coexist in the human body and consists of about 100 trillion (10^{20}) cells. (Peterson et al., 2009; Gever et al., 2012). The study of the microbiome plays an increasingly important role in understanding complex diseases: there is, in fact, ample evidence of the association between the composition of the microbial communities and the state of an individual's health, providing information on the investigated pathology, chances of prevention and the use of new drugs. (Lloyd-Price et al., 2016; Moffatt et al., 2017; Zeeuwen et al.,2013; Sedghi et al., 2021; Kim et l.,2019). In the last decade, numerous studies have been undertaken internationally: the American National Institutes of Health launched the "the Human Microbiome Project (HMP)", aiming at identifying the microorganisms that are normally present in healthy human subjects and trying to understand how they vary in pathologic contexts (Li et al., 2014; HMP Research Network Consortium 2014; HMP Research Network Consortium 2019). In Europe, the METAHIT project (METAgenomics of the Human Intestinal Tract) was funded through the 7th Framework Program (FP7), which examined the microbial communities present in the feces of healthy individuals, or with inflammatory bowel disease and, overweight and obese subjects.

This field of research is also beginning to be applied to obstetrics: it has been found, indeed, that the colonization of microorganisms of the fetus-placental unit, with the consequent development of infectious processes, could contribute to pregnancy complications or pathologies (preterm birth, abortion, premature rupture of membranes, chorionamnionitis, etc.) (Mysorekar et al., 2014; Taddei et al., 2018).

1.1. Uterine Microbiome

The presence of a microbiome in healthy uterus has long been a matter of debate. Over the last 15 years, many studies have examined uterine microbiome and the reproductive tract microbiome, but most studies involved women with obstetric pathologies. Additionally, most studies utilized culture techniques, while it is now known that bacteria that grow in culture represent only a small part of all pathogens: most of them do not grow in culture or are suppressed in culture by other fast growing bacteria. The concept of a sterile uterus in healthy women has changed thanks to the advent of new sequencing techniques based on metagenomics and 16S rRNA gene amplicon sequencing (Seeferovic et al., 2019; Koedooder et al., 2019; Baker et al., 2018). The dominant taxa identified in human non pregnant endometrial samples were *Bacteroidetes,* which is commonly found in the gut microbiome, conversely, another study found *Lactobacillus iners, Prevotella spp.,* and *Lactobacillus crispatus,* which are present in the vaginal microbiome (Verstraelen et al., 2016, Moreno et al., 2016; Koedooder et al., 2019). In fact, the comparison of uterine microbiome with the bacterial composition of other body sites reveals great similarities not only to vaginal microbiome, but also to oral and intestinal microbiome (Aagaard et al., 2014; Gomez-Arango et al., 2017). Subsequent studies have demonstrated the association of alterations in the uterine microbiome with some endometrial pathologies. A study conducted on the endometrial microbiota and chronic endometritis reported that *Lactobacillus crispatus* was less abundant in women with chronic endometritis, suggesting that this microorganism could play a protective role (Fang et al., 2016; Liu et al., 2019).

There are tangible reports that demonstrated how the uterine microbiome can have an impact on conception, and suggested that endometrial microbiome may interact with the endometrial epithelium and the endometrial immune cells, resulting in impaired endometrial receptivity and defective implantation (Tao et al., 2017; Campisciano et al., 2018). Moreno

et al. showed a correlation between low levels of Lactobacillus species (<90%) and poor pregnancy outcomes regarding implantation success and ongoing and term pregnancy rates (Moreno et al., 2016).

Additional studies have found a connection between the endometrial microbiome and improved success of assisted reproductive technologies (ART). The detection of certain bacterial taxa, such as *Acinetobacter, Lactobacillus* on catheter tips after insertion of an embryo correlated with increased pregnancy success rates (Pelzer et al., 2013; Franasiak et al., 2016). In contrast, whenever *Enterobacteriaceae* and *Staphylococcus* species, as well as *Streptococcus viridans*, were detected on catheter tips, decreased pregnancy rates were observed. (Selman et al., 2007; Moore et al., 2000)

1.2. Placental microbiome

The key components of the pregnant intrauterine environment include uterus, placentas, fetal membranes and umbilical cord. The placenta is a complex and heterogeneous organ responsible for transfer of nutrients and respiratory gases from maternal blood to the fetus, removal of fetal waste, and supporting maternal pregnancy physiology and fetal growth and development. In addition, the placenta protects the fetus from toxins and pathogens that may be present in maternal circulation. Until a few years ago, the placenta was thought to be a sterile tissue, therefore, the amniotic cavity and the fetus were also supposed to be sterile, with any different condition being the indication of a pathological state (Bushman et al., 2019). The concept that the placenta might harbor a microbiome gained great attention in 2014, when Aagaard et al., by conducting metagenomic analysis, identified in placental specimens bacterial DNA sequences belonged to non- pathogenic commensal microbiota from *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes* and *Fusobacteria phyla* (Aagaard et al., 2014). These findings opened a debate about the existence of a prenatal

microbiome and the sterility of placental tissue, amniotic fluid, and fetus. Subsequent studies investigated the potential differences in placental microbiome in both the presence and absence of negative pregnancy outcomes. The results of these studies indicated that the placental microbiome profile was significantly different in pregnancy with preterm delivery. Moreover, in placenta of women with chorioamnnionitis, the most frequently isolated pathogens were *Bacteroides species*, *E. coli, Gardnerella vaginalis*, *Mycoplasmas hominis, Peptostreptococci, Streptococci,* and *Ureaplasma urealyticum*, suggesting that pathological bacteria can invade amnion and chorion from other body sites such as the vagina (Hyman et al., 2014; Fettweis et al., 2019; Brown et al., 2019). The hypothesis of a prenatal microbiome has also been supported by experimental evidence that reported the presence of bacterial microorganisms in the meconium, and the same bacteria were detected in amniotic fluid and placenta, suggesting a maternal-fetal transfer and that the acquisition and colonization of the new-born gastrointestinal tract begin in the uterus (Collaudo et al., 2016; Perez-Munoz et al., 2017; Martinez et al., 2018; Antony et al., 2015; Prince et al., 2016; Seferovic et al., 2019). The finding, in the placenta, of DNA sequences belonging to bacteria species of human oral cavity suggested a bond between oral dysbiosis and pregnancy complications (Aagaard et al., 2014; Shanthi et al., 2012). Subsequent studies reported an association between dissemination of pathogenic bacteria associated with moderate and severe periodontitis and adverse outcomes of pregnancy (Shewale et al., 2016; Gogeneni et al., 2015). A possible involvement of the maternal gut microbiome was suggested, following the detection in placenta and amniotic fluid of intestinal bacteria, including *E. coli* and *Enterococcus faecalis* (Stout et al., 2013; Zhu et al., 2018). Then, it was supported by several studies that showed the crucial role of maternal gut microbiome to healthy pregnancy and offspring health (Nyangahu et al., 2019; Gomez de Aguero et al., 2016; Ferrocino et al., 2018).

Thanks to the application of 16S ribosomal DNA-based and whole-genome shotgun metagenomic sequencing analyses, researchers have observed that changes in the placental microbiome that influence delivery outcomes are accompanied by variations in microbiome-induced metabolic pathways. It has been observed that women with chorioamnionitis showed alterations of lipid metabolism associated with increased abundance of oral commensal bacteria, such as *Streptococcus thermophilus* and *Fusobacterium sp* (Antony et al., 2015; Prince et al., 2015). Gomez-de Aguero et al. reported that the existing bacterial communities in the placenta were metabolically enriched with genes associated with fatty acid metabolism, playing an important role in supplying energy-yielding substrates to the fetus, and tryptophan metabolism (Gomez de Aguero et al., 2016). Placental tryptophan metabolism is essential for fetal neural development, its catabolism enhances the establishment and maintenance of maternal-fetal immune tolerance, placental circulation, growth, and modulation of antimicrobial activity against infections (Sedlmayr et al.;2014). Therefore, these evidences suggest that the placenta has its own endogenous microbiome, the nature of this colonization may differ between healthy and complicated pregnancies, and the contact between the fetus and microorganisms is a physiological phenomenon, whose role is still to be determined.

1.3. Evidence against a placental microbiome

Conversely**,** several studies have assessed that the human placenta does not have a distinct microbiome, suggesting that the findings of bacteria in the intrauterine environment were due to contamination by reagents used in 16S gene sequencing or by sample processing (Lauder et al., 2016; De Goffau et al., 2019; Leon et al., 2018). Later studies reported that DNA reagent kits have their own distinct microbiome called a" kitome". In body sites with a high biomass, such as the intestines, low levels of the "kitome" is not detected. Since the

placenta or uterus host a ultra-low biomass, the use of proper contamination controls is needed (Theis et al., 2019). Some researchers found that bacterial communities in placental samples were similar to negative controls, denying the existence of an intrauterine microbiome (de Goffau et al., 2019; Kuperman et al., 2020).

The mainly contaminants usually identified in sequencing experiments are water and soil-associated bacteria including *Acinetobcter*, *Alcaligenes, Bacillus, Delphia, Herbaspirillum, Legionella, Leifsonia, Pseudomonas, Ralstonia*, *Sphingomonas* and *Xanthomonas* (Salter et al., 2014; De Goffau et al., 2018). The introduction of methods for removing contamination from sequencing reagents combined with a carefully performed magnetic bead-based extraction minimize contamination in microbiome workflows to the point that it is barely detectable (Stinson et al., 2019), Therefore, studies that adopted this methodology found a genetic signatures that were distinguishable from respective controls, confirming the hypothesis of a distinct microbial community in the placenta (Chen et al., 2017; Walther-Antonio et al., 2016; Franasiak et al., 2016)

1.4. Possible sources of intrauterine microbiome

It remains unclear how bacteria are transmitted to the intrauterine environment. Recent studies reported a resemblance between the microbial communities found in the intrauterine environment and bacterial composition of other body sites, such as that of the oral cavity, the gut, and the vagina (Baker et al., 2018) (**Figure 1**). Two primary mechanisms of transmission have been hypothesized: direct ascension from the vaginal canal or hematogenous spread from distal sites such as the oral cavity and the gut. In support of the hypothesis that microbiome ascends from the vaginal canal through the cervix to reach the intrauterine environment, studies on human and rodent uteri have shown that radioactively labeled particles and bioluminescent bacteria ascend from the vagina (Suff et al., 2018).

Moreover, the vaginal microbiome has been implicated in shaping the gut microbiome of the newborn, presumably during delivery (Jasarevic et al., 2017; Jasarevic et al., 2021).

The hematogenous source is supported by experimental evidence from bovine models that reported the presence of similar pathogens between blood and uterus, supporting the idea that these bacteria could enter the uterus through bloodstream (Jeon et al., 2017). To investigate hematogenous source, subsequent studies have been conducted using experimentally inoculated rodents, to trace bacteria to intrauterine environment. Injections of human salivary and subgingival plaque samples into the tail veins of pregnant mice resulted in the presence of bacterial DNA in the placenta that resembled the bacteria identified in salivary and plaque samples. When bacteria were inoculated in oral cavity of pregnant mice, the same bacteria were detected into the amniotic fluid, placenta and fetus (Tan et al., 2013), suggesting a transmission of bacteria from the oral cavity to the intrauterine environment (Fardini et al., 2010; Boutigny et al., 2016). Additionally, Rautava et al. studied pregnant women taking probiotics orally, and these bacteria were found in the placenta (Rautava et al., 2019).

The hypothesis of gut origin of the intrauterine microbiome is supported by experimental evidence showing an increased permeability of the gut epithelial barrier during pregnancy, this may favor bacteria to escape from the lumen of the gastrointestinal tract and, via the bloodstream, are capable of reaching the uterus (Gomez-Arango et al., 2017; Kiacolt-Glaser et al., 2018).

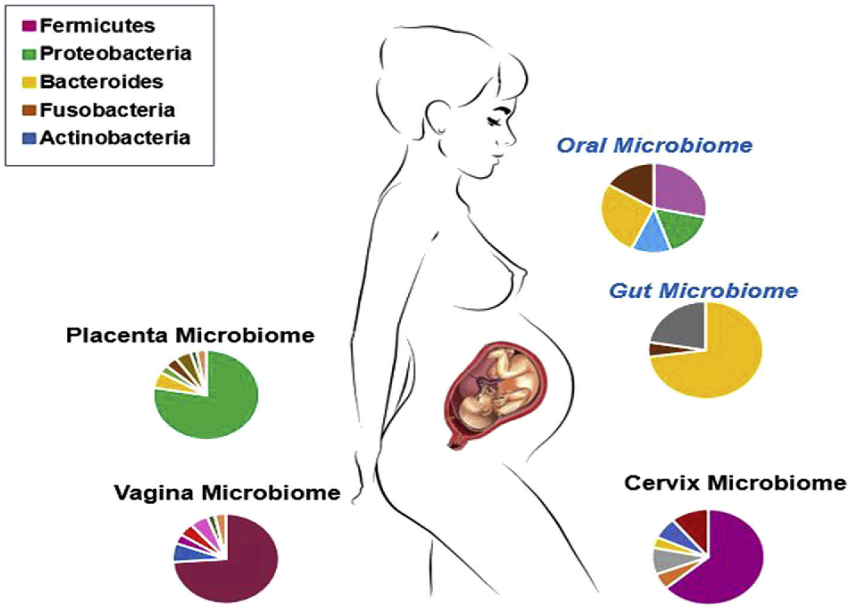


Figure 1. Seeding of placental microbiome; microbes ascend from the vagina, gut, and oral cavity which are internalized and translocated by hematogenous spread to the placenta. DOI:https://doi.org/10.1016/j.siny.2016.02.004.

1.5. The maternal microbiome and pregnancy outcomes

1.5.1. The vaginal microbiome

The vaginal microbiome changes throughout a woman's reproductive life from puberty to menopause. Thanks to the use of metagenomics DNA sequencing, it has been shown that the healthy vaginal microbiome of women during fertile age is characterized by a predominance of *Lactobacillus spp*., that helps maintaining a stable vaginal equilibrium and prevents infective states in the healthy reproductive tract (Chen et al., 2017; Srinivasan et al., 2012; Kroon et al., 2018). Other commensal inhabitants of the vaginal environment include *Escherichia spp.*, *Staphylococcus spp., Gardnerella spp., Streptococcus spp., Mycoplasma spp., Prevotella spp.* and *Atopobium spp.* (Pietrzak et al., 2013). Maintaining this balance during pregnancy is a complex process and must be appropriate for the gestational age. During early stage of pregnancy, due to a variation of sex hormonal levels,

the vaginal microbiome undergoes significant changes by increasing its stability, and decreasing its overall diversity (Aagaard et al., 2012). During later stages of pregnancy and the puerperium, the vaginal microbiome gets back to baseline, with an increase in diversity, a decrease in *Lactobacillus*, and an enrichment of commensal bacteria associated (Macintyre et al., 2015). Romero et al. using 16sRNA gene sequencing, demonstrated that vaginal microbiome of pregnancy was more stable than that of non-pregnant state, with a greater abundance of *L. vaginalis*, *L. crispatus, L. gasseri* and *L jensenii*, resulting in reduction of vaginal pH, and thus creating an unfavorable environment for the growth of pathogenic bacteria (Romero et al., 2014). In particular, it has been observed a key role of *L. crispatus* in maintaining the stability of vaginal environment during pregnancy. An alteration in vaginal microbiome composition, called dysbiosis, may make a woman susceptible to genital tract infections, which can result in adverse gestational outcomes like preterm delivery, preterm rupture of membranes, pre-eclampsia, miscarriage, fetal growth restriction, low birth weight and neonatal sepsis (Romero et al., 2014; Abdelmaksoud et al., 2016; Stout et al., 2017). Recently, it has been observed that vaginal dysbiosis causes the increase of pathogens which are able to enter the uterine cavity, inducing the release of pro-inflammatory cytokines, prostaglandins, and metalloprotease (Campisciano et al., 2018; Anahtar et al., 2018; Hyman et al., 2014; Fettweis et al., 2019; Dominguez-Bello et al., 2010). These molecules can trigger cervical ripening and shortening, weakening of membranes and rupture, uterine contractility, and therefore increase the risk of a negative pregnancy outcome (Ramos et al., 2015). Fettweis et al., observed that levels of the vaginal inflammatory cytokines CXCL10 were related to the *L. crispatus/L. iners* ratio in patients at increased risk of preterm delivery, indicating a cytokines/Lactobacillus ratio as a possible marker for pregnancy outcomes (Fettweis et al., 2019).

Currently, researchers are increasingly interested in the transition of the vaginal flora of the mother to the intestinal flora of the fetus. Recent studies showed that the bacterial flora of

vaginally-delivered infants was similar to that of the mother's vagina, while the microbiome of cesarean-delivered children is similar to the microbiota of maternal skin (Gomez de Aguero et al., 2016). Additionally, it has been suggested that the maternal vaginal microbiome results in a certain regulatory effect on the infant's intestinal flora (Dominguez-Bello et al., 2016), influencing the growth and the development of innate immune system of the newborn (Gomez et al., 2016).

1.5.2. The gastrointestinal microbiome

The composition of the maternal intestinal microbiome undergoes profound changes during pregnancy. Although there is experimental evidence on the maternal gut microbiome in the third trimester of pregnancy, data on the changes during early pregnancy are scarce (Nuriel-Ohayon et al., 2016; Mesa et al., 2020; Koren et al., 2012). Several studies support the hypothesis that changes in the gut microbiome during early pregnancy are associated with an increased risk of gestational diabetes and hypertension (Wang et al., 2018; Zheng et al., 2020; Ma et al., 2020). An important association between gut dysbiosis and early-onset pre-eclampsia (PE) was observed, and the composition of gut microbiota in patients with early PE differed significantly from that in healthy pregnant women. In addition, the bacteria associated with PE were also associated with other maternal morbidities, including obesity, glucose metabolic disorders, proinflammatory state and intestinal barrier dysfunction (LV et al., 2019). These microorganisms correlated with host immune parameters, such as IL-6, and lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, and the latter can be considered a marker of increased bacterial translocation across the intestinal epithelium (LV et al., 2019). These evidences were supported by subsequent studies that observed an association between an increased intestinal permeability during pregnancy and high levels of lipopolysaccharide (LPS) at the endometrial level, with an

excessive production of pro-inflammatory cytokine (Tersigni et al., 2019; D' Ippolito et al., 2016). Studies conducted on animal models have shown that LPS binds Toll-like receptor 4 (TLR4), the activation of TLR4 induces an increase of pro-inflammatory cytokines and chemokines such as tumor necrosis alpha (TNF a), and interleukin 6 (IL-6), both systemically and in the placenta, increasing the risk of pregnancy loss and preterm birth (Zhao et al., 2013; Szarka et al., 2010).

1.5.3. The oral microbiome

Studies based on the traditional culture and PCR methods identified a limited number of gram-negative anaerobic bacteria as periodontal pathogens, in particular *Porphyromonas gingivalis*, *Tannerella forsythia, Fusobacterium nucleatum* and *Prevotella intermedia*, which were considered as markers of periodontitis (Hajishengallis et al., 2012; Socransky. et al., 1998; Salminen et al., 2015). With the advent of metagenomics sequencing technologies, recent studies revealed a greater degree of complexity in the oral microbiome (Park et al., 2015; Hong et al., 2015). During the pregnancy, the composition of the oral microbiome undergoes changes due to numerous factors, including pH, nutrition and hormone levels (Balan et al., 2018; Wu et al., 2015). The presence of both subgingival *Porphyromonas gingivalis* and *Prevotella Intermedia* increased during pregnancy and was positively correlated with maternal hormone levels (Carrillo-De-Albornoz et al., 2010). Recently, it has been revealed that although the microbial diversity remains stable during the course of pregnancy, the composition of the oral microbiome undergoes a pathogenic shift during pregnancy that reverts back to baseline during the post-partum period (Balan et al., 2018). Lin et al. reported that the genera *Neisseria spp., Porphyromonas spp.* and *Treponema spp.* were over- represented in the oral cavity of pregnant group, whereas *Streptococcus* and *Veillonella* were less represented compared with that of the non-pregnant group (Lin et al.,

2018). By contrast, other studies reported that the genera most abundant during pregnancy were *Fusobacteria*, whereas *Neisseria* and *Streptococcus* were less abundant (Aas et al., 2005). However, the compositional shift during pregnancy may cause a greater risk of periodontal diseases, or preexisting maternal gingivitis or periodontitis can worsen during pregnancy (Balan et al., 2018; Tilakatatne et al., 2000). Clinical studies indicate that maternal periodontitis may be a potential risk factor of adverse pregnancy outcome (Ryu et al., 2010; Ye et al., 2022; Ha et al., 2011; Wang et al., 2018). Higher amounts of *P. gingivalis* in subgingival plaque increase the risk of preterm birth, while *Prevotella intermedia* was more prevalent in subgingival sample of women with preeclampsia (Ye et al., 2022; Hirano et al., 2012). Oral microbes have been detected in the placental fetal units, where they might be involved in the development and progression of inflammatory state. The most prevalent periodontal pathogens in placental fetal units are *P. gingivalis* and Fusobacterium *nucleatum* (Liang et al., 2018; Ao et al., 2015). Additionally, studies conducted on animal models confirmed that the oral infection with *P.gingivalis* and *F. nucleatum*, leads to colonization in the mouse placenta, causing localized infection and increased serum level of pro-inflammatory mediators such as TNF alpha, IL-1, IL-6 and IL-8, leading to preterm and still births (Konishi et al., 2019).

1.6. Intrauterine inflammation during pregnancy

Pregnancy represents a unique physiological phenomenon in nature, consisting in the symbiosis between two semi-allogeneic individuals. Years of study and research have only partially clarified this immunological paradox. Initially, to explain the tolerance of the semi-allogeneicity of the fetus it was hypothesized that the pregnancy was characterized by a state of immune depression, however, this hypothesis was not subsequently confirmed. Subsequent studies have highlighted that the immune system rather undergoes an

immunological remodeling, which varies depending on the gestational stage. Experimental evidences have shown that, both at the decidual level and in the maternal peripheral blood, during pregnancy the secretory pattern of T-helper type 2 (Th2) predominates, with the secretion of anti-inflammatory cytokines, such as IL-4 and IL-10, and reversal to T-helper 1 (Th1) pro-inflammatory cytokine dominance is associated with labor (Abioye et al., 2019; Sykes et al., 2012). The shift between Th1 and Th2 cytokines is crucial factor for healthy pregnancy, alterations of this immunological state can lead induce antifetal rejection, placental damage and pregnancy complications (Antony et al., 2015; De Goffau et al., 2019; Prince et al., 2016).

Increased expression of pro-inflammatory cytokines in the placenta, cord blood, maternal blood and cervico-vaginal fluid are associated with poor pregnancy outcomes including placental dysfunction, intrauterine growth restriction and pre-eclampsia. Placentas from women with PROM (premature rupture of membranes*)* and preterm birth indicate a bias towards Th1 cytokines IFN-y, IL-2, and IL-12, compared with term placentas that show higher TH2 levels (Gargano et al., 2008; Nadeau-Vallee et al., 2016). Emerging data have shown that uterine maternal microbiota, in appropriate amounts, can contribute to the anti-inflammatory stage (Ramos et al., 2015; Nuriel-Ohayon et al., 2016). In fact, commensal bacteria present at the epithelium of the uterus promote the induction of regulatory cytokines by trophoblast and macrophages. Macrophages secrete antimicrobial products that mitigate commensal overgrowth and prevent invasion of pathogenic bacteria (Mor et al., 2015).

Choriodecidual colonization by bacteria with the release of pathogen-associated molecular patterns activate toll-like receptors (TLR) that are expressed by amnion epithelial cells, macrophages and neutrophils, inducing the synthesis and release of prostaglandins, contractile associated proteins and matrix metalloproteinases in the placental tissue and fetal membranes (Sweeney et al.,2017).

Recent studies reported a link between preterm birth and increased chorionic expression of TLR-1 and TLR-2 and TLR-4. TLR-2 recognizes microbial products of gram-positive bacteria, genital mycoplasmas, while TLR-4 recognizes bacterial endotoxin (LPS). The junction of LPS-TLR4 actives NF-kb inflammasone pathway, inducing a metabolic endotoxemia capable of modulating pro-inflammatory cytokines (Pelzer et al., 2017). The anti-inflammatory cytokines, such as IL-10 and IL-4, have been shown to have an important role in the pregnancy immunological profile, inhibiting the activity of NF-kb inflammasone pathway, and inducing a down-regulation of Th1- associated cytokines. High levels of IL-10 and IL-4 have been observed in placentas of women who delivered at term, while placental IL-10 and IL-4 expression are reduced in pregnancies complicated by chorioamnionitis and preterm birth (Iyer et al., 2012; Stinson et al., 2019; Tsiartas et al., 2012; Gustafsson et al., 2018) (**Figure 2**).

Recently, intrauterine inflammation has been associated with alteration in fetal neurodevelopment and offspring behavior in adulthood. Studies conducted on animal models have shown that high levels of IL-1B and IL-6 in placentas are associated with anxiety-like behavior and hyperactivity in murinae newborn. Furthermore, the injection of LPS induced the increase of pro-inflammatory cytokines not only in placental tissue, but high levels of pro-inflammatory cytokines were detected in the fetal brain, compromising blood-brain barrier integrity (Kemp et al., 2014;(Hodyl et al., 2017).

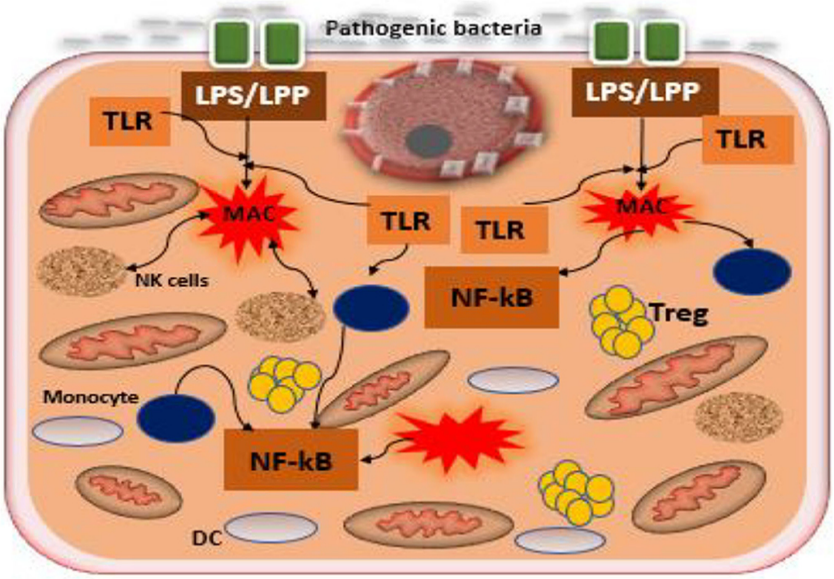


Figure 2. Interaction between commensal bacteria and toll like receptors of the trophoblast in the formation of regulatory cytokines. DOI: 10.3389/fcimb.2020.00413.

2. AIMS

The belief that during pregnancy the uterus, whether physiological, is sterile is now being questioned. The presence of microorganisms in the human placenta in a non-pathological pregnancy at the end of gestation suggests that the contact between the fetus and microorganisms is a physiological phenomenon. Therefore, it is necessary to understand when placental colonization begins, and what role it may have both in the course of a physiological pregnancy and in the development of some maternal and fetal-neonatal pathologies. Current knowledge is based solely on the results obtained from placentas sampled at the time of delivery (term or pre-term), therefore it is not possible to assume in what period of pregnancy the colonization occurred. In addition, considering the interpersonal variability, as well as the influence that factors such as obesity, ethnicity, age, state of health exerted on the microbiome, these results remain of doubtful interpretation, in the absence of a comparison between placental and amniotic fluid microbiome with microbiomes of other body districts coming from the same subjects.

In this context, the primary aim of this study is to characterize the placental microbiome in the first and second trimester of pregnancy, analyzing the microbial composition of the chorionic villi and amniotic fluids, respectively, using the Next-Generation Sequencing technique.

The secondary objective is to investigate whether there are associations between the profile of the fetus-placental microbiome and that present in other areas of the body of the same pregnant woman, such as the vaginal, rectal and oral environments.

Finally, to study the maternal-placental-fetal interaction in antimicrobial immunity, we have analyzed the local immune response in vagina, in chorionic villi samples and in amniotic fluids.

Ascertaining possible colonization of the fetus-placental unit in the early stages of pregnancy could provide new information on fetal development and on the immunomodulatory role of this early exposure to microorganisms. Furthermore, correlating the fetal microbiome with that of other areas of the pregnant woman's body could help identify preventive biomarkers to detect pregnancy pathologies and to predict negative pregnancy outcomes.

3. METHODS

3.1 Study population and sampling

In this prospective longitudinal study, women with singleton pregnancy afferent to the Departmental Research Structure of Fetal Medicine and Prenatal Diagnostics of the IRCCS Burlo Garofolo Hospital in Trieste, were enrolled for the execution of villocentesis or amniocentesis. Samples consisted in chorionic villous (CVS) in the first trimester or amniotic fluid (AF) in the second trimester of pregnancy. The women enrolled were subjected to invasive procedures for clinical reasons, such as advanced age, previous medical history (genetic pathologies), or with a prenatal screening tests showing a high risk of major aneuploidies. Women with sexually transmitted infections, hormonal or antibiotic/probiotic therapy in the previous 6 months to the enrollment, a history of chronic or infectious diseases, periodontal diseases, or with documented risk factors (smoking, obesity or drug use) were excluded from the study. All enrolled patients provided an informed consent.

Chorionic villi or amniotic fluid samples were collected from each pregnant woman, together with vaginal, rectal and sputum samples. Vaginal samples were collected using 200 mm polyethylene swabs with transport medium (Cliniswab, Aptaca S.p.A, Italy), by a single gentle 360° rotation of the swab at the vaginal wall, under speculum examination. Rectal samples were collected using 200 mm polyethylene swabs with transport medium (Cliniswab, Aptaca S.p.A, Italy), by inserting the swab two/three cm into the rectal sphincter, and gently rotating. The sputum was collected at least 1 hour after the last meal and after subsequent oral hygiene, and stored in a sterile container.

CVS is an invasive procedure performed in the first trimester of pregnancy (11-14 weeks). It is carried out trans-abdominally, involves the insertion of a 18 G guide needle into the placenta. Subsequently, a second 20 G needle is introduced to aspirate the chorionic villi. The quantification of the sampled villi is performed only at the end of the procedure.

Approximately 15mg of villi are sufficient for standard cytogenetic analysis. Cytogenetic analysis was privileged, in the case of sufficient material, the chorionic villi samples were deposited in a sterile container with 1ml of sterile physiological solution and immediately sent to the SSD Advanced Microbiological Translational Diagnostics laboratory.

Amniocentesis is an invasive procedure that is performed starting from the 15th week of gestation, is carried out trans-abdominally, and involves the insertion of a single 21 g needle for the aspiration of amniotic fluid. 16-18 cc of liquid are sufficient for cytogenetic analysis. The excess quantity withdrawn was used for the study and immediately sent in a sterile container to the SSD Advanced Microbiological Translational Diagnostics laboratory.

3.2. Microbiome characterization

3.2.1. DNA extraction and library preparation

Amniotic fluid and chorionic villus samples were stored until the time of analysis. As for the swabs, they were suspended in 1.5ml of saline solution and stored at -80 °C. After being thawed, samples were vortexed and total DNA was extracted from 300 µL of each sample in a final elution volume of 50 µL by the automatic extractor Maxwell CSC DNA Blood Kit (Promega, Madison, WI, USA), according to manufacturer's instruction. The characterization of the bacterial composition of the samples was performed with Ion Torrent next generation high-throughput sequencing (NGS) of the 16s rRNA, in particular, region V3 of the 16S rRNA gene was sequenced. The steps of the sequencing are summarized in **Figure 3**.

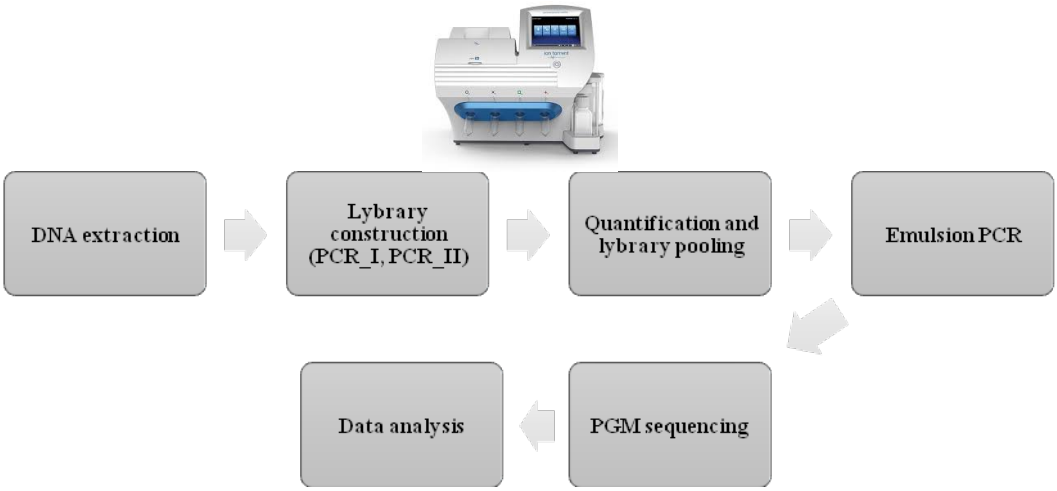


Figure 3. Schematic representation of the NGS analyses.

A qPCR targeting the V1–V3 region 16S rRNA gene (500 bp) was performed by employing the U534R primer and the degenerated primer 27FYM (PCR_I). A nested PCR was subsequently carried out with the primers B338F_P1-adaptor and U534R_Aadaptor_barcode, targeting the V3 region (200 bp) of the 16S rRNA gene (PCR_II), with a different barcode for each sample linked to the reverse primer (Sundquist et al., 2007). PCR reactions were performed using EvaGreen® dye (Fisher Molecular Biology, Waltham, MA, USA), the Kapa 2G HiFi Hotstart ready mix 2X (Kapa Biosystems, Wilmington, MA, USA), 0.5 µM of each primer and 400 ng/µL of Bovine Serum Albumin (BSA), in a final volume of 10 µL.

Negative controls including no template and no bacterial DNA were processed with clinical samples, starting from the pre-analytic phase of samples manipulation. A total absence of amplification signal at the end of PCR runs (I and II step of PCR) was successfully obtained. The correct size of the amplicons (560 bp for PCR_I and 260 bp for the PCR_II) was assessed on a 2% agarose gel. The amount of dsDNA of each sample after PCR_II was

quantified with a Qubit® 2.0 Fluorimeter (Invitrogen, Carlsbad, California, USA) using the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and an equal amount of each sample (100 ng) was mixed into a single batch to generate a pooled library at a final concentration of 100 pM, according to manufacturer's instructions. Template preparation was performed by emulsion PCR using The Ion OneTouch™ 2System (Life Technologies, Gran Island, New York, USA), with the Ion PGM Hi-Q View OT2 200 kit (Life Technologies, New York, USA) and a subsequent quality control was carried out on Qubit® 2.0 Fluorimeter. Sequencing was performed with the Ion PGM™ System technology by using the Ion PGM Hi-Q View sequencing kit (Life Technologies, New York, USA). The high-throughput sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME 1.9.1.) software (Caporaso et al., 2010), (available at: https://www.nature.com/articles/nmeth.f.303 Last access 30 0ctober 2020). High quality sequences (Q > 25) were demultiplexed and filtered by quality using split_libraries_fastq.py with default parameters, retaining sequences with a minimum length of 150 bp. Sequences with homopolymer length >8 or ambiguous bases were removed. Operational Taxonomic Units (OTUs) were picked at 97% similarity and clustered against the reference taxonomy database SILVA V.132 (Quast et al., 2013).

3.3. Immune profile analysis

3.3.1. Dosage of immune soluble proteins

The Bio-Plex suspension microarray system features three essential elements of Luminex-xMAP technology: Microspheres labeled with fluorophores, each with a different color code or spectral value; two laser cytofluorimeter with associated optics to measure the different molecules on the surface of the microspheres; and high-speed digital signal that efficiently processes fluorescence data. The magnetic bead is covalently linked to a specific antibody capture for each cytokine chemokine; the marbles react with the sample containing the molecules of interest (cell supernatant, serum and plasma, saliva or other biological fluids) and, after a series of washes that eliminate unbound proteins, a biotinylated antibody detection is added which creates a "sandwich" complex. The detection of the final complex is obtained by adding a streptavidin-phycoerythrin (SA-PE) conjugate, in which phycoerythrin acts as a fluorescent reporter. When the suspension following the reaction is directed to the reader, a red laser beam (635 nm) illuminates the fluorescent dye of each microsphere which provides an initial classification of the beads; simultaneously, a green laser beam (532 nm) excites the phycoerythrin (PE) generating a reporter signal which is detected by a photomultiplier tube (PMT). A high-speed digital processor then organizes an output of the data, which is processed by the Bio-PlexManager TM software providing data with median fluorescence intensity (MFI) values. Each cytokine is identified on the basis of the two fluorescence signals described above and reported in a graph in which each of them is represented in different areas (**Figure 4**).

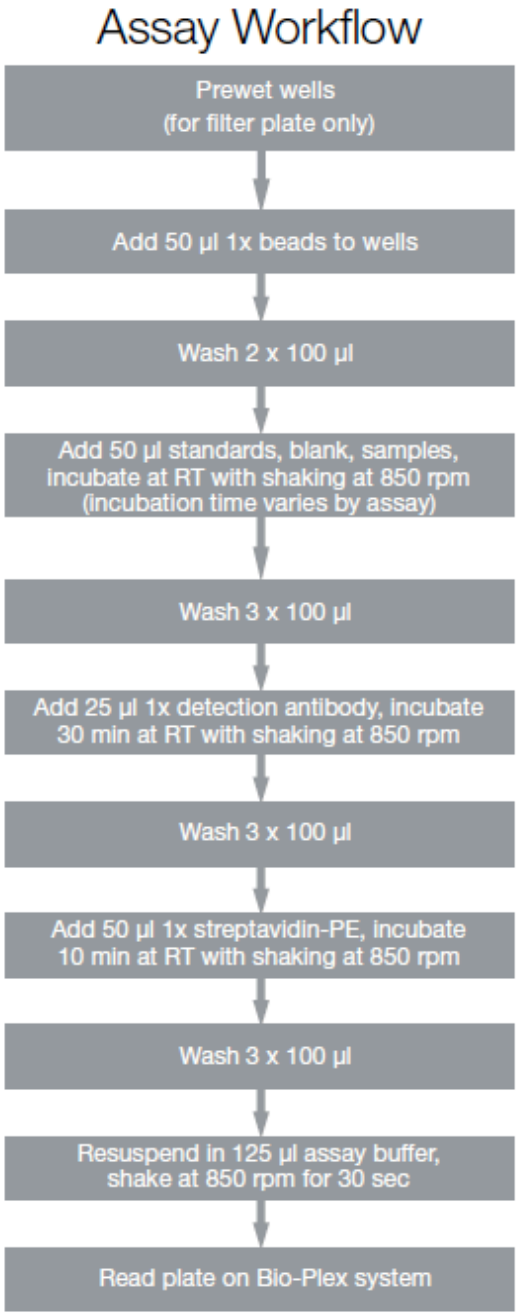


Figure 4. Assay Workflow of the cytokines analysis.

In this study, a soluble concentration of 27 immune proteins, including cytokines, chemokines, and growth factors (**Table 1**) was assessed in duplicate in all 60 vaginal swabs and in 37 amniotic fluids and in 23 chorionic villi samples using magnetic bead-based multiplex immunoassays (Bioplex ProTM human cytokine 27-plex panel, Bio-Rad Laboratories, Milan, Italy) according to the pre-optimized protocol (Zanotta et al., 2019).

Regarding vaginal swabs, after centrifugation at 3000 rpm, the undiluted samples (50 µl) were mixed with biomagnetic beads in 96-well flat-bottom plates, with the addition of 0,5% of BSA. The amniotic fluid and chorionic villi samples were centrifuged at 1000 x g, and, before analysis, they were diluted 1:4 using Bioplex Sample Diluent. Then 50ul of diluted samples were mixed with biomagnetic beads in 96-well flat-bottom plates. After incubation for 30 min at room temperature followed by washing plate with Bio-Plex wash buffer, 25 µl of the antibody–biotin reporter was added. After the addition of 50 µl of streptavidin–phycoerythrin (PE) and following incubation for 10 min, the concentrations of the cytokines were determined using the Bio-Plex-200 system (Bio-Rad Corp., United States) and Bio-Plex Manager software (v.6, Bio-Rad). The data were expressed as median fluorescence intensity (MFI) and concentration (pg/ml).

Cytokines	**Chemokines**	**Trophic factors**
IL-1β; IL-1Ra; IL-2; IL-4; IL-5; IL-6; IL-9; IL-10; IL-12 (p70); IL-13; IL-15; IL-17; IFN-γ; TNF-α;	IL-8/CXCL8; Eotaxin/CCL11; MCP-1/CCL2 (MCAF); IP-10/CXCL10; MIP-1α/CCL3; MIP-1β/CCL4; RANTES/CCL5;	IL-7; basic FGF; G-CSF;PDGF-BB; VEGF GM-CSF

Table 1. **Immune soluble proteins analyzed (27-plex (BioRad).** IL-1β, Interlukin 1 subunit β; IL-1Ra, Interleukin 1 receptor alpha; IL-2, Interleukin 2; IL-4, Interleukin 4; IL-5, Interleukin 5; IL-6, Interleukin 6; IL-9, Interleukin 9; IL-10, Interleukin 10; IL-12p70, Interleukin-12 subunit p70; IL-13, Interleukin 13; IL-15, Interleukin 15; IL-17, Interleukin 17; IFN-γ, Interferon gamma; TNF-α, Tumor necrosis factor alfa; IL-8, Interleukin 8; Eotaxin, eosinophil chemotactic protein; MCP-1, monocyte chemoattractant protein1 (CCL2); MIP-1α, Macrophage inflammatory proteins 1 subunit α (CCL3); IP-10, 10 kDa interferon gamma-induced protein (CXCL10); MIP-1β, Macrophage inflammatory proteins 1 subunit β (CCL4); RANTES, regulated on activation T cell expressed and secreted (CCL5); IL-7, Interleukin 7; FGF basic, Basic fibroblast growth factor; G-CSF, Granulocyte colony-stimulating factor; PDGF-bb, Platelet derived growth factor; VEGF, Vascular endothelial growth factor; GM-CSF, Granulocyte-Macrophage Colony Stimulating.

3.4. Statistical analysis

In order to establish the sample size, we used the platform https://fedematt.shinyapps.io/shinyMB/ (Mattiello et al., 2016, Bioinformatics). The data required to make this estimate are: the maximum number of bacterial species expected in our samples and the extent of the variation in the quantities of these bacteria between the various samples analyzed. Therefore, it is estimated that the analysis of a minimum of 25 samples for the villi and 20 samples for the amniotic fluids provides the study with a statistical power > 90% for the description of the microbiome.

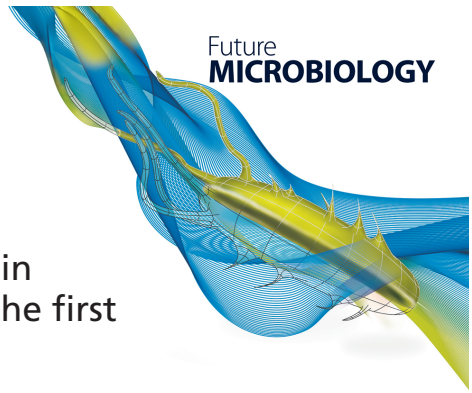
For the big data microbiome analysis, using QIIME 2.22.2, evenness and observed ASVs metrics were calculated to assess the alpha diversity and compared by means of Kruskal-Wallis test. Bray Curtis dissimilarity index was calculated to assess the beta diversity, visualized by the principal coordinate analysis (PcoA) and compared by the PERMANOVA test. To highlight the differences in the microbial composition, we performed the differential abundance testing using the ANCOM test. Using MicrobiomeAnalyst, we applied the LEfSE test to identify microbiological biomarkers (Chong et al., 2020). To test the differences in the immune soluble factors, GraphPad Prism (v. 5, San Diego, CA USA) was used. Specifically, the Kruskal-Wallis one-way analysis of variance was used for comparisons between groups. When a significant p-value was observed ($p < 0.05$), a multiple comparison test was used to determine which groups were different

.

4. RESULTS

4.1. Preliminary results

A preliminary study was conducted on 53 pregnant women to analyze microbiome of chorionic villi (n=24) or amniotic fluids (n=29). To distinguish between DNA deriving from biological samples and that deriving from environmental contaminants, in parallel with biological sample sequencing, we have included the sequencing from unused swabs, and we have compared sequencing data from skin swabs of the area punctured during amniocentesis after disinfection with the bacterial composition of amniotic fluid. The preliminary results showed that the 37,5% of CVS and the 14% of AF tested positive for bacterial DNA. We detected a specific microbial population in CVS and AF, which differed from the bacteria identified in the sterile swab. In addition, the skin swabs performed after the conventional disinfection excluded a systematic inoculation of bacterial DNA during the invasive procedures. Out of ten skin swabs, eight tested negative for the presence of bacterial DNA. With regard to the two positive skin swabs, one was coupled to a negative AF and the other was coupled to a nearly negative AF. The preliminary results were published in Frontiers Microbiology (Campisciano et al., 2020).



Short Communication

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Evidence of bacterial DNA presence in chorionic villi and amniotic fluid in the first and second trimester of pregnancy

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The sterile-womb dogma in uncomplicated pregnancy has been lively debated. Data regarding the *in utero* microbiome environment are based mainly on studies performed at the time of delivery. **Aim:** To determine whether human placenta and amniotic fluid are populated by a bacterial microbiota in the first and second trimesters of pregnancy. **Materials & methods:** We analyzed by next-generation sequencing method 24 and 29 samples from chorionic villus sampling (CVS) and amniocentesis (AC), respectively. The V3 region of the *16S rRNA* gene was sequenced. **Results:** 37.5% of CVS and 14% of AC samples showed the presence of bacterial DNA. **Conclusion:** Our study suggests that bacterial DNA can be identified in the placenta and amniotic fluid during early prenatal life.

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Keywords: amniocentesis • chorionic villous sampling • microbiome • placenta

For many years, the placenta was thought to be a sterile tissue, allowing the passage of oxygen and nutrients from the mother to the fetus, but acting as a barrier for infections. Consequently, also the amniotic cavity and the fetus had to be sterile unless of an occurrence of a pathological state [1].

Recent studies, based on metagenomics and *16S rRNA* gene amplicon sequencing, have challenged this concept by detecting bacterial DNA in placental tissue [2–7], in amniotic fluid [8] and even in the developing fetus [9–11].

Collado *et al.* reported the presence of a distinct microbiota in the meconium of infants delivered via C-section, which shares its taxa with the placenta and amniotic fluid, suggesting a maternal–fetal transfer [8]. Although the presence of microorganisms in fetal membranes is highly correlated with intrauterine infections that often lead to preterm birth [12–14], there is also evidence supporting the fact that bacteria and inflammatory cells do not always cause preterm birth [15]. Thus, a rising hypothesis is that there might be a microbial community *in utero* toward the end of noncomplicated pregnancy, suggesting that the contact between a fetus and microorganisms is a physiological phenomenon that may be initiated prenatally and whose role is still to be determined [16].

On the other hand, several studies have assessed that the human placenta does not have a distinct microbiome. An exception can be made only for *Streptococcus agalactiae* (group B *Streptococcus*), which has been detected in the placenta, and that represents a potential perinatal infection pathogen [17]. In general, the studies that failed to detect a placental microbiome applied sequencing-based methods taking into account the potential for false-positive results due to the presence of contaminating bacterial DNA in the reagents used for the sequencing procedures. Indeed,

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4.2. Microbiome characterization by NGS

4.2.1. Sequencing results

In the present study, 64 Caucasian women carrying singleton pregnancy were included, with a mean age 38 ± 4 years. The suggestions to undergo invasive procedures were fetal malformation (21/64, 32.8%), advanced maternal age (22/64, 34.4%), high risk screening test (8/64, 12.5%), anamnestic risk (8/64, 12.5%), combined advanced maternal age and high risk screening test (2/64, 3.1%), combined advanced maternal age and fetal malformation (1/64, 1.6%), and combined advanced maternal age and anamnestic risk (2/64, 3.1%). Before further analysis, four women were excluded from the study as the chorionic villous/amniotic fluid sampling was not sufficient for downstream analyses. A total of 240 biological samples were sequenced, including chorionic villi or amniotic fluid, vaginal swabs, rectal swabs, and saliva samples from 60 pregnant women.

After the DADA2 filtering, the sequencing of the V3 region of the 16S rRNA gene produced a total of 9,916,147 reads, identifying 15,207 features. The 24 no template controls produced a total of 223,186 reads, identifying 1,560 features.

For the analyses, we grouped the samples as follows: chorionic villi (CVS, n= 23) and the matched samples including vaginal swabs (Vag.CVS), rectal swabs (Rect.CVS), and saliva samples (Sal.CVS). The same sample grouping was performed for the amniotic fluid samples (AF, n=37) and matched samples including vaginal swabs (Vag.AF), rectal swabs (Rect.AF), and saliva sample (Sal.AF) (**Table 2**).

Total of 240 samples			
	Vaginal swabs	Rectal swabs	Saliva samples
Amniocentesis samples n.37	37	37	37
Villocentesis samples n.23	23	23	23

Table 2. Analyzed Samples

As next step, we computed the core microbiome of the negative controls, identifying 10 features present in 30% of samples, including 4 features corresponding to *Homo sapiens* sequences and the remaining features corresponding to *Ralstonia pickettii, Escherichia coli, Bacillus pseudofirmus, Cutibacterium acnes* and *Pseudomonas fulva* (2 different features) sequences. No common features were identified at a higher percentage of presence. The **Table 3** shows the unique bacteria identified in one or more no template controls and that was identified in less than 30% of these samples. We filtered these bacteria from taxonomic assignment of the samples. This allowed us to sort the CVS and AF samples into true positive samples for the presence of bacterial DNA and false positive samples for the presence of DNA. In these last samples, the bacteria identified overlapped those present in the negative controls. This event depends on the presence of contaminating DNA in the commercial kits used for the library construction, thus being an unavoidable problem. After the sorting, 12/37 (32%) AF samples tested positive for the presence of bacterial DNA and 10/23 (44%) CVS samples tested positive for the presence of bacterial DNA.

Bacteria
Stenotrophomonas spp.
Methylobacterium-Methylorubrum spp.
Chryseobacterium spp.
Cloacibacterium spp.
Empedobacter spp.
Pedobacter spp.
Posterioochromonas spp.
Desulfovibrio spp.
Anaerobacillus spp.
Exiguobacterium spp.
Weissella paramesenteroides
Saccharimonadales spp.
Azospirillum spp.
Brevundimonas spp.
Devosia spp.
Novosphingobium spp.
Sphingomonas spp.
Delftia spp.
Diaphorobacter spp.
Herbaspirillum spp.
Dechloromonas spp.
Enhydrobacter spp.

Table 3. The kitome. Unique bacteria identified in one or more no template controls (n=24) and identified in less than 30% of these samples. The names of bacteria are reported, several features corresponded to each one.

4.2.2. Bacterial species relative abundance and prevalence

Including the positive samples, we tested the alpha diversity (microbiome diversity within a community) by the Evenness and Observed ASVs metrics, retaining 5,000 reads per each sample. Regarding the evenness metric, which quantifies how equally distributed are the species within a community, when comparing the positive CVS/AF samples with the matched vaginal, rectal and saliva samples there were not significant differences. An evenness value = 1 is reached when all species have the same abundance. Only one significant difference was observed when comparing the CVS positive samples for bacterial DNA and the matched saliva samples, which showed a higher evenness (Kruskal-Wallis test, FDR p value= 0.008) (**Figure 5A).** The same trend was observed for the ASVs metric, which accounts for the total number of species in the samples, showing no significant differences among samples groups (**Figure 5B)**.

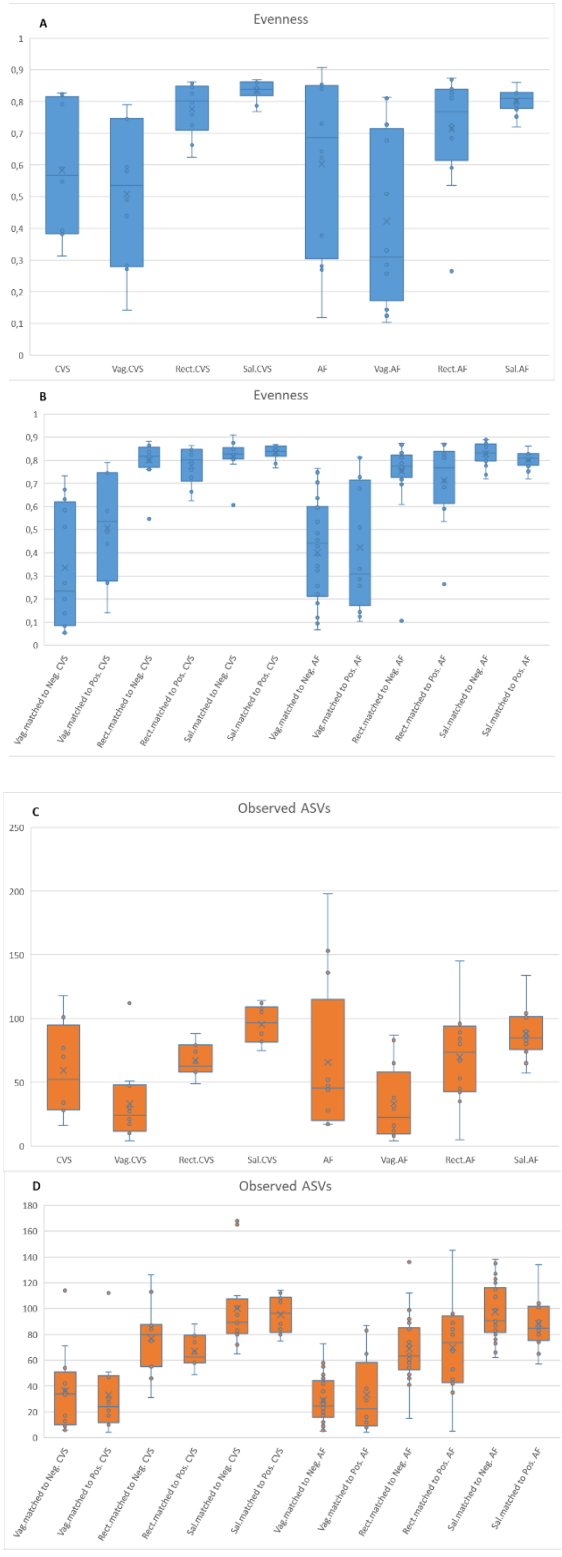

Figure 5. A/B) Alpha diversity in AF and CVS samples. The alpha diversity metrics measured by means of the evenness and observed ASVs metrics. CVS = chorionic villus samples; Vag.CVS = matched vaginal swabs of CVS; Rect.CVS = matched rectal swabs of CVS samples; Sal.CVS = matched saliva samples of CVS; AF = amniotic fluid; Vag.AF = matched vaginal swabs of AF; Rect.AF = matched rectal swabs of AF samples; Sal.AF = matched saliva samples of AF. The comparisons among groups were performed by the Kruskal-Wallis test. **C/D) Alpha diversity in different body sites.** The alpha diversity metrics measured by means of the evenness and observed ASVs metrics. The comparisons among groups were performed by the Kruskal-Wallis test.

Then, we compared the alpha diversity of the vaginal, rectal and saliva samples matched to the positive CVS/AF samples with the vaginal, rectal and saliva samples matched to the negative CVS/AF samples. Concerning both evenness and observed ASVs metrics, there were not significant differences (**Figure 5C/5D**).

After, we performed a beta diversity analysis which measures the similarity or dissimilarity of the analyzed groups. We used the Bray Curtis dissimilarity index to calculate the distance matrices that are visualized by the principal coordinate analysis (PCoA). **The Figure 6** shows that both CVS and AF clustered near of the vaginal and rectal samples and were more distant from the saliva samples. Regardless of the graphical clustering, the pairwise PERMANOVA highlighted significant differences in these samples' groups (**Table 4**).

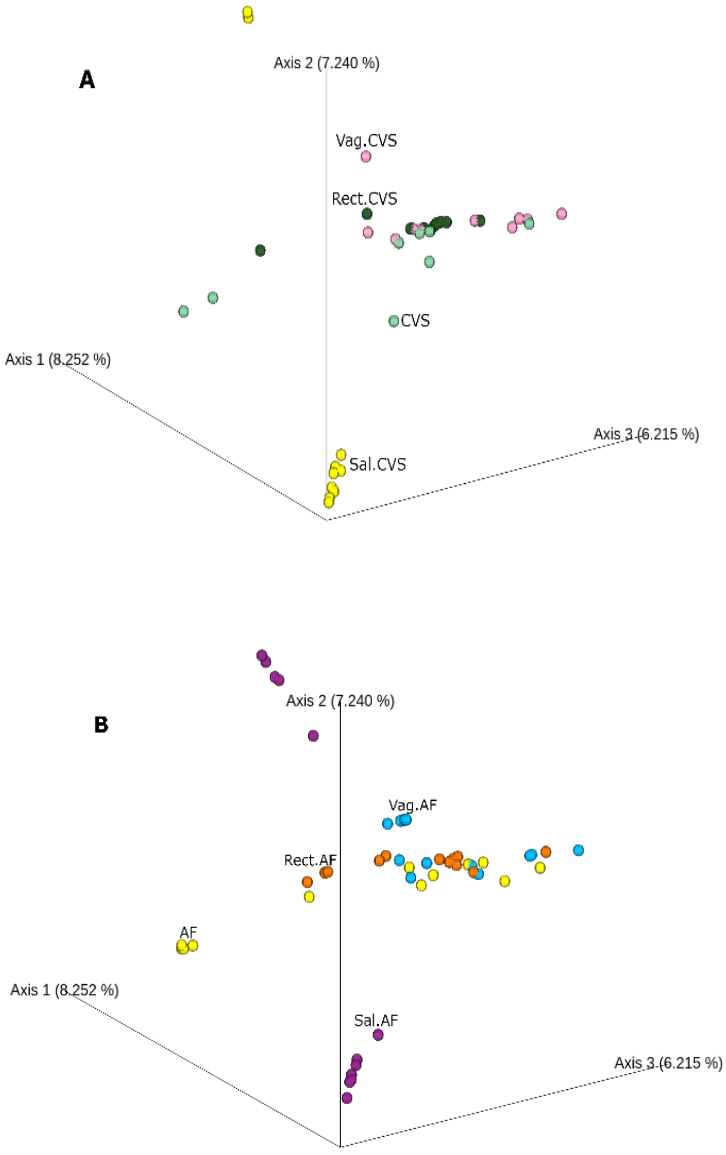

Figure 6. The beta diversity. Principal coordinate analysis (PcoA) based on Bray-Curtis dissimilarity matrix of bacterial communities in the analyzed groups. **A)** CVS samples positive for bacterial DNA (light green) and matched vaginal (pink), rectal (dark green), saliva (yellow) samples. **B)** AF samples (yellow) and matched vaginal (light blue), rectal (orange), saliva (purple) samples. Not all the analyzed samples are visible, hiding samples in an ordination can be misleading.

PAIRWISE PERMANOVA

CVS *vs*		Pseudo-F	FDR p value
	Vag.CVS	1.6	0.03
	Rect.CVS	2	0.001
	Sal.CVS	3.6	0.001
AF *vs*		Pseudo-F	FDR p value
	Vag.AF	2.8	0.001
	Rect.AF	2.3	0.003
	Sal.AF	3.9	0.001

Table 4. PERMANOVA. Results of the pairwise PERMANOVA of the Bray Curtis dissimilarity matrix. CVS = chorionic villus samples; Vag.CVS = matched vaginal swabs of CVS; Rect.CVS = matched rectal swabs of CVS samples; Sal.CVS = matched saliva samples of CVS; AF = amniotic fluid; Vag.AF = matched vaginal swabs of AF; Rect.AF = matched rectal swabs of AF samples; Sal.AF = matched saliva samples of AF.

4.2.3. Microbiome profile of fetus-placental complex and other maternal body districts.

To compare the differential abundance of the microbial taxa between analyzed groups , we used the ANCOM test (analysis of composition of microbiomes). The bacteria that significantly changed between the compared groups were *Anaerococcus* (W=551), *Corynebacterium* (W=560), *Dialister* (W=561), *Gemella* (W=554), *Haemophilus* (W=556), *Lactobacillus* (W=561), *Mobiluncus* (W=520), *Peptoniphilus* (W=552), *Porphyromonas* (W=561), *Prevotella* (W=561), *Streptococcus* (W=560), *Staphylococcus* (W=543), and *Veillonella* (W=558).

Figure 7 shows the distribution and the relative abundances of the significantly modulated bacteria in the positive AF and CVS samples and the matched vaginal, rectal, and saliva

samples. In AF positive samples, the most frequently identified bacterial DNA belonged to *Lactobacillus* (n=5) which was shared with the vaginal samples and *Streptococcus* (n=5) which was shared with saliva samples. The remaining bacteria were present in more than one body site and were less frequently identified.

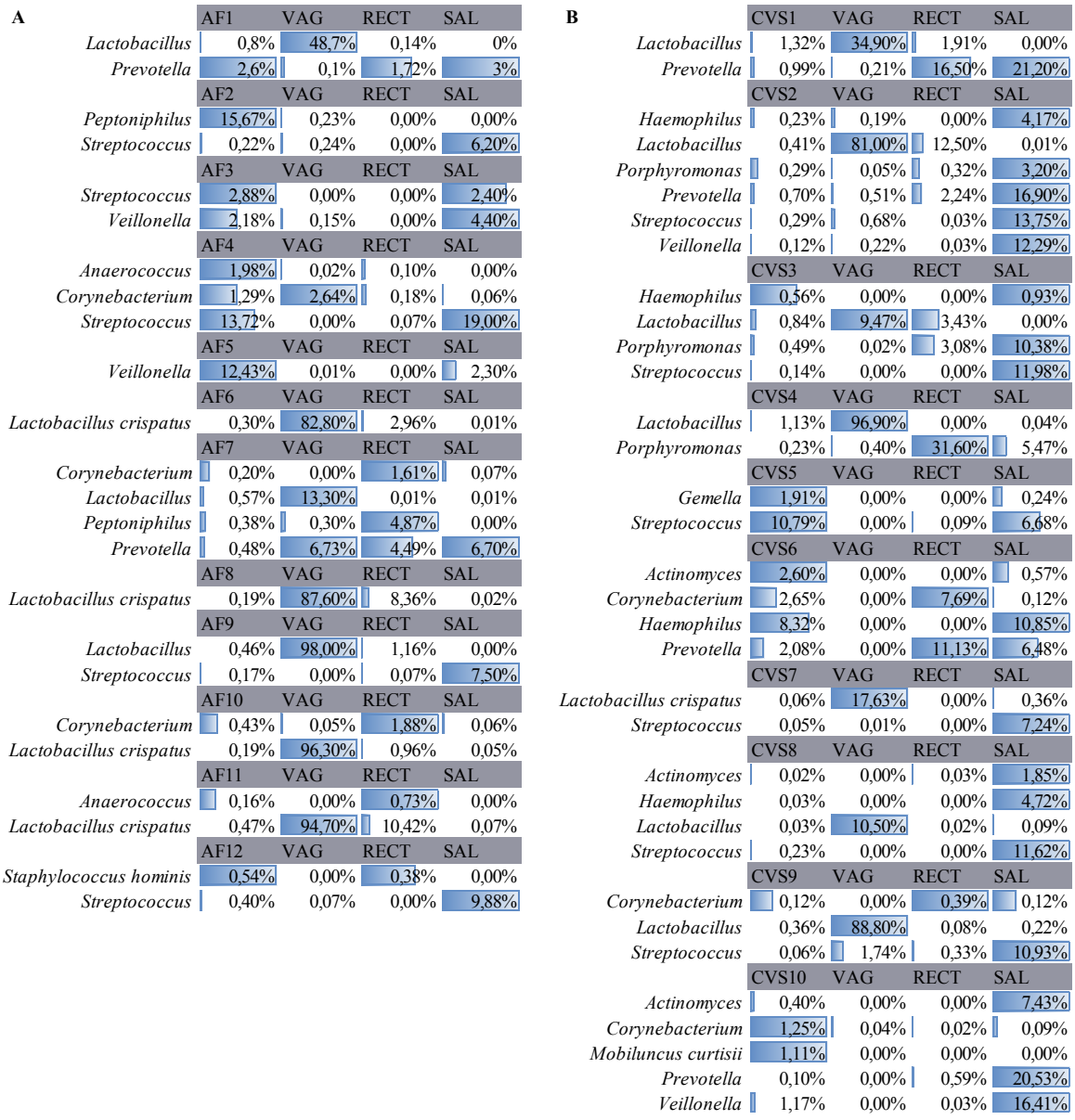

Figure 7. Bacteria in positive AF/CVS and matched samples. The identified bacteria in the amniotic fluid samples (AF) and chorionic villi samples (CVS) positive for the presence of bacterial DNA and in the matched vaginal (VAG), rectal (RECT), and saliva (SAL) samples. Data are presented as relative abundances

A higher microbial heterogeneity was observed in positive CVS samples, showing the presence of DNA belonging to 10 different bacteria. The most frequently identified DNA belonged to *Lactobacillus* (n=7), shared with vaginal samples, and *Streptococcus* (n=6), shared with vaginal and saliva samples (**Figure 7**).

Then, we applied the LEfSE test to identify microbiological biomarkers in the vaginal, rectal and saliva samples matched to the negative CVS/AF compared with those matched to the positive CVS/AF. LEfSE test identified several bacterial genera most likely to explain the differences among sample groups (**Table 5**).

Biomarker	**LDA score**	**FDR p value**
Alloprevotella	5.15	<0.001
Campylobacter	6.02	<0.001
Dialister	5.66	0.001
Fusobacterium	5.65	<0.001
Gemella	5.46	<0.001
Granulicatella	5.66	<0.001
Haemophilus	5.85	<0.001
Lactobacillus	6.68	<0.001
Peptoniphilus	5.97	<0.001
Prevotella	6.04	0.002
Rothia	5.03	<0.001
Staphylococcus	5.74	<0.001
Streptococcus	6.37	<0.001
TM7x	5.19	<0.001
Veillonella	5.94	<0.001

Table 5. Biomarkers at the genus level. Biomarkers identified by the LEfSe test at the genus level in the vaginal, rectal and saliva samples matched to the negative CVS/AF compared with those matched to the positive CVS/AF.

The **Figure 8A** shows the median abundances of the biomarkers identified by the LEfSE test in the samples matched to the positive and negative CVS samples. The saliva samples matched to the positive CVS showed an increased abundance of A*lloprevotella, Fusobacterium, Granulicatella, Haemophilus, Prevotella, Streptococcus* and *Veillonella* compared to the saliva samples matched to the negative CVS. Concerning the rectal samples matched to the positive CVS samples, there was higher *Campylobacter* and *Peptoniphilus* while lower *Prevotella* compared to the rectal samples matched to the negative CVS. In the vaginal samples matched to positive CVS, an increase of lactobacilli was observed compared to the vaginal samples matched to negative CVS.

A

	Saliva		Rectal swab		Vaginal swab	
	Positive CVS	Negative CVS	Positive CVS	Negative CVS	Positive CVS	Negative CVS
Alloprevotella	2,23%	1,74%	0,00%	0,00%	0,00%	0,00%
Campylobacter	0,00%	0,00%	2,00%	0,00%	0,00%	0,00%
Dialister	0,13%	0,08%	1,38%	1,55%	0,10%	0,03%
Fusobacterium	3,22%	0,91%	0,01%	0,00%	0,02%	0,00%
Gemella	1,42%	1,41%	0,00%	0,00%	0,00%	0,00%
Granulicatella	3,49%	2,56%	0,00%	0,00%	0,00%	0,00%
Haemophilus	5,55%	4,69%	0,00%	0,00%	0,00%	0,00%
Lactobacillus	0,00%	0,01%	0,10%	0,23%	38,68%	28,80%
Peptoniphilus	0,00%	0,00%	1,63%	0,69%	0,04%	0,00%
Prevotella	15,88%	13,35%	0,45%	4,55%	0,44%	0,18%
Rothia	1,99%	0,21%	0,00%	0,00%	0,00%	0,00%
Staphylococcus	0,00%	0,00%	0,02%	0,00%	0,02%	0,03%
Streptococcus	15,31%	10,29%	0,14%	0,24%	0,03%	0,02%
TM7x	0,48%	0,15%	0,00%	0,00%	0,00%	0,00%
Veillonella	7,70%	6,88%	0,01%	0,00%	0,00%	0,00%

B

	Saliva		Rectal swab		Vaginal swab	
	Positive AF	Negative AF	Positive AF	Negative AF	Positive AF	Negative AF
Alloprevotella	2,29%	4,10%	0,00%	0,00%	0,00%	0,00%
Campylobacter	0,00%	0,00%	0,00%	0,00%	0,00%	0,00%
Dialister	0,15%	0,22%	0,64%	2,35%	0,00%	0,03%
Fusobacterium	1,70%	0,48%	0,00%	0,00%	0,00%	0,00%
Gemella	2,08%	0,00%	0,00%	0,00%	0,00%	0,00%
Granulicatella	4,24%	2,86%	0,00%	0,00%	0,00%	0,00%
Haemophilus	5,44%	4,72%	0,00%	0,00%	0,00%	0,00%
Lactobacillus	0,00%	0,02%	0,65%	0,04%	33,86%	42,62%
Peptoniphilus	0,00%	0,00%	0,46%	0,43%	0,00%	0,00%
Prevotella	7,62%	17,45%	2,52%	11,42%	0,02%	0,28%
Rothia	0,77%	0,23%	0,00%	0,00%	0,00%	0,00%
Staphylococcus	0,00%	0,00%	0,52%	0,02%	0,02%	0,00%
Streptococcus	12,43%	13,91%	0,07%	0,22%	0,02%	0,03%
TM7x	0,20%	0,19%	0,00%	0,00%	0,00%	0,00%
Veillonella	0,43%	7,84%	0,00%	0,00%	0,00%	0,00%

Figure 8. Significantly different bacterial genera among samples matched to positive and negative CVS and AF samples. Biomarkers identified by the LEfSe test at the genus level in the vaginal, rectal and saliva samples matched to the negative and positive CVS (panel A) and AF samples (panel B). Data are shown as median relative abundances.

The **Figure 8B** shows the median abundances of the biomarkers identified by the LEfSE test in the samples matched to the negative and positive AF samples. The saliva samples matched to the positive AF samples showed increased *Fusobacterium, Granulicatella, Haemophilu*s and *Streptococcus* while *Prevotella* decreased compared to the saliva samples matched to the negative AF. Rectal and vaginal samples matched to positive AF samples showed increased *Prevotella* and *Lactobacillus*, respectively, compared to the samples matched to the negative AF samples.

The same analysis was performed at the species level. *Campylobacter ureolyticus, Lactobacillus crispatus, Prevotella bivia, Staphylococcus epidermidis* and *S. salivarius* were identified as features most likely to explain the differences among sample groups (**Table 6)**.

Biomarker	**LDA score**	**FDR p value**
Campylobacter ureolyticus	6.02	<0.001
Lactobacillus crispatus	6.44	<0.001
Prevotella bivia	5.94	<0.001
Staphylococcus epidermidis	5.74	<0.001
Streptococcus salivarius	5.62	<0.001

Table 6. Biomarkers at the species level. Biomarkers identified by the LEfSe test at the species level in the vaginal, rectal and saliva samples matched to the negative CVS/AF compared with those matched to the positive CVS/AF.

The **Figure 9A** shows the increase of *S. salivarius* in the saliva samples and of *L. crispatus* in the vaginal samples matched to the negative CVS samples when compared to the samples matched to the positive CVS. *C. ureolyticus* was increased in the rectal samples matched to the positive CVS samples while, in these samples, *P. bivia* decreased compared to the rectal samples matched to the negative CVS.

A

	Saliva		Rectal swab		Vaginal swab	
	Positive CVS	Negative CVS	Positive CVS	Negative CVS	Positive CVS	Negative CVS
Campylobacter ureolyticus	0,00%	0,00%	1,53%	0,13%	0,02%	0,00%
Lactobacillus crispatus	0,00%	0,01%	0,01%	0,14%	0,45%	28,57%
Prevotella bivia	0,00%	0,00%	0,35%	1,28%	0,07%	0,04%
Staphylococcus epidermidis	0,00%	0,00%	0,00%	0,00%	0,02%	0,03%
Streptococcus salivarius	0,99%	1,31%	0,02%	0,06%	0,00%	0,00%

B

	Saliva		Rectal swab		Vaginal swab	
	Positive AF	Negative AF	Positive AF	Negative AF	Positive AF	Negative AF
Campylobacter ureolyticus	0,00%	0,00%	0,06%	0,08%	0,00%	0,00%
Lactobacillus crispatus	0,00%	0,00%	0,65%	0,04%	21,40%	28,57%
Prevotella bivia	0,00%	0,00%	0,12%	0,14%	0,02%	0,04%
Staphylococcus epidermidis	0,00%	0,00%	0,47%	0,02%	0,02%	0,03%
Streptococcus salivarius	0,73%	1,06%	0,03%	0,00%	0,00%	0,00%

Figure 9. Significantly different bacterial species among samples matched to positive and negative CVS and AF samples. Biomarkers identified by the LEfSe test at the species level in the vaginal, rectal and saliva samples matched to the negative and positive CVS (panel A) and AF samples (panel B). Data are shown as median relative abundances.

The **figure 9B** revealed a similar trend to that observed for the saliva and vaginal samples matched to the CVS samples concerning *S. salivarius* and *L. crispatus*. With regard to the rectal samples, higher *L. crispatus*, *S. epidermidis* and *S. salivarius* increased in the rectal samples matched to the positive AF samples compared to the samples matched to the negative AF.

4.3. Immuno profile analisys

To analyze the possible interaction between intrauterine and vaginal immune profile, correlated with the presence of bacterial microorganisms, the concentration of 27 soluble immune proteins, including Th1/pro‐inflammatory and Th2/anti‐inflammatory cytokines, chemokines, and trophic factors, was measured in the AF/CVS samples and vaginal swabs. By comparing the immunological profile of the CVS tested positive for bacterial DNA with that of the CVS tested negative, no significant difference in the concentration of analyzed immune proteins was observed. While, in amniotic fluids tested positive for the detection of bacteria, a statistically significant increase in concentration of two pro-inflammatory proteins, the cytokine IL-8, and grow factor G-CSF, was observed (**Figure 10**).

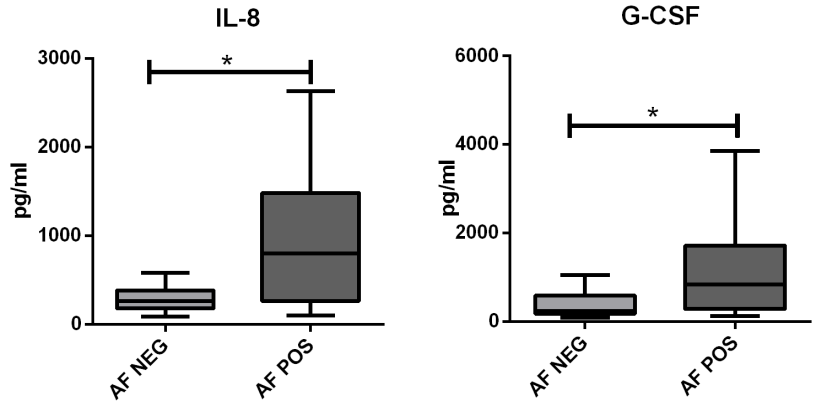

Figure 10. **Immune soluble factors in AF samples.** The significantly modulated immune soluble factors between AF samples positive for bacterial DNA and AF samples negative for bacterial DNA. Differences were calculated by means of a non-parametric T test for pairwise comparisons (GraphPad Prism v. 5). *$p < 0.05$, ** $p < 0.01$.

When accounting for the immune soluble factors dosed in the vaginal swabs, we did not observe significant differences in the vaginal swabs matching to the CVS samples. On the contrary, we observed significantly modulated factors in the vaginal swabs matched to the AF samples positive for the bacterial DNA compared to the vaginal swabs matched to the AF samples negative for the bacterial DNA. Namely, IL-4, IL-10, IFN-γ and IL-1ra were significantly down-regulated in the vaginal swabs matched to the AF samples positive for the bacterial DNA (**Figure 11**).

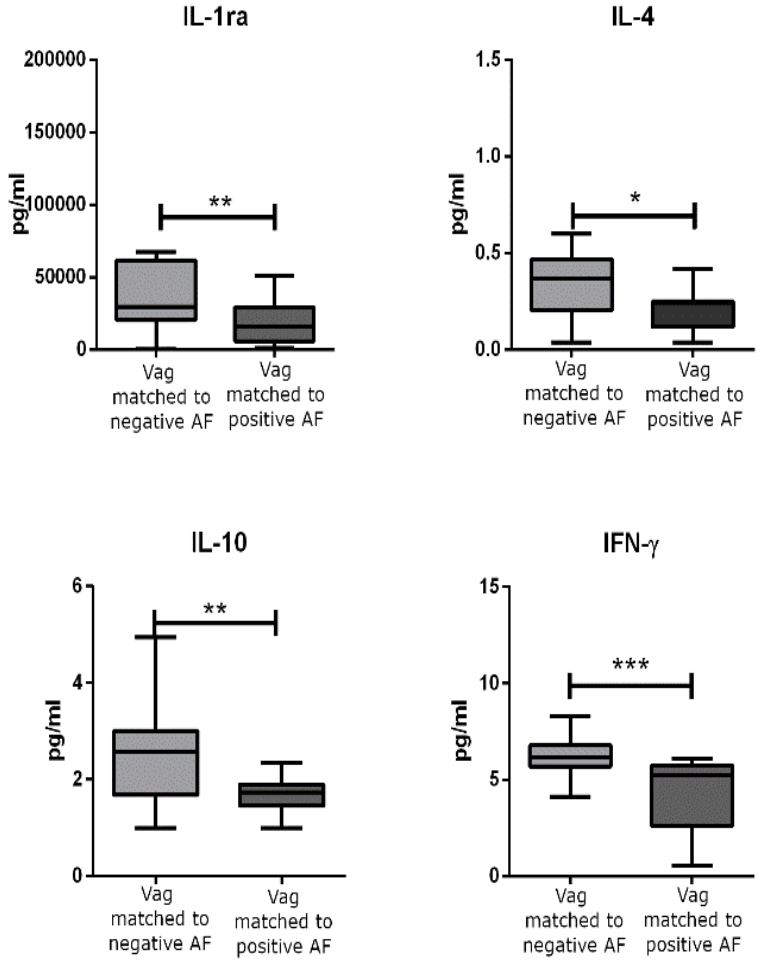

Figure 11. Immune soluble factors in vaginal samples. The significantly modulated immune soluble factors between vaginal swabs matched to negative and positive AF samples for bacterial DNA. Differences were calculated by means of a non-parametric T test for pairwise comparisons (GraphPad Prism v. 5). *$p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

5. DISCUSSION

In the last years, there is a growing interest of researchers in the potential importance of the placental microbiome and microbiome-metabolite interactions in immune response and subsequent pregnancy outcome. Numerous reports have documented the presence of bacteria in the placental membranes, also in absence of immunopathology. It has been suggested that poor pregnancy outcomes result from an inflammatory response originating from the mother, fetus, and placenta. In addition, dysbiosis of the vagina, gut or oral microbiome has been shown to be important in the development of infection, inflammation, and negative pregnancy outcome. These reports have been accepted by the scientific community with both enthusiasm and criticism, leading to an ongoing controversy on the real existence of the "*in utero"* microbiome. Despite this evidence, there is currently no consensus regarding the existence of a placental microbiome in healthy full-term pregnancy, suggesting that the presence of bacteria was due to contamination from the environment and gene sequencing reagents. Contamination of biological samples, especially of low-biomass specimen types, with trace amounts of bacterial DNA from sampling sites, clinical or laboratory environments, reagents and consumables (the kitome) is a common technical issue as a source of false positive microbiome signals (Eisenhofer et al., 2019; Minich et al., 2019). To handle this issue, a good practice is that of implementing proper process controls as well as biological and technical replicates.

In the present study, we attempted to profile the "*in utero"* microbiome by analyzing the chorionic villi sampling and the amniotic fluids of 60 singletons pregnancies. In addition, in order to identify the possible origin of the identified bacterial DNA, at the same time of the invasive procedure, we have taken vaginal and rectal swabs, and saliva samples.

Exploiting the removal of the kitome by the elimination of the bacteria identified in the 24 no template controls that have been processed in parallel with the biological samples, we

observed that several AF and CVS did not retain a specific microbiome. This selection allowed us to discriminate samples showing the presence of bacterial DNA and samples negative for the presence of bacterial DNA.

In the CVS and AF microbiome, we did not observe the predominance of bacterial DNA from a specific bacterium. Indeed, when accounting for the alpha diversity of the positive CVS/AF samples, metrics values did not significantly differ from the other matched body districts. Thus, suggesting the presence of a heterogeneous microbiome in CVS /AF positive samples likely partially overlapping the microbial composition of the other analyzed body sites (**Figure 5A/5B**). Focusing on the alpha diversity of the vaginal, rectal and saliva samples matched to the positive CVS/AF samples and those matched to the negative CVS/AF samples, there were not differences (**Figure 5C/5D**). Though, a slightly higher uneven distribution of the microbial relative abundances was evident, from the evenness metric, in the vaginal samples matched to the positive CVS samples, likely, supporting the possibility of a predisposing vaginal microbiome to the CVS colonization. Indeed, a lower evenness number is consistent with the composition of an eubiotic vaginal microbiome which is usually dominated by *Lactobacillus* spp. alongside several other bacteria at very low amounts (Lehtoranta et al., 2021).

The similarity suggested by the alpha diversity between CVS/AF samples and the matched samples from the other body districts was partially confirmed when accounting for the bacterial identities, taken into consideration by the beta diversity analysis. CVS-positive samples clustered near the matched vaginal and rectal samples. Though, being identified as significantly different by the PERMANOVA test, we can speculate that only some bacterial species are shared between these body districts. The same observation was confirmed for the AF positive samples which clustered near of vaginal and rectal samples (**Figure 6).**

When accounting for the significantly modulated bacteria, several bacteria were identified by the ANCOM test. The identified bacteria in the CVS and AF positive samples belonged

to commensal and opportunistic pathogens of the reproductive tract and of the oral cavity (Di Stefano et al., 2022; Lehtoranta et al., 2021) that were not identified in the negative samples. This identification was consistent with the presence of these bacteria in one or more matched samples. Our results show that CVS samples harbor a greater microbial heterogeneity, in particular regarding the possibly derived oral species, suggesting that the placenta could be colonized not only from the urogenital route but also from the oral route through the hematogenous access (low-grade bacteremia) (Fardini et al., 2010). Opposite, the amniotic fluid is less exposed by the hematogenous access. Thus, it is less prone to colonization by oral microbes (**Figure 7**). However, the hematogenous source is supported by experimental evidence only on animal models, suggesting that oral bacteria could enter the uterus through the bloodstream.

We looked at possible predisposing microbiome to the colonization of CVS and AF from several maternal body districts. We observed that in saliva samples matched to the positive CVS samples and, less pronounced, in the oral microbiome of the colonized AF samples, *Fusobacterium, Prevotella, Streptococcus* and *Veillonella* increased compared to the saliva samples matched to the negative CVS/AF samples. Previous data detected, during pregnancy, an increase in pathogenic taxa in the genus *Prevotella, Fusobacterium* and *Veillonella* (Balan et al., 2018; Lin et al., 2018). While, the decrease of probiotic strains, such as *S. salivarius*, has been observed (MacDonald et al., 2021). In our cohort, in saliva samples matched to positive CVS/AF samples, *S. salivarius* decreased compared with the saliva samples matched to negative CVS/AF samples. This probiotic species is able to inhibit immune activation by periodontal disease pathogens. Considering that it is not only the most present species that determine the balance of the ecosystem, rather the complex interactions between the components, the decrease of probiotic species and the increase of keystone low-abundance microbial pathogens can orchestrate inflammatory disease by remodeling a normally eubiotic microbiota into a dysbiotic one (Hajishengallis et al., 2012). This dysbiotic

state, even at a subclinical level, could favor the low-grade bacteremia leading in particular to the colonization of CVS.

In vaginal samples, the most evident result was the absence and the decrease, respectively, of *L. crisptaus* in the samples matched to the positive CVS and AF samples compared to the samples matched to the negative CVS/AF samples (**Figures 8-9**). As shown by previous studies, vaginal microbial communities state type (CSTs) are defined by the dominance of one of four *Lactobacillus* species (*L. crispatus*, *L.gasseri, L. iners*, *L. jensenni*). In particular, *L. crispatus* shows a potential role to inhibit dysbiotic vaginal microbiome and infectious inflammation. Therefore, *L. crispatus*-dominated microbiome (CST I) has been considered important to maintain a healthy state of the vaginal environment (Walther-Antonio et al., 2014; Ravel et al., 2011). In pregnancy, healthy obstetric outcomes are associated with an enrichment of *L. crispatus,* while a decrease in the abundance of this bacterium in the vaginal environment has been associated with a higher risk of infection and preterm delivery (Odogwu et al., 2021; Aagaard et al., 2012). In our study, the significant decrease of *L. crispatus* observed in vaginal swabs matched to positive AF samples could suggest the protective role of this bacterium in the vaginal environment, and indirectly, in the fetal-placental complex colonization.

As shown in the last years, pregnancy has a unique and dynamic immunological milieu that is required to support a healthy pregnancy. Initially, a pro-inflammatory state is required for implantation which involves a release of pro-inflammatory Th1 cytokines. Next, a shift toward the release of anti-inflammatory Th2 immune proteins is required to maintain the success of normal pregnancy (Fan et al., 2017, Liuet al., 2017). Emerging evidence demonstrated the intrauterine microbiome as a key modulator of local inflammatory and immune pathways throughout pregnancy. In addition, the DNA bacterial translocation from other maternal body sites could be elicited or accompanied with an altered local immune response. Our results support the presence of significant differences between the AF samples

positive and those negative for the presence of bacterial DNA. In particular, markers of intraamniotic inflammation, such as IL-8 and G-CSF, were increased in presence of bacterial DNA in the AF samples (**Figure 10**) (Calhoun et al., 2001; Kacerovsky et al., 2009). Increased of pro-inflammatory IL-8 has been previously reported in association with pre-term delivery (Fettweis et al., 2019), and is predicted to play a role in the induction of labor. Additionally, recent studies reported that high IL-8 levels in amniotic fluid during the third trimester could be useful to identify pregnant women at risk of developing preeclampsia (Black et al., 2018; Kenny et al., 2014). It has been observed that in case of the acute inflammatory response, neutrophilic migration to the chorioamnion is induced by elevated concentrations of intra-amniotic neutrophil cytokines such as IL-8 (Kim et al., 2015).

Regarding G-CSF, it is a secretory cytokine that can be expressed in trophoblasts and endometrium. There are studies that suggest a positive effect of this factor on trophoblast growth, embryonic implantation and placental metabolism (Smith et al., 2017). On the other hand, the involvement of this growth factor in the activation of pro-inflammatory cell signaling pathways is known. Therefore, there are contrasting findings about the role of G-CSF in pregnancy, and further studies are required.

Other immune factors, such as IL-4, IL-10, IFN-γ, and IL-1ra, were downregulated in the vaginal swabs matched to the positive AF samples (**Figure 11**). To note, an increase in Th2 cytokines, including IL-4 and IL-10, and IL-1ra has fundamental role in the regulation and control of inflammation, to promote a healthy, successful pregnancy by suppressing inflammation (Chatterjee et al., 2014; Equils et al., 2020). In this regard, recent studies observed that the production of IL-4 was enhanced at the feto-maternal interface and a decrease of this production has been found in pregnant with preterm delivery. (Chatterjee et al., 2014) Likewise, in normal pregnancy, IL-10 plays a key role in Th2 response, controlling inflammation. It has been shown that the levels of IL-10 are increased in human placental tissue during a normal pregnancy, and remain high until delivery (Cheng et al., 2015; Cubro

et al., 2018). While, the placental IL-10 level is reduced in complicated pregnancies. (Romero et al., 2007). IFN- γ plays critical roles that include the activation of innate and adaptive immune responses to pathogens. Alterations in these processes are believed to contribute to gestational complications (Szabo et al., 2003). These previous studies analyzed the levels of these cytokines only in the intrauterine environment, not in the vaginal one, and were based on the pregnancy outcomes (preterm vs full term pregnancy).

In the current study, in which the pregnancy outcomes were not considered because of limited number of patients, IL-8 and G-CSF were not significantly modulated in the vaginal swabs matched to positive AF. The discrepancy in the concentration of certain immune proteins between the two body districts, according to previous studies (Mei et al., 2019), confirms that mother, placenta, and fetus all possess unique innate immune systems.

Thus, we can speculate that immune hyporesponsiveness, as represented by low cervico-vaginal concentrations of various inflammatory cytokines, and high intraamniotic concentrations of cytokines associated with inflammation could constitute an increased risk for bacterial DNA translocation among women with lower genital tract altered microbial composition (Kalinka et al., 2005).

6. CONCLUSIONS

Taken together, our results support the presence of bacterial DNA in CVS and AF samples. Thus, supporting the hypothesis of an *in utero* microbiome. Though, we cannot speculate on the clinical relevance of the presence of bacterial DNA in terms of pregnancy progression and outcomes.

To note, we had the possibility to collect CVS samples and to study a microbial composition which is less explored compared to AF samples and placentas of at term pregnancies. In this regard, our results support the hypothesis of a hematogenous spread of bacterial DNA belonging to oral microbes to the placenta, as we have observed an overlap between CVS and saliva microbiomes of the same pregnant woman. Instead, the presence of bacterial DNA overlapping vaginal bacteria seemed equally common in AF and CVS. A predisposing vaginal microbiome for this colonization seemed possible. Namely, a reduction of lactobacilli and, in particular, of *L. crispatus* seemed a risk factor. In addition, an immune hyporesponsiveness in the vaginal milieu could contribute to the bacterial DNA translocation in the amniotic fluid where, in absence of an ongoing infection, the upregulation of inflammatory cytokines was revealed. Further studies are needed to understand, the variations in fetus-placental microbiome-induced metabolic pathways and their role in pregnancy outcomes.

7. BIBLIOGRAPHY

Aagaard K, Riehle K, Ma J, Segata N, Mistretta TA, Coarfa C et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PLoS One. 2012; 7(6): e36466. doi: 10.1371/journal.pone.0036466

Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J.The placenta harbors a unique microbiome. Sci Transl Med. 2014; 21; 6(237):237ra65. doi:10.1126/scitranslmed.3008599.

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005; 43(11):5721-32. doi: 10.1128/JCM.43.11.5721-5732.2005

Abdelmaksoud AA, Koparde VN, Sheth NU, Serrano MG, Glascock AL, Fettweis JM et al. Comparison of Lactobacillus crispatus isolates from Lactobacillus-dominated vaginal microbiomes with isolates from microbiomes containing bacterial vaginosis-associated bacteria. Microbiology. 2016; 62(3): 466-475. doi: 10.1099/mic.0.000238.

Abioye AI, McDonald EA, Park S, Joshi A, Kurtis JD, Wu H et al. Maternal, placental and cord blood cytokines and the risk of adverse birth outcomes among pregnant women infected with Schistosoma japonicum in the Philippines. PLoS Negl Trop Dis. 2019; 13(6): e0007371. doi: 10.1371/journal.pntd.0007371.

AnahtarMN, Gootenberg DB, Mitchell CM, KwonDS. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. Cell Host Microbe. 2018; 23(2):159-168. doi: 10.1016/j.chom.2018.01.013.

Antony KM, Ma J, Mitchell KB, Racusin DA, Versalovic J, Aagaard K. The preterm placental microbiome varies in association with excess maternal gestational weight gain. Am J Obstet Gynecol. 2015; 212(5):653.e1-16. doi: 10.1016/j.ajog.2014.12.041

Ao M, Miyauchi M, Furusho H, Inubushi T, Kitagawa M, Nagasaki A et al. Dental Infection of Porphyromonas gingivalis Induces Preterm Birth in Mice. PLoS One. 2015; 10(8): e0137249. doi: 10.1371/journal.pone.0137249

Balan P, Chong YS, Umashankar S, Swarup S, Loke WM, Lopez V, He HG, Seneviratne CJ. Keystone Species in Pregnancy Gingivitis: A Snapshot of Oral Microbiome During Pregnancy and Postpartum Period. Front Microbiol. 2018; 9:2360. doi: 10.3389/fmicb.2018.02360

Baker JM, Chase DM, Herbst-Kralovetz MN. Uterine microbiota: residents, tourist or invaders? Front Immunol 2018; 9: 2008

Black KD, Horowitz JA. Inflammatory Markers and Preeclampsia: A Systematic Review. Nurs Res. 2018; 67(3): 242-251. doi: 10.1097/NNR.0000000000000285.

Boutigny H, de Moegen ML, Egea L, Badran Z, Boschin F et al, Oral infections and pregnancy: knowledge of Gynecologists/Obstetricians, midwives and dentists. Oral Health Prev Dent. 2016; 14: 41-7

Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D et al. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Transl Res.2019; 207: 30–43.doi:10.1016/j.trsl.2018.12.005

Bushman FD. De-Discovery of the Placenta Microbiome. Am J Obstet Gynecol. 2019; 220(3): 213-214. doi: 10.1016/j.ajog.2018.11.1093.

Calhoun DA, Chegini N, Polliotti BM, Gersting JA, Miller RK, Christensen RD et al. Granulocyte colony-stimulating factor in preterm and term pregnancy, parturition, and intra-amniotic infection. . Obstet Gynecol. 2001; 97(2): 229-34. doi: 10.1016/s0029-7844(00)01120-0

Campisciano G, Zanotta N, Licastro D, De Seta F, Comar M. In vivo microbiome and associated immune markers: New insights into the pathogenesis of vaginal dysbiosis. Sci Rep. 2018; 8(1): 2307. doi: 10.1038/s41598-018-20649-x

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010; 7(5):335-6. doi: 10.1038/nmeth.f.303

Carrillo-de-Albornoz A, Figuero E, Herrera D, Bascones-Martínez A. J Clin Periodontol. 2010; 37(3): 230-40. doi: 10.1111/j.1600-051X.2009.01514.x

Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM.. Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. Front Immunol. 2014; 5: 253. doi: 10.3389/fimmu.2014.00253

Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. Nat Commun. 2017; 8: 875. doi:10.1038/s41467-017-00901-0.

Cheng SB, Sharma S. Interleukin-10: a pleiotropic regulator in pregnancy. Am J Reprod Immunol. 2015; 73(6): 487-500. doi: 10.1111/aji.12329
Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc. 2020; 15(3): 799-821. doi: 10.1038/s41596-019-0264-1.

Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep. 2016; 6: 23129. doi: 10.1038/srep23129.

Cubro H, Kashyap S, Nath CM, Ackerman AW, Garovic.VD. The Role of Interleukin-10 in the Pathophysiology of Preeclampsia. Curr Hypertens Rep. 2018; 20(4): 36. doi: 10.1007/s11906-018-0833-7.

de Goffau MC, Lager S, Salter SJ et al. Recognizing the reagent microbiome. Nat.Microbiol. 2018; 3(8): 851-853

de Goffau MC, Lager S, Sovio U, Gaccioli F, Cook E, Peacock SJ et al. Human placenta has no microbiome but can contain potential pathogens. Nature. 2019; 572(7769): 329–34. doi: 10.1038/s41586-019-1451-5

D'Ippolito S, Tersigni C, Marana R, Di Nicuolo F, Gaglione R, Rossi ED et al. Inflammosome in the human endometrium: further step in the evaluation of the "maternal side". Fertil Steril. 2016; 105(1): 111-8.e1-4. doi: 10.1016/j.fertnstert.2015.09.027

Di Stefano M, Polizzi A, Santonocito S, Romano A, Lombardi T, Isola G. Impact of Oral Microbiome in Periodontal Health and Periodontitis: A Critical Review on Prevention and Treatment. Int J Mol Sci. 2022; 23(9): 5142. doi: 10.3390/ijms23095142.

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 2010; 107(26): 11971-5. doi: 10.1073/pnas.1002601107.

Dominguez-Bello MG, De Jesus-Laboy KM, Shen N, Cox LM, Amir A, Gonzalez A et al. Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer. JC. Nat Med. 2016; 22(3): 250-3. doi: 10.1038/nm.4039.

Eisenhofer R, Minich JJ, Marotz C, Cooper A, Knight R, Weyrich LS et al. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. Trends Microbiol. 2019; 27(2): 105-117. doi: 10.1016/j.tim.2018.11.003.

Equils O, Kellogg C, McGregor J, Gravett M, Neal-Perry G, Gabay C et al. The role of the IL-1 system in pregnancy and the use of IL-1 system markers to identify women at risk for pregnancy complication. Biol Reprod. 2020; 103(4): 684-694. doi: 10.1093/biolre/ioaa102.

Fang RI, Chen LX, Shu WS, Yao SZ, Wang SW et al. Barcoded sequencing reveals diverse intrauterine microbiome in patients suffering with endometrial polyps. Am J Transl Res. 2016; 8: 1581-92

Fan DM, Wang Y, Liu XL, et al.. Polymorphisms in interleukin-6 and interleukin-10 may be associated with risk of preeclampsia. Genet Mol Res. 2017; 16:gmr16018588

Fardini Y, Chung P, Dumm R, Joshi N, Han YW. Transmission of Diverse Oral Bacteria to Murine Placenta: Evidence for the Oral Microbiome as a Potential Source of Intrauterine Infection. Infect Immun. 2010; 78(4): 1789–1796. doi:10.1128/IAI.01395-09

Ferrocino I, Ponzo V, Gambino R, Zarovska A, Leone F, Monzeglio C et al. Changes in the gut microbiota composition during pregnancy in patients with gestational diabetes mellitus (GDM). Sci Rep. 2018; 8: 12216. doi:10.1038/s41598-018-30735-9

Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI et al. The vaginal microbiome and preterm birth. Nat Med. 2019; 25(6): 1012–1021. doi:10.1038/s41591-019-0450-2

Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. J Assist Reprod Genet. 2016;33(1): 129–136. doi:10.1007/s10815-015-0614-z

Gargano JW, Holzman C, Senagore P, Thorsen P, Skogstrand K, Hougaard DM et al. Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. J Reprod Immunol. 2008; 79(1): 100-10. doi: 10.1016/j.jri.2008.08.006.

Gever D, Knight R, Petrosino JF, Huang K, McGuire AL, Birren BW, et al. The Human microbiome project: a community resource for the healthy hman microbiome. Plos Biol. 2012; 10: e1001377.

Gogeneni H, Buduneli N, Ceyhan-Öztürk B,Gümüş P, Akcali A, Zeller I et al. Increased infection with key periodontal pathogens during gestational diabetes mellitus. J Clin Periodontol. J Clin Periodontol. 2015; 42(6): 506–512. doi: 10.1111/jcpe.12418

Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Nitert MD. Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. Sci Rep. 2017; 7: 2860. doi:10.1038/s41598-017-03066-4

Gomez-de Aguero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. Science. 2016; 351: 1296–1302. doi:10.1126/science.aad2571

Gustafsson AM, Fransson E, Dubicke A, Hjelmstedt AK, Ekman-Ordeberg G, Silfverdal SAet al. Low levels of anti-secretory factor in placenta are associated with preterm birth and inflammation. Acta Obstet Gynecol Scand. 2018; 97(3): 349-356. doi: 10.1111/aogs.13282

Ha JE, Oh KJ, Yang HJ, Jun JK, Jin BH, Paik DI et al. Oral health behaviors, periodontal disease, and pathogens in preeclampsia: a case-control study in Korea. J Periodontol. 2011; 82(12): 1685-92. doi: 10.1902/jop.2011.110035

Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol. 2012; 27(6): 409-19. doi: 10.1111/j.2041-1014.2012.00663.x

Hajishengallis G, Lambris JD. Complement and dysbiosis in periodontal disease. Immunobiology. 2012; 217(11): 1111-6. doi: 10.1016/j.imbio.2012.07.007

Hirano E, Sugita N, Kikuchi A, Shimada Y, Sasahara J, Iwanaga R et al. The association of Aggregatibacter actinomycetemcomitans with preeclampsia in a subset of Japanese pregnant women. J Clin Periodontol. 2012; 39(3): 229-38. doi: 10.1111/j.1600-051x.2011.01845.x.

Hodyl NA, Aboustate N, Bianco-Miotto T, Roberts CT, Clifton VL, Stark MJ. Child neurodevelopmental outcomes following preterm and term birth: What can the placenta tell us? Placenta. 2017; 57:79-86. doi: 10.1016/j.placenta.2017.06.009.

Hong BY, Furtado Araujo MV, Strausbaugh LD, Terzi E, Ioannidou E, Diaz PI. Microbiome profiles in periodontitis in relation to host and disease characteristics. PLoS One. 2015; 10(5):e0127077. doi: 10.1371/journal.pone.0127077

Hyman RW, Fukushima M, Jiang HJ, Fung E, Rand L, Johnson B et al. Diversity of the Vaginal Microbiome Correlates With Preterm Birth. Reprod Sci.2014; 21(1): 32–40.doi:10.1177/1933719113488838

Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health and disease.
Cell Host Microbe. 2014; 16(3): 276-89. doi: 10.1016/j.chom.2014.08.014.

Integrative HMP (iHMP) Research Network Consortium. Integrative Human Microbiome Project. Nature. 2019; 569(7758): 641-648. doi: 10.1038/s41586-019-1238-8.

Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol. 2012; 32(1): 23-63. doi: 10.1615/critrevimmunol.v32.i1.30.

Jašarević E, Howard CD, Misic AM, Beiting DP, Bale TL. Stress during pregnancy alters temporal and spatial dynamics of the maternal and offspring microbiome in a sex-specific manner. Sci Rep. 2017; 7: 44182. doi:10.1038/srep44182

Jašarević E, Hill EM, Kane PJ, Rutt L, Gyles T, Folts L et al. The composition of human vaginal microbiota transferred at birth affects offspring health in a mouse model. Nat Commun. 2021; 12: 6289. doi: 10.1038/s41467-021-26634-9

Jeon SJ, Cunha F, Vieira-Neto A, Bicalho RC, Lima S, Bicalho ML, Galvão KN. Blood as a route of transmission of uterine pathogens from the gut to the uterus in cows. Microbiome.2017;5: 109. doi:10.1186/s40168-017-0328-9

Kacerovsky M, Drahosova M, Hornychova H, Pliskova L, Bolehovska R, Forstl M et al. Value of amniotic fluid interleukin-8 for the prediction of histological chorioamnionitis in preterm premature rupture of membranes. Neuro Endocrinol Lett. 2009; 30(6): 733-8.

Kalinka J, Sobala W, Wasiela M, Brzezińska-Błaszczyk E.Decreased proinflammatory cytokines in cervicovaginal fluid, as measured in midgestation, are associated with preterm delivery. Am J Reprod Immunol. 2005; 54(2): 70-6. doi: 10.1111/j.1600-0897.2005.00289.x.

Kemp MW. Preterm birth, intrauterine infection, and fetal inflammation. Front Immunol. 2014; 5: 574. doi: 10.3389/fimmu.2014.00574

Kenny LC, Black MA, Poston L, Taylor R, Myers JE, Baker PN et al. Early pregnancy prediction of preeclampsia in nulliparous women, combining clinical risk and biomarkers: the Screening for Pregnancy Endpoints (SCOPE) international cohort study. Hypertension. 2014; 64(3): 644-52. doi: 10.1161/HYPERTENSIONAHA.114.03578.

Kiecolt-GlaserJK, Wilson SJ, Bailey ML, Andridge R, Peng J, Jaremka LM et al. Marital Distress, Depression, and a Leaky Gut: Translocation of Bacterial Endotoxin as a Pathway to Inflammation. Psychoneuroendocrinology. 2018; 98: 52–60. doi: 10.1016/j.psyneuen.2018.08.007

Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. Am J Obstet Gynecol. 2015; 213(4 Suppl):S29-52. doi: 10.1016/j.ajog.2015.08.040.

Kim SK, Guevarra RB, Kim YT, Kwon J, Kim H, Cho JH, et al. The Role of Probiotics in Human Gut Microbiome-Associated Diseases. J Microbiol Biotechnol. 2019; 28; 29(9): 1335-1340. doi: 10.4014/jmb.1906.06064.

Koedooder R, Mackens S, Budding A, Fares D, Bloockeel C, et al. Identification and evaluation of the microbiome in the female and male reproductive tracts. Hum Reprod Update. 2019; 25: 298-325

Konishi H, Urabe S, Miyoshi H, Teraoka Y, Maki T, Furusho H et al. Fetal Membrane Inflammation Induces Preterm Birth Via Toll-Like Receptor 2 in Mice With Chronic Gingivitis. S. Reprod Sci. 2019; 26(7): 869-878. doi: 10.1177/1933719118792097

Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 2012; 150(3):470-80. doi: 10.1016/j.cell.2012.07.008

Kroon SJ, Ravel J, Huston WM. Cervicovaginal microbiota, women's health and reproductive outcomes. Fertil Steril. 2018; 110: 327-36 doi: 10.1016/j.fertnstert.2018.06.036

Kuperman AA, Zimmerman A, Hamadia S, Ziv O, Gurevich V, Fichtman B et al. Deep microbial analysis of multiple placentas shows no evidence for a placental microbiome. BJOG. 2020; 127(2):159–69. doi: 10.1111/1471-0528.15896

Lauder AP, Roche AM, Sherrill-Mix S, Bailey A, Laughlin AL, Bittinger K et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome. 2016; 4: 29.doi:10.1186/s40168-016-0172-3

Lehtoranta L, Hibberd AA, Yeung N, Laitila A, Maukonen J, Ouwehand AC. Characterization of vaginal fungal communities in healthy women and women with bacterial vaginosis (BV); a pilot study. Microb Pathog. 2021; 161(Pt A): 105055. doi: 10.1016/j.micpath.2021.105055

Leon LJ, Doyle R, Diez-Benavente E, Clark TG, Klein N, Stanier P et al. Enrichment of Clinically Relevant Organisms in Spontaneous Preterm-Delivered Placentas and Reagent Contamination across All Clinical Groups in a Large Pregnancy Cohort in the United Kingdom. Appl Environ Microbiol. 2018; 84(14): e00483-18.doi:10.1128/AEM.00483-18

Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, et al. An integrated catalog of reference genes in the human gut microbiome. Nat Biotechnol. 2014; 32(8): 834-41. doi: 10.1038/nbt.2942.

Liang S, Ren H, Guo H, Xing W, Liu C et al. Periodontal infection with Porphyromonas gingivalis induces preterm birth and lower birth weight in rats. Mol Oral Microbiol. 2018; 33(4): 312-321. doi: 10.1111/omi.1222

Lin W, Jiang W, Hu X, Gao L, Ai D, Pan H et al. Ecological Shifts of Supragingival Microbiota in Association with Pregnancy. Front Cell Infect Microbiol. 2018; 5;8:24. doi: 10.3389/fcimb.2018.00024

Liu M, Zhen X, Song H, et al.. Low-dose lymphocyte immunotherapy rebalances the peripheral blood Th1/Th2/Treg paradigm in patients with unexplained recurrent miscarriage. Reprod Biol Endocrinol. 2017;15:95

Liu Y, Ko EY, Wong KK, Chen X, Cheung WC et al. Endometrial microbiota in infertile women with and without chronic endometritis as diagnodes using a quantitative and reference range-baed method. Fertil Steril. 2019; 112: 707-17

Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. Genome Med. 2016; 27;8(1): 51. doi: 10.1186/s13073-016-0307-y.

Lv LJ, Li SH, Li SC, Zhong ZC, Duan HL, Tian C et al. Early-Onset Preeclampsia Is Associated With Gut Microbial Alterations in Antepartum and Postpartum Women. Front Cell Infect Microbiol. 2019; 9: 224. doi: 10.3389/fcimb.2019.00224

Ma S, You Y, Huang L, Long S, Zhang J, Guo C et al. Alterations in Gut Microbiota of Gestational Diabetes Patients During the First Trimester of Pregnancy. Front Cell Infect Microbiol. 2020; 10: 58. doi:10.3389/fcimb.2020.00058

MacDonald KW, Chanyi RM, Macklaim JM, Cadieux PA, Reid G, Burton JP. Streptococcus salivarius inhibits immune activation by periodontal disease pathogens. BMC Oral Health. 2021; 21(1): 245. doi: 10.1186/s12903-021-01606-z.

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

Martinez KA, Romano-Keeler J, Zackular JP, Moore DJ, Brucker RM, Hooper C et al. Bacterial DNA is present in the fetal intestine and overlaps with that in the placenta in mice. PLoS One. 2018;13(5):e0197439. doi: 10.1371/journal.pone.0197439.

Mattiello F, Verbist B, Faust K, Raes J, Shannon WD, Bijnens L, Thas O. A web application for sample size and power calculation in case-control microbiome studies. Bioinformatics. 2016; 32(13): 2038-40. doi: 10.1093/bioinformatics/btw099

Mei C, Yang W, Wei X , Wu K, Huang D. The Unique Microbiome and Innate Immunity During Pregnancy. Front Immunol. 2019; 10: 2886. doi: 10.3389/fimmu.2019.02886.

Mesa MD, Loureiro B, Iglesia I, Fernandez Gonzalez S, Llurba Olivé E, García Algar O et al. The Evolving Microbiome from Pregnancy to Early Infancy: A Comprehensive Review. Nutrients. 2020; 12(1):133. doi: 10.3390/nu12010133

Minich JJ, Sanders JG, Amir A, Humphrey G, Gilbert JA, Knight R. Quantifying and Understanding Well-to-Well Contamination in Microbiome Research. mSystems. 2019; 4(4): e00186-19. doi: 10.1128/mSystems.00186-19.

Moffatt MF, Cookson WO. The lung microbiome in health and disease. Clin Med (Lond). 2017; 17(6): 525-529. doi: 10.7861/clinmedicine.17-6-525.

Moore DE, Soules MR, Klein NA, Fujimoto VY, Agnew KJ, Eschenbach DA. Bacteria in the transfer catheter tip influence the live birth rate after in vitro fertilization. Fertil Steril. 2000; 74: 1118-24

Mor G, Kwon JY. Trophoblast-microbiome interaction: a new paradigm on immune regulation. Am J Obstet Gynecol 2015; 6: 133–140. 10.1016/j.ajog.2015.06.03

Moreno I, Codofier FM, Vilella F, Valbuena D, et al. Evidence that the endometrial microbiome has an effect on implantation success or failure. Am J Obstet Gynecol. 2016; 215: 684-701

Mysorekar IU, Cao B. Microbiome in parturition and preterm birth. Semin Reprod Med. 2014; 32: 50-5

Nadeau-Vallée M, Obari D, Palacios J, Brien MÈ, Duval C, Chemtob S et al. Sterile inflammation and pregnancy complications: a review. Reproduction. 2016; 152(6):R277-R292. doi: 10.1530/REP-16-0453.

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

Nyangahu DD, Jaspan HB. Influence of maternal microbiota during pregnancy on infant immunity. Clin Exp Immunol. 2019; 198(1): 47–56. doi: 10.1111/cei.13331

Odogwu NM, Onebunne CA, Chen J, Ayeni FA, Walther-Antonio MRS, Olayemi OO et al. Lactobacillus crispatus thrives in pregnancy hormonal milieu in a Nigerian patient cohort. Sci Rep. 2021; 11(1):18152. doi: 10.1038/s41598-021-96339-y.

Park OJ, Yi H, Jeon JH, Kang SS, Koo KT, Kum KY et al. Pyrosequencing Analysis of Subgingival Microbiota in Distinct Periodontal Conditions. J Dent Res. 2015; 94(7):921-7. doi: 10.1177/0022034515583531

Pelzer ES, Allan JA, Waterhouse MA, Ross T, Beagley KW, Knox CI. Microorganisms within human follicolar fluid: Effects on IFV. PLOS ONE. 2013; 8: e59062

Pelzer ES, Gomez-Arango LF, Barrett HL, Nitert MD. (2017). Maternal health and the placental microbiome. Placenta. 2017; 54: 30–37. 10.1016/j.placenta.2016.12.003

Perez-Muñoz ME, Arrieta MC, Ramer-Tait AE, Walter J. A critical assessment of the hypotheses: implications for research on the pioneer infant microbiome. Microbiome. 2017; 5(1): 48. doi: 10.1186/s40168-017-0268-4

Peterson J, NIH HMP Working Group, Garges S, Giovanni M, McInnes P, Wang L, et al. The NIH Human Microbiome Project. M. Genome Res. 2009; 19(12): 2317-23. doi: 10.1101/gr.096651.109.

Pietrzak B. Biocenosis the vagina in a variety of periods of a woman's life. In: Wielgos M, Gdansk VM, Editors. Vaginal Infections. 2013; p. 1-5

Prince AL, Chu DM, Seferovic MD, Antony KM, Ma J, Kjersti M. Aagaard KM.The Perinatal Microbiome and Pregnancy: Moving Beyond the Vaginal Microbiome. Cold Spring Harb Perspect Med. 2015; 5(6): a023051. doi:10.1101/cshperspect.a023051

Prince AL, Ma J, Kannan PS, Alvarez M, Gisslen T, Harris RA et al. The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. Am J Obstet Gynecol. 2016; 214(5):627.e1-627.e16. doi: 10.1016/j.ajog.2016.01.193

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013; 41: D590-596. doi:10.1093/nar/gks1219.

Ramos BA, Kanninen TT, Sisti G, Wjtkin SS. Microrganisms in the female genital tract during pregnancy: tolerance versus pathogenesis. Am. J.Reprod. Immunol. 2015; 73: 383-9 doi: 10.1111/aji.12326

Rautava S, Tuominen H, Collado MC, Rautava J, Syrjänen S et al. Composition and maternal origin of the neonatal oral cavity microbiota. J Oral Microbiol. 2019; 11(1): 1663084. doi:10.1080/20002297.2019.1663084

Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011; 108 Suppl 1(Suppl 1):4680-7. doi: 10.1073/pnas.1002611107.

Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. Microbiome. 2014; 2(1):4. doi: 10.1186/2049-2618-2-4.

Ryu JI, Oh K, Yang H, Choi BK, Ha JE, Jin BH et al. Health behaviors, periodontal conditions, and periodontal pathogens in spontaneous preterm birth: a case-control study in Korea. J Periodontol; 2010; 81(6): 855-63. doi: 10.1902/jop.2010.090667

Salminen A, Kopra KA, Hyvärinen K, Paju S, Mäntylä P, Buhlin K, Nieminen MS, Sinisalo J, Pussinen PJ. Quantitative PCR analysis of salivary pathogen burden in periodontitis. Front Cell Infect Microbiol. 2015; 5:69. doi: 10.3389/fcimb.2015.00069

Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014; 12: 87.doi:10.1186/s12915-014-0087-z

Sedghi L, Di Massa V, Harrington A, Lynch SV, Kapila YL. The oral microbiome: Role of key organisms and complex networks in oral health and disease. Periodontol 2000. 2021; 87(1): 107-131. doi: 10.1111/prd.12393.

Sedlmayr P, Blaschitz A, Stocker R. (2014). The role of placental tryptophan catabolism. Front. Immunol. 2014; 5:230. doi:10.3389/fimmu.2014.00230

Seferovic MD, Pace RM, Carroll M, Belfort B, Major AM, Chu DM et al. Visualization of microbes by 16S in situ hybridization in term and preterm placentas without intraamniotic

infection. Am J Obstet Gynecol. 2019; 221(2):146.e1-146.e23. doi: 10.1016/j.ajog.2019.04.036

Selman H, Mariani M, Barnocchi N, Mencacci A, Bistoni F, Arena S et al. Examination of the bacterial contamination at the time of embryo transfer, and its impact on the IFV/pregnancy outcomes. J Assist Reprod Genet. 2007; 24: 395-9

Severovic MD, Pace RM, Carroll M, et al. Visualizations of microbes by 16S in situ hybridization in term and preterm placentas withouth intraamniotic infection. Am J Obstet Gynecol. 2019; 146 e1–146 e 23

Shanthi V, Vanka A, Bhambal A, Saxena V, Saxena S, Kumar SS. Association of pregnant women periodontal status to preterm and low-birth weight babies: A systematic and evidence-based review. Dent Res J. 2012 ;9(4): 368–380.

Shewale AH, Gattani DR, Bhatia N, Mahajan R, Saravanan SP. Prevalence of Periodontal Disease in the General Population of India-A Systematic Review. J Clin Diagn Res. 2016; 10(6): ZE04–ZE09 doi:10.7860/JCDR/2016/17958.7962

Smith A, Witte E, McGee D, Knott J, Narang K, Racicot K. Cortisol inhibits CSF2 and CSF3 via DNA methylation and inhibits invasion in first-trimester trophoblast cells. Am J Reprod Immunol. 2017; 78(5): doi:10.1111/aji.12741.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25(2):134-44. doi: 10.1111/j.1600-051x.1998.tb02419.x.

Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW et al. Bacterial Communities in Women with Bacterial Vaginosis: High Resolution Phylogenetic Analyses Reveal Relationships of Microbiota to Clinical Criteria. PLoS One. 2012; 7(6): e37818. doi:10.1371/journal.pone.0037818

StinsonLF, Boyce MC, Payne MS, Keelan JA. The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. Front Microbiol. 2019; 10: 1124.doi:10.3389/fmicb.2019.01124

Stinson LF, Payne MS, Keelan JA. Placental and intra-amniotic inflammation are associated with altered fetal immune responses at birth. Placenta. 2019; 85:15-23. doi: 10.1016/j.placenta.2019.08.079.

Stout MJ, Conlon B, Landeau M, Lee I, Bower C, Zhao Q et al. Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. Am J Obstet Gynecol. 2013; 208(3): 226.e1 226.e7. doi:10.1016/j.ajog.2013.01.018

Stout MJ, Zhou Y, Wylie KM, Tarr PI, Macones GA, Tuuli MG. Early pregnancy vaginal microbiome trends and preterm birth. Am J Obstet Gynecol. 2017; 217(3):356.e1-356.e18. doi: 10.1016/j.ajog.2017.05.030.

Suff N, Karda R, Diaz JA, Joanne NG, Baruteau J, Perocheau D et al. Ascending Vaginal Infection Using Bioluminescent Bacteria Evokes Intrauterine Inflammation, Preterm Birth,

and Neonatal Brain Injury in Pregnant Mice. Am J Pathol. 2018 188(10): 2164–2176. doi:10.1016/j.ajpath.2018.06.016

Sundquist A, Bigdeli S, Jalili R, Druzin ML, Waller S, Pullen KM et al. Bacterial flora-typing with targeted, chip-based Pyrosequencing. M BMC Microbiol. 2007; 7:108. doi: 10.1186/1471-2180-7-108.

Sweeney EL, Kallapur SG, Meawad S, Gisslen T, Stephenson SA, Jobe AH et al. Ureaplasma Species Multiple Banded Antigen (MBA) Variation Is Associated with the Severity of Inflammation In vivo and In vitro in Human Placentae. Front Cell Infect Microbiol. 2017; 7:123. doi: 10.3389/fcimb.2017.00123.

Sykes L, MacIntyre DA, Yap XJ, Teoh TG, Bennett PR et al. The Th1:th2 dichotomy of pregnancy and preterm labour. Mediators Inflamm. 2012; 2012:967629. doi: 10.1155/2012/967629.

Szabó I, Vizer M, Ertl T. Fetal betamethasone treatment and neonatal outcome in preeclampsia and intrauterine growth restriction. Am J Obstet Gynecol. 2003; 189(6): 1812-3. doi: 10.1016/s0002-9378(03)00923-2.

Szarka A, Rigó J Jr, Lázár L, Beko G, Molvarec A. Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. BMC Immunol. 2010; 11:59. doi: 10.1186/1471-2172-11-59.

Tan Q, Xu H, Aguilar ZP, Xu F, Yang Y, Dong S et al. Survival, distribution, and translocation of Enterococcus faecalis and implications for pregnant mice. FEMS Microbiol Lett. 2013; 349: 32–39

Taddei CR, Cortez RV, Mattar R, Torloni MR, Daher S. Microbiome in normal and pathological pregnancies: A literature overview. Am J Reprod Immunol. 2018; 80(2): e12993. doi: 10.1111/aji.12993.

Tao X, Franasiak JM, Zhan Y, Scott RT, Rajchel J et al. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips IVF cycle: Next generation sequencing (NGS) analysis of the ribosomial gene. Hum Microbiol J. 2017; 3: 15-21

Tersigni C, D'Ippolito S, Di Nicuolo F, Marana R, Valenza V, Masciullo V et al. Recurrent pregnancy loss is associated to leaky gut: a novel pathogenic model of endometrium inflammation? Transl Med. 2019; 17(1):83. doi: 10.1186/s12967-019-1823-5.

Tilakaratne A, Soory M, Ranasinghe AW, Corea SM, Ekanayake SL, de Silva M et al. Periodontal disease status during pregnancy and 3 months post-partum, in a rural population of Sri-Lankan women. J Clin Periodontol. 2000; 27(10): 787-92. doi: 10.1034/j.1600-051x.2000.027010787.x.

Tsiartas P, Holst RM, Wennerholm UB, Hagberg H, Hougaard DM, Skogstrand K et al. Prediction of spontaneous preterm delivery in women with threatened preterm labour: a prospective cohort study of multiple proteins in maternal serum. BJOG. 2012; 119(7):866-73. doi: 10.1111/j.1471-0528.2012.03328.x.

Verstraelen H, Vilchez-Vargas R, Desimped F, et al. Characterization of the human uterine microbiome in non pregnant women through deep sequencing of the V2-2 region of the 16S RNA gene. PEERL. 2016; 4: E1602.

Walther-António RS, Chen J, Multinu F, Hokenstad A, Distad TJ, Cheek EH, Keeney GL, Creedon DJ, Nelson H, Mariani A, Chia N. Potential contribution of the uterine microbiome in the development of endometrial cancer. Genome Med. 2016; 8: 122. doi:10.1186/s13073-016-0368-y

Wang J, Zheng J, Shi W, Du N, Xu X, Zhang Y et al. Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. Gut. 2018; 67(9):1614-1625. doi: 10.1136/gutjnl-2018-315988

Wu M, Chen SW, Jiang SY. Relationship between gingival inflammation and pregnancy. Mediators Inflamm. 2015; 2015:623427. doi: 10.1155/2015/623427

Ye CC, Wu YF. Oral microbiome dysbiosis triggers gestational periodontal disease and adverse pregnancy outcomes. Zhonghua Kou Qiang Yi Xue Za Zhi. 2022; 9;57(6): 635-641. doi: 10.3760/cma.j.cn112144-20220410-00166

Zanotta N, Monasta L, Skerk K, Luppi S, Martinelli M, Ricci G, Comar M. Cervico-vaginal secretion cytokine profile: A non-invasive approach to study the endometrial receptivity in IVF cycles. Am J Reprod Immunol. 2019; 81(1): e13064. doi: 10.1111/aji.1306

Zeeuwen PL, Kleerebezem M, Timmerman HM, Schalkwijk J. Microbiome and skin diseases. Curr Opin Allergy Clin Immunol. 2013; 13(5): 514-20. doi: 10.1097/ACI.0b013e328364.

Zhao M, Chen YH, Dong XT, Zhou J, Chen X, Wang H et al. Folic acid protects against lipopolysaccharide-induced preterm delivery and intrauterine growth restriction through its anti-inflammatory effect in mice. PLoS One. 2013; 8(12):e82713. doi: 10.1371/journal.pone.0082713

Zheng W, Xu Q, Huang W, Yan Q, Chen Y, Zhang L et al. Gestational Diabetes Mellitus Is Associated with Reduced Dynamics of Gut Microbiota during the First Half of Pregnancy. mSystems. 2020;5(2):e00109-20. doi: 10.1128/mSystems.00109-20

Zhu L, Luo F, Hu W, Han Y, Wang Y, Zheng H et al. Bacterial Communities in the Womb During Healthy Pregnancy. Front Microbiol.2018; 9: 2163.doi:10.3389/fmicb.2018.02163