How Does Microbial Community Composition and Metabolism Influence Inflammation-Induced Preterm Birth?

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A thesis submitted to the Faculty of Medicine, Dentistry and Health, University of Sheffield for the degree of Doctor of Philosophy (PhD)

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September 2022

Abstract

Preterm birth (PTB) is a global issue which effects more than 14 million babies per year resulting in increased infant mortality and lifelong diseases. The vaginal microbiome (VMB) has been associated with PTB and preterm prelabour rupture of fetal membranes (PPROM) however, the mechanisms remain unclear. Key bacteria such as *Streptococcus agalactiae* (GBS) and *Gardnerella vaginalis* have been extensively studied however there are many other bacteria commonly present in the vaginal microbial community which remain relatively unstudied. Currently prediction of which women will deliver prematurely (<37 weeks gestation) is limited.

This study aimed to identify reliable predictive biomarkers of preterm birth from the vaginal microbiome (chapter 2), metabolome (chapter 3) and their interactions. Additionally, we aimed to assess how the VMB may play a role in PPROM through fetal membrane degradation via bacterial products (chapter 4).

We were able to successfully optimise dynamic shear analysis, a novel technique for the study of fetal membrane integrity. We observed that infection-associated molecules reduced the visco-elastic properties of fetal membrane samples which may increase risk of PPROM *in vivo*. Additionally, we were able to characterise the cervico-vaginal microbiome and metabolome of asymptomatic women with and without sPTB.

We identified bacteria and metabolites which are promising biomarkers for the prediction of PTB. *Atopobium vaginae, Gardnerella vaginalis* were found to be predictive of PTB at gestational timepoint (GTP) 1. At GTP2 pantothenate and phytoene were predictive of PTB. The change in abundance from GTP1 to GTP2 was also found to be predictive of PTB for pantothenate, phytoene, adenosine, dehydrosafynol, giganin, nonacosane and urate.

Presentations & Publications

Society of Reproductive Investigations Annual Meetings - Boston 2021 - *Mid-Trimester Changes in Cervicovaginal Metabolites Are Distinct in Women with and without Preterm Birth*

Preterm Birth Dialogues Virtual Conference March 2021 - *Mechanical assessment of fetal membrane integrity after infection and identification of potential biomarkers of preterm prelabour rupture of membranes*

Frontiers Abstract 2021 - Mechanical assessment of fetal membrane integrity after infection and identification of potential biomarkers of preterm prelabour rupture of membranes (DOI:10.3389/978-2-88966-550-1)

Frontiers Abstract 2021 - *Mid-trimester changes in cervicovaginal metabolites are distinct in women with and without preterm birth* (DOI:10.3389/978-2-88966-550-1)

In preparation for publication 2023 - *Assessment of vaginal microbiome-metabolite and host immune interactions associated with spontaneous preterm birth in a predominantly Caucasian population*

Acknowledgements

Firstly, I'd like to thank Professor Dilly Anumba and Emmanuel Amabebe, without whom this PhD would not be possible. I also want to thank the rest of the PRIME team for their support and inspiring work.

I want to express my gratitude to the University staff in the medical school and department of Reproductive Medicine, especially Mark Fenwick, Elspeth Whitby and Sarah Waite. I extend my gratitude to Heather Walker and the SiTran Nanopore team for their assistance and collaboration.

I'd like to thank Chris Holland and Natural materials team for their assistance with the novel fetal membrane dynamic shear analysis experiments.

Mostly I'd like to thank the mothers who took part in this study. Also, I'd like to thank all the staff at Jessop Wing including the midwives on labour ward who assisted with placenta collection and the research midwives who greatly assisted in sample collection.

I want to thank my second supervisor Paul Heath for his support and routinely checking in with me during lockdown.

I also want to thank room 11 ladies for your friendship over the last 4 years. I am lucky to have made good friends who made my days brighter. I'd like to give a special mention to Neha who helped me with many bioinformatic troubles and Brenda who encouraged me to pursue research collaborations and was a brilliant conference buddy.

I want to thank my new friends and colleagues at QIAGEN who have supported me over the last 9 months with the difficult task of working full time whilst also writing up my thesis.

I also want to thank Shona and Dafydd for providing much needed respite from PhD work. Thank you for the best times and thank you for being the best lockdown bubble buddies.

I'd like to thank my family for their unwavering support and always being available on the phone no matter the time or where in the world they were.

Finally, I would like to express my massive appreciation for my PhD and life partner Reece. Thank you for being there every step of the way during this PhD journey and supporting me no matter what. I wanted to say that I couldn't have done this without you, the truth is I could, but I'm so glad we did it together. You've made the experience significantly more enjoyable (p<0.00000001).

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Abbreviations

Name	Abbreviation
Bacterial Vaginosis	BV
Bacterial Vaginosis Associated Bacterium 1	BVAB1
Cervical Length	CL
Community State Type	CST
Cervico-vaginal Fluid	CVF
Extracellular Matrix	ECM
Epithelial To Mesenchymal Transition	EMT
Fetal Fibronectin	FFN
Group B Streptococcus or *Streptococcus agalactiae*	GBS
Human Immunodeficiency Virus	HIV
Intrauterine Growth Restriction	IUGR
In Vitro Fertilisation	IVF
Low- and Middle-Income Countries	LMIC
Matrix Metalloproteases	MMP
Placental Alpha Microglobulin-1	PAMG-1
Polymerase Chain Reaction	PCR
Phosphorylated Insulin-Like Growth Factor Binding Protein-1	phIGFBP-1
Preterm Prelabour Rupture of Fetal Membranes	PPROM
Prelabour Rupture of The Membranes	PROM
Preterm Birth	PTB
Spontaneous Preterm Labour	sPTL
Regulated On Activation, Normal T Cell Expressed and Secreted	RANTES
Small For Gestational Age	SGA
Single Nucleotide Polymorphisms	SNP
Sexually Transmitted Infection	STI
Tissue Inhibitor Matrix Metalloproteases	TIMPS
The University of Sheffield	TUOS
Toll-Like Receptors	TLR
Vaginal Microbiome	VMB

1 Chapter 1 - Introduction

Globally, 10% of all live births are preterm (<37 weeks gestation) (Chawanpaiboon et al., 2019). That equates to more than 14 million babies and their families effected every year. Being born prematurely initially increases the risk of infant mortality, neonatal infection and the fetal inflammatory response syndrome (Romero & Chaiworapongsa, 2002). Beyond immediate complications, survivors of preterm birth (PTB) may have lifelong problems such as: visual impairment (O'Connor et al., 2007), neurological and respiratory disorders (Soleimani et al., 2014; Greenough, 2012) throughout later life. In addition to infants, it has been shown that mothers of premature babies have significantly more health issues such as anxiety and flashbacks compared to term (Henderson et al., 2016).

The economic cost of PTB is substantial: it was estimated that in England and Wales the medical cost of a child born preterm over their lifetime was £22,764 more compared to term. The increased cost substantially increased for the very preterm (£61,509) and extremely preterm (£94,190) groups (Mangham et al., 2009). A more recent study from the US estimated the cost to be $64,815 per preterm birth over the babies lifetime (Waitzman et al., 2021). Studies from Finland (Tommiska et al., 2003), UK (Khan et al., 2015), Sweden (Ringborg et al., 2006) and USA (Walsh et al., 2019) also show the economic cost of PTB compared to term infants is significantly increased. However, PTB rates are unevenly distributed, with Asia and Sub-Saharan Africa accounting for approximately 81.1% of preterm births globally (Chawanpaiboon et al., 2019) (Figure 1.1 & Table 1.1). These settings have much lower incomes, hence, shoulder most of the global health burden.

In the light of the considerable health and economic problems associated with PTB, it is crucial that we improve our understanding and work towards predictive and preventive interventions to save lives and improve outcomes *around the world*. As most of the research is conducted in higher income countries, it is important to consider how the findings can be applied to other less resourced settings. Ideally, we should not assume that predictive and preventive methods developed in places such as the UK will show the same results or be applicable in low- and middle-income countries (LMIC). Research should consider all settings and, where possible, attempt to have data representative of high-, middle- and low-income settings. Investigating differences and similarities in PTB between and within settings will help us better understand the mechanisms and improve the global health burden.

Figure 1.1. Heat map of estimated preterm birth rates in 2014 (preterm births per 100 live births). Reproduced with permission (Chawanpaiboon et al., 2019).

Table 1.1 Estimated preterm birth (PTB) rates in 2014.

	Estimated PTB rate (%) *	Proportion of Global livebirths (%)	Proportion of Global PTB (%)
Asia	10.4%	53.9%	52.9%
Europe	8.7%	5.7%	4.7%
Latin America & the Caribbean	9.8%	7.7%	7.2%
North America	11.2%	3.1%	3.3%
North Africa	13.4%	4.1%	5.2%
Oceania	10.0%	0.5%	0.4%
Sub-Saharan Africa	12.0%	25%	28.2%
Global	10.6%	100%	100%

*Preterm births per 100 livebirths. Reproduced with permission (Chawanpaiboon et al., 2019).

1.1 Preterm birth Aetiology

PTB can be categorised as either spontaneous or indicated (maternal or fetal) which includes syndromes such as preeclampsia and intrauterine growth restriction (Figure 1.2). Approximately 30% of PTB are considered indicated (Goldenberg et al., 2008). Spontaneous PTB is usually preceded by spontaneous preterm labour (sPTL) which accounts for approximately 45% of PTB or preterm prelabour rupture of fetal membranes (PPROM) which accounts for approximately 25% of PTB (Goldenberg et al., 2008). PPROM is defined as rupture of membranes <37 weeks gestation before the onset of labour (Walker & Morley, 2018). This should not be confused with prelabour rupture of the membranes (PROM) defined as rupture of membranes before the onset of labour >37 weeks gestation. To understand the mechanisms of PTB we need to understand how term labour is initiated. Although there are several theories, the details of how the mechanism of PTB differs from term labour are still unclear.

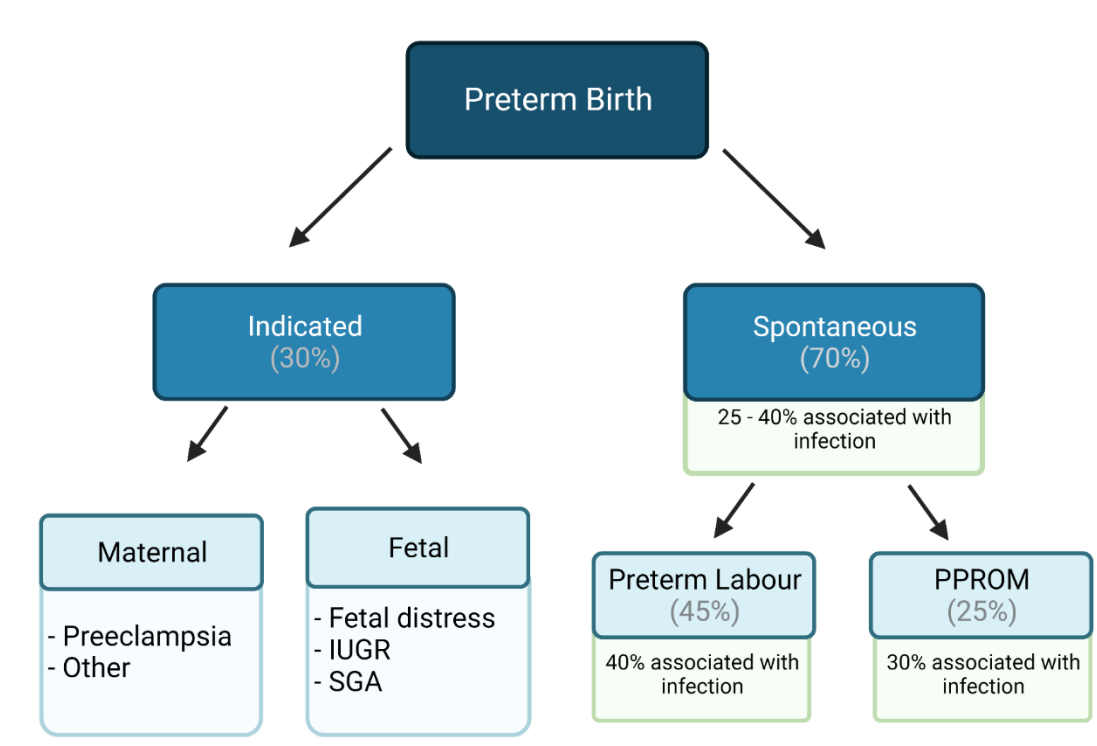


Figure 1.2. Preterm birth categories and percentage of spontaneous cases that are associated with infection. IUGR = Intrauterine growth restriction, SGA= Small for gestational age, PPROM= preterm prelabour rupture of fetal membranes. % of spontaneous PTB associated with infection from (Romero, Dey, et al., 2014; Klein & Gibbs, 2005; Goldenberg & Culhane, 2003; Mercer, 2003), % of spontaneous or indicated PTB from (Goldenberg et al., 2008). Created with BioRender.com

1.1.1 Term Labour

The initiation of term labour is not a single pathway but a web of **1)** endocrine, **2)** inflammatory and **3)** structural changes which ultimately lead to cervical ripening and dilation, myometrial contractions and rupture of the membranes, which are all needed for labour (Figure 1.3, Figure 1.4).

Firstly, endocrine regulation; oestrogen and progesterone are both produced by the placenta and increase throughout gestation. For most of the pregnancy, the ratio favours progesterone, contributing to the uterus' quiescent state. Toward the end of pregnancy, the ratio begins to favour oestrogen which in contrast, upregulates factors needed for labour (Table 1.2 & Figure 1.3) (Vannuccini et al., 2016).

Progesterone promotes myometrial quiescence by downregulating oxytocin receptor, calcium ion channel and prostaglandin production, increasing production of Tissue inhibitor of metalloproteases (TIMPS) which inhibit collagen degradation (Keelan, 2018; Vannuccini et al., 2016; Byrns, 2014). In early pregnancy progesterone is produced by the corpus luteum, around 32 weeks gestation. Progesterone levels rise gradually due to placental utilisation of fetal precursors (Magon & Kumar, 2012). By term gestations, progesterone levels range from 100-200 ng/ml and the placenta produces about 250 mg/day (Magon & Kumar, 2012).

Oestrogen contributes to the initiation of labour by stimulating cervical ripening, upregulating prostaglandin and oxytocin receptors and increasing cytokine production (Keelan, 2018; Vannuccini et al., 2016; Andersson et al., 2008). Oxytocin directly induces myometrial contraction but also upregulates prostaglandin production resulting in a positive feedback loop (Vannuccini et al., 2016). Oestradiol and oestrone are synthesized by placental aromatization of DHEAS with the majority of estriol derived from fetal sources (Magon & Kumar, 2012). Circulating levels of all oestrogens increase throughout pregnancy, peaking at term and concentrations correlate with the increasing size of the fetus (Costa, 2016; Loriaux et al., 1972).

Finally**, prostaglandins** which are found at increased levels before and during labour, play a role in cervical ripening and stimulate myometrial contraction (Vannuccini et al., 2016; Challis et al., 1997).

Table 1.2. The influence of progesterone and oestrogens on labour inducing factors. TIMP = Tissue inhibitor of metalloproteases.

Progesterone	Oestrogen
- Prostaglandin	+ Prostaglandin
- Oxytocin receptors	+ Oxytocin receptors
+ TIMPS (inhibits collagen degradation)	Contributes to cervical ripening
- Calcium ion channels (needed for contractility)	

Secondly, inflammatory signals; during term labour there is recruitment of immune cells and cytokines to the myometrium and cervix (Osman et al., 2003). The number of circulating peripheral blood neutrophils increases during healthy pregnancy (Gimeno-Molina et al., 2022) (Figure 1.3). Neutrophils secrete matrix metalloproteases (MMPs) which degrade collagen in the membranes and cervix (Gomez-Lopez et al., 2014). Cytokines indirectly contribute to cervical ripening (Sennstrom, 2000) and myometrial contractions via prostaglandins (Menon & Fortunato, 2004). A recent systematic review established seven pro-inflammatory markers that are present during healthy term labour: COX-2, PGE_2 hCAP18 and the cytokines IL-6, IL-8, IL-1β and TNF-α (Hadley et al., 2018). As these factors increase throughout pregnancy the maternal inflammatory burden increases, upregulating more pro-labour pathways leading to structural changes and eventually sPTL or PPROM (Figure 1.4). The concentration of inflammatory markers such as cytokines vary depending on location. For example, vaginal concentrations of IL-6 and IL-8 were observed to increase roughly 10-fold and 5-fold respectively in third trimester (Donders et al., 2003). Whereas, placental concentration of IL-6 was shown to increase roughly 50-fold (Hadley et al., 2018). Overall, maternal inflammatory burden is thought to increase in the weeks leading up to delivery (Figure 1.4). However, compared to the inflammatory increase during sPTL and PPROM the increase seen at term is relatively small. Placental concentrations of IL-2/IL- 4 was 600 fold higher in preterm patients and IFN-γ/IL-4 was increased 1000 fold (El-Shazly et al., 2004).

Thirdly, structural changes; weeks before labour is initiated structural changes begin in the cervix and membranes (Menon & Fortunato, 2004). A weak zone can be observed in the area of the membranes overlying the cervix (Figure **1.5**). This area is significantly thinner than the rest of the membranes (McLaren et al., 1999) and at later gestations is further distended leading to increased risk of rupture (Millar et al., 2000). Weakening in the membranes is thought to be caused by the degradation of collagen by enzymes such as MMPs (Dang et al., 2013; Li et al., 2004). At term, membranes usually rupture during labour when myometrial contractions increase the pressure on the already weakened and strained membranes.

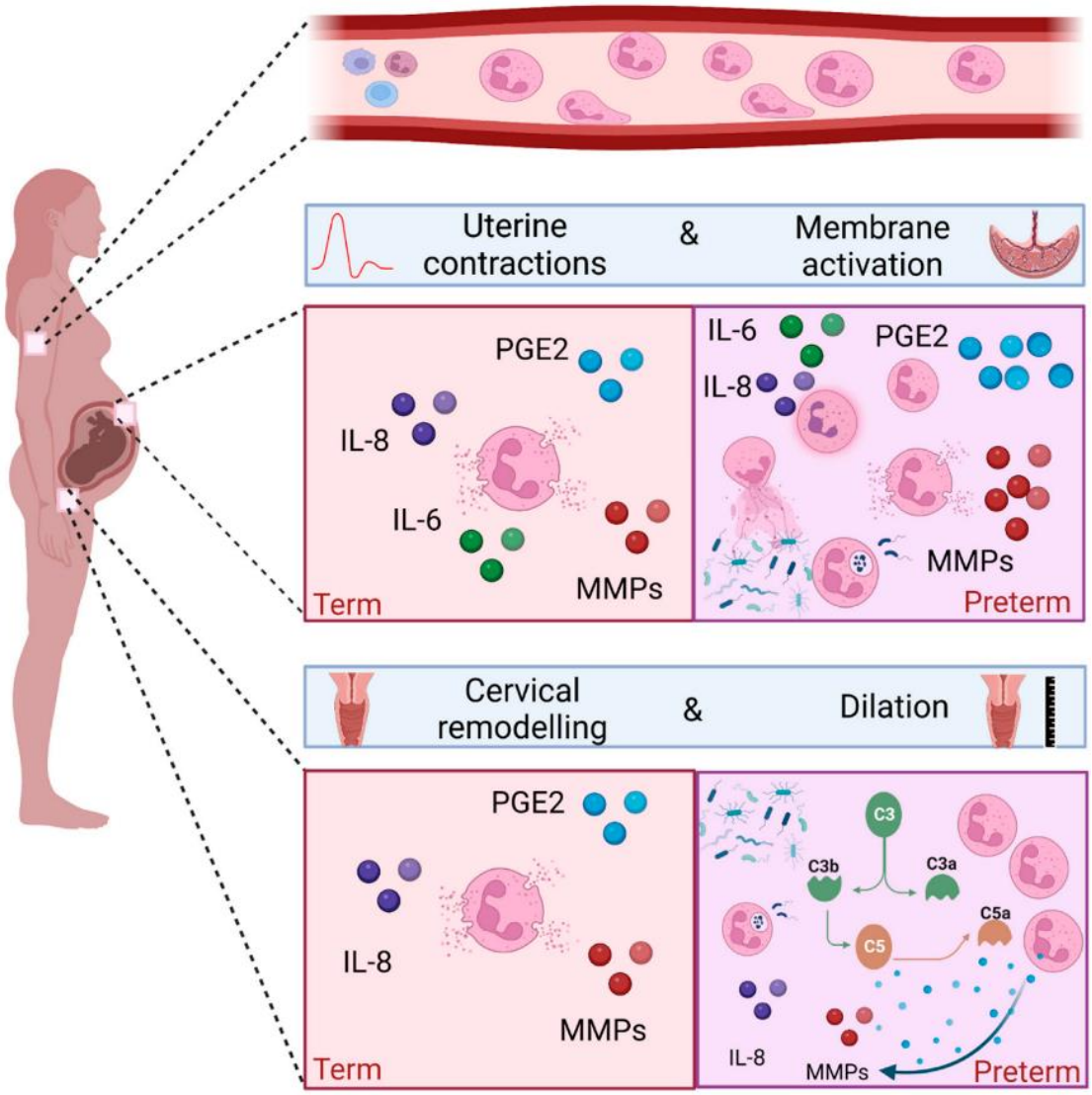


Figure 1.3 The role of neutrophils in term birth and microbe-driven preterm birth. The number of circulating peripheral blood neutrophils increase during healthy pregnancy. Neutrophils can migrate into tissue following a chemotactic gradient. Term labour (left) is associated with infiltration of neutrophils in the lower uterine segment, fetal membranes and cervix. Their detection is accompanied by an increase in cytokines such as IL-8 and MMPs participating in the degradation of the extracellular matrix, therefore contributing to tissue remodelling. Neutrophils are also a source of COX-2 and PGE2, therefore can contribute to uterine contractility membrane activation and cervical dilation. In cases of infection (right), neutrophils are detected in fetal membranes and amniotic fluid, along with an increase in pro-inflammatory mediators. Ex vivo experiments using neutrophils demonstrate their capacity to perform phagocytosis, NETosis and release of pro-inflammatory mediators. Finally, an adverse vaginal microbial composition is associated with an increase in cervical neutrophils, inflammatory mediators and complement activation. (Gimeno-Molina et al., 2022) reproduced under creative commons licence. Image created with Biorender.com.

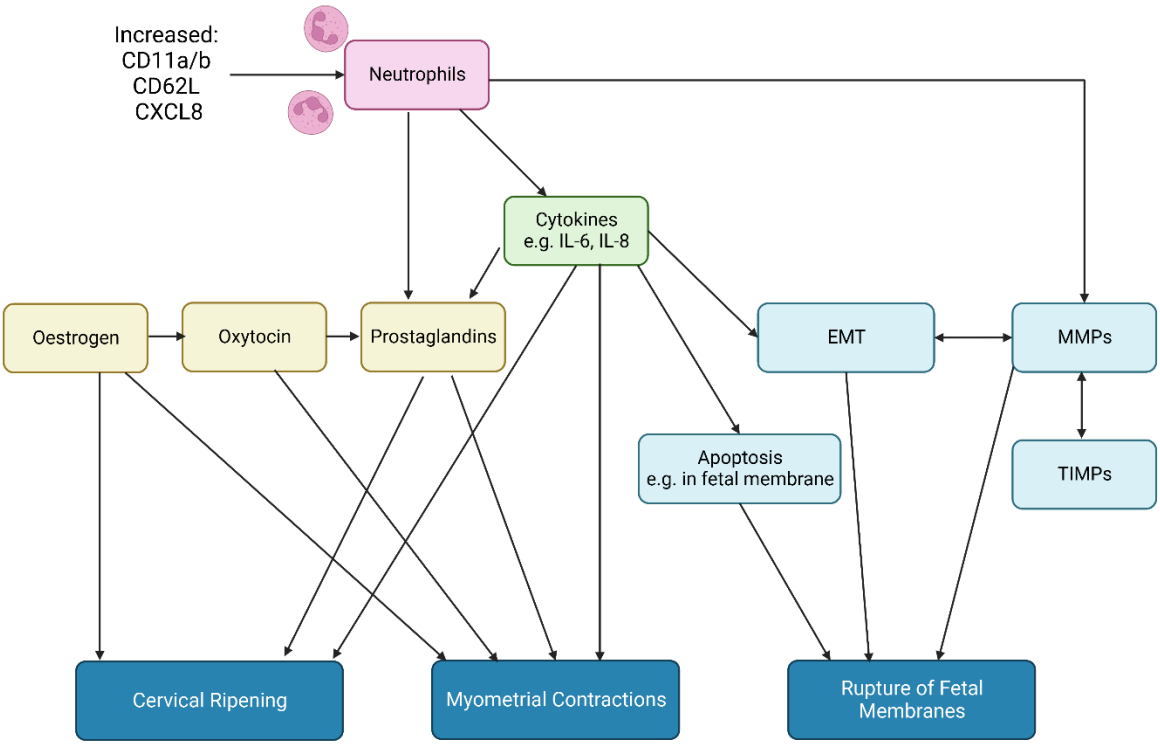


Figure 1.4. Initiation of term labour. Cervical ripening, myometrial contractions and rupture of fetal membranes are required for term labour (dark blue boxes). Initiation of labour is associated with increased migration markers (CD11a/b & CD62L), increased expression of *CXCL8* and neutrophilia. Neutrophils are able to produce pro-labour mediators such as prostaglandins, MMPs, cytokines e.g. IL-8 & IL-6. Oestrogen contributes labour initiation via cervical ripening, upregulating prostaglandin and oxytocin receptors and increasing cytokine production. Increased cytokines and inflammatory signalling directly contribute to cervical ripening and myometrial contractions but can also increase apoptosis and EMT in fetal membrane which contributes to the weakened zone seen at term. Increased MMPs contributes to tissue remodelling. Arrows indicate upregulation/ recruitment/ induction/ stimulation. EMT = Epithelial to Mesenchymal Transition, MMP = matrix metalloproteinases, TIMP = tissue inhibitor of metalloproteases. Created with BioRender.com

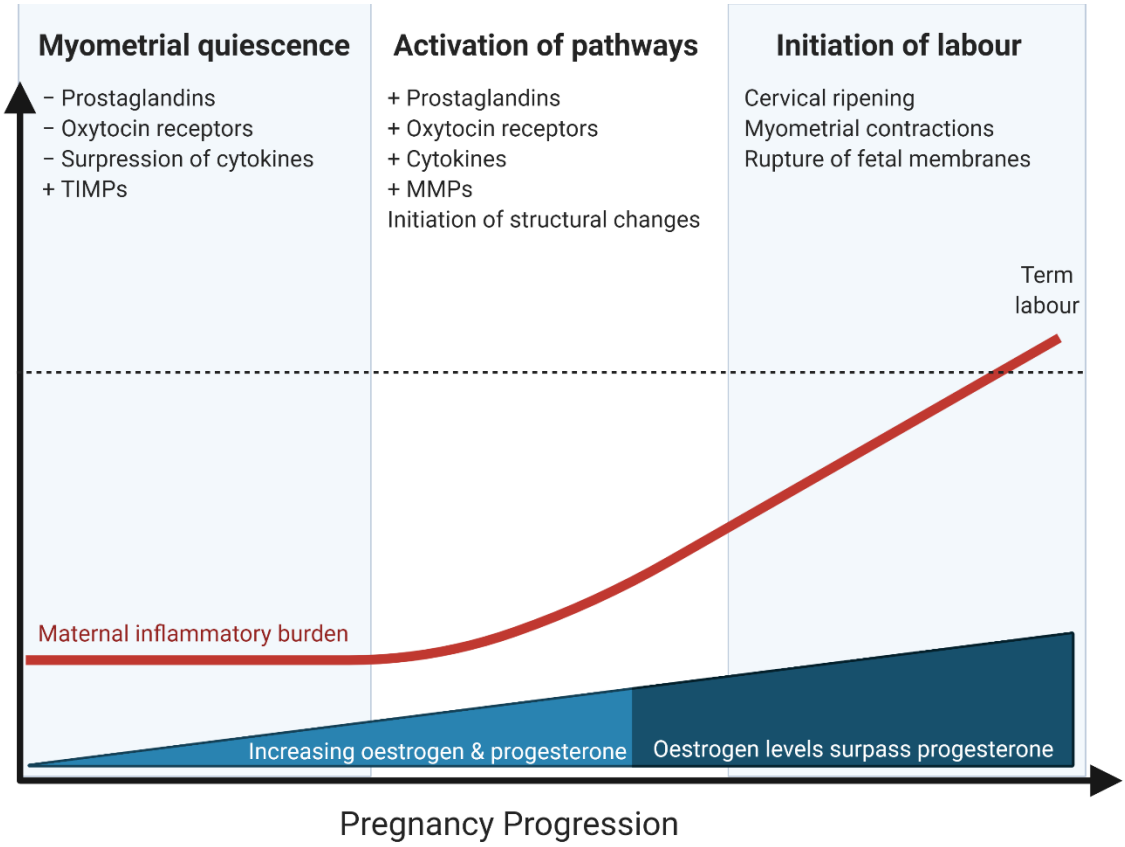


Figure 1.5. Initiation of term labour. Graph illustrating the change in labour inducing factors throughout pregnancy. MMP = matrix metalloproteinases, TIMP = tissue inhibitor of metalloproteases. Dotted line represents the threshold of inflammatory signals needed for labour. Created with BioRender.com

1.1.2 Spontaneous preterm labour

The cause of spontaneous preterm labour is largely unknown. In parallel to term labour upregulation of inflammatory pathways lead to cervical ripening, myometrial contractions and rupture of membranes before 37 weeks' gestation, resulting in a preterm delivery (Voltolini et al., 2013; Menon & Fortunato, 2004).

1.1.3 Preterm premature rupture of the fetal membranes (PPROM)

PPROM is a subcategory of spontaneous PTB and affects an estimated one third of PTBs (Mercer, 2003). In contrast to term labour, where myometrial contractions increase intrauterine pressure aiding rupture of the fetal membranes, patients with PROM or PPROM appear to have structural changes in the amniotic membrane overlying the cervix at much earlier gestations (Figure 1.5). These changes compromise membrane integrity leading to rupture without the presence of labour and are often associated with infection (McParland & Bell, 2004).

Over half of mothers with PPROM will deliver within one week of membrane rupture (Mercer, 2003). This latency period during which microbes have more opportunity to ascend into the amniotic cavity and many women have oligohydramnios (low levels of amniotic fluid) often leads to complications such as infection, compression of the umbilical cord and respiratory distress syndrome from premature birth (Lovereen et al., 2018). The risk of complications such as retinopathy of prematurity and bronchopulmonary dysplasia are higher at earlier gestational ages (Mercer, 2003).

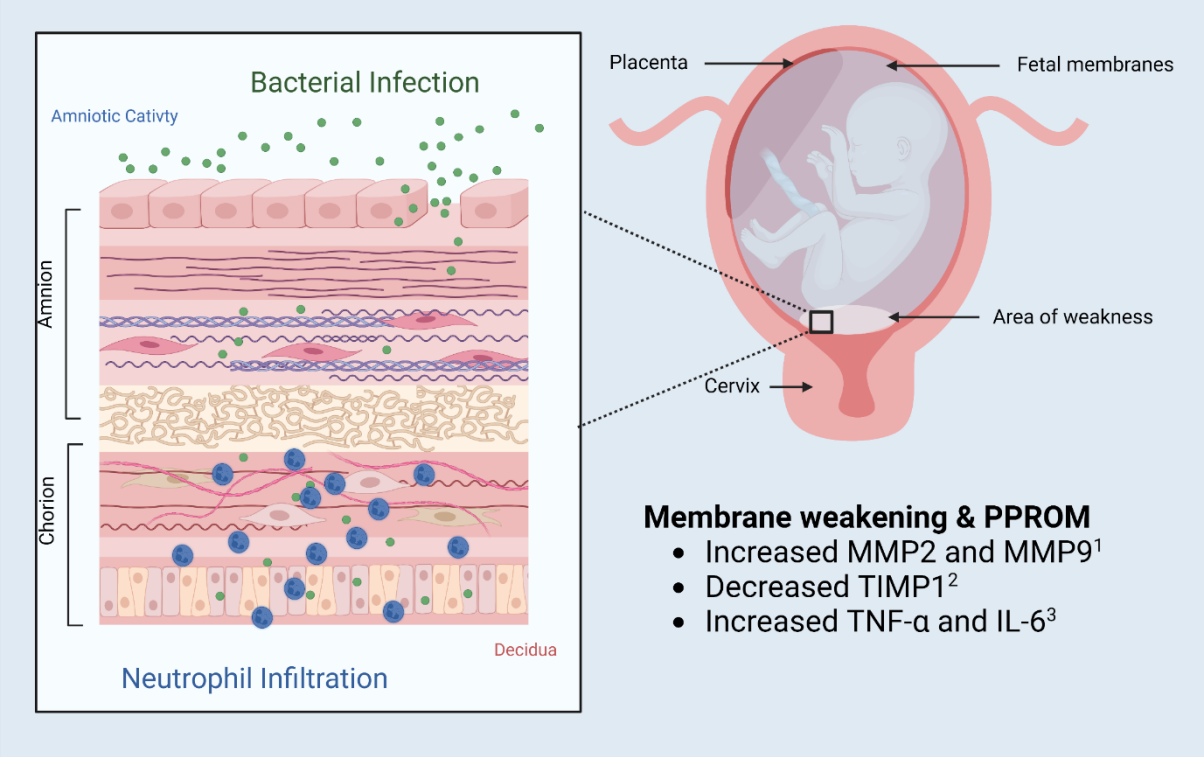


Figure 1.6 Weakened area of fetal membrane above cervix during infection/ microbial invasion of fetal membrane. Neutrophils respond to bacterial infection by initiating an immune response, releasing cytokines and MMPs. MMP= Matrix metalloprotease, TIMP= Tissue inhibitors of metalloproteinases, TNF-α= tumour Necrosis Factor alpha, IL-6= Interleukin 6. [1](Fortunato et al., 1997; Vadillo-Ortega et al., 1996), [2](Weiss et al., 2007), [3](Li et al., 2020). Created with BioRender.com

1.1.3.1 Physiology of the fetal membranes

The amnion is the inner surface of the fetal membranes and is made up of an epithelial cell layer attached to a basement membrane (Bourne & Lacy, 1960), underneath which, is a dense compact layer made up of a network of collagen embedded in an extracellular matrix (ECM) (Figure 1.6). This is the source of tensile strength in the amnion (Bryant-Greenwood, 1998). The compact layer is followed by a fibroblast layer similar to the compact layer but with embedded mesenchymal cells which help to produce the ECM (Malak et al., 1993). Between the amnion and chorion is a spongey layer rich in proteoglycan, preventing friction between the two structures (Bryant-Greenwood, 1998). The chorion is composed of a reticular layer attached to a basement membrane, under which are trophoblast cells. Basal trophoblast cells adhere to the basement membrane and are highly structured, connected with tight junctions. Chorion is tightly adherent to the decidua (Bryant-Greenwood, 1998).

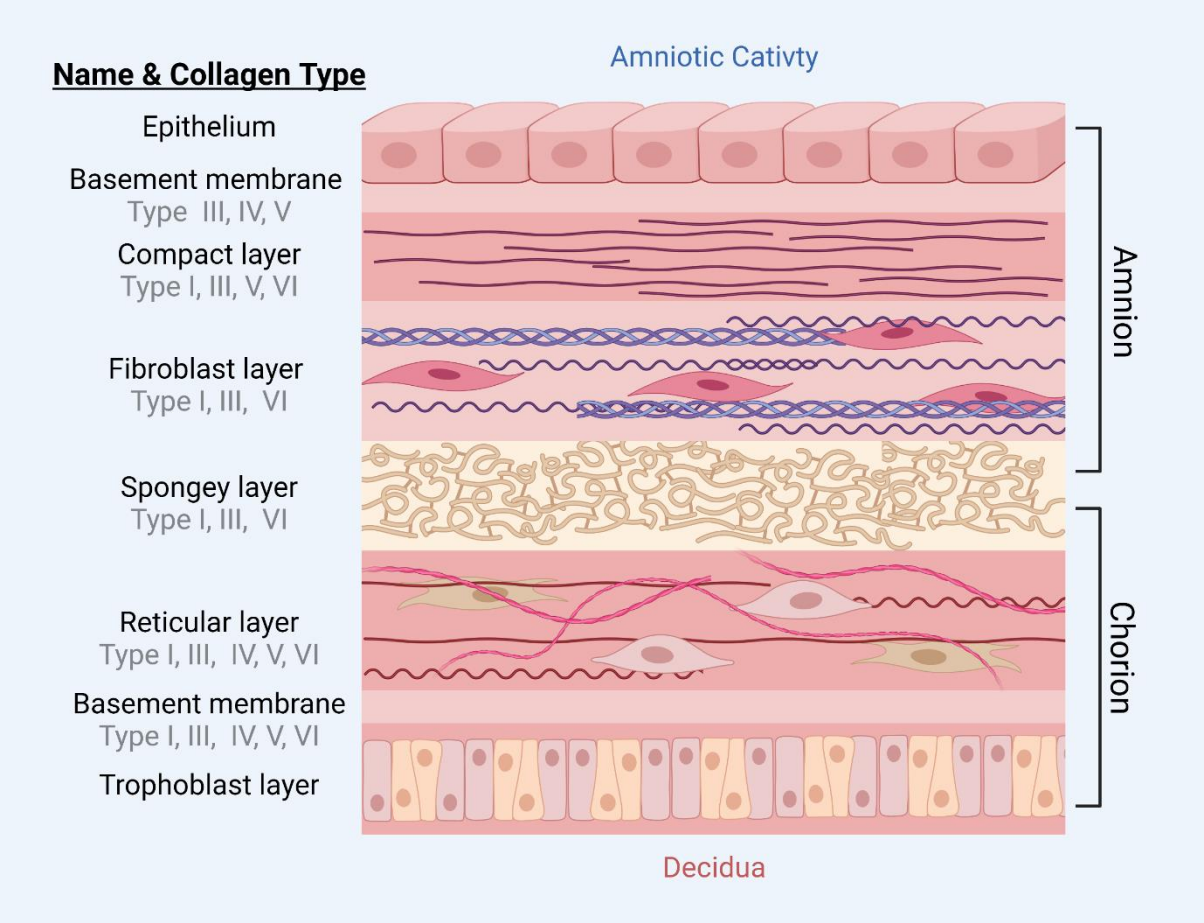


Figure 1.7 Cross section structure of human fetal membranes. Created with BioRender.com

1.1.3.2 Fetal membrane relevant enzymes

MMPs are important enzymes in tissue remodelling including the changes seen in the cervix and fetal membranes during pregnancy (Woessner, 1991). The MMPs are a family of proteolytic enzymes which are able to degrade a range of substrates including: ECM proteins and glycoproteins, membrane receptors, cytokines, and growth factors (Laronha & Caldeira, 2020). MMPs can be divided according to their structural and functional properties including: Collagenases (MMP1, -8, -13), Gelatinases (MMP-2 and MMP-9) and Stromelysins (MMP-3, -7, -10, -11). Gelatinases are able to degrade denatured collagen, specifically, gelatin, collagen (types IV, V, VIII, X, XI, XIV),elastin, proteoglycan core proteins, fibronectin, laminin, fibrilin-1, and TNF-α and IL-1b precursor (Laronha & Caldeira, 2020). While collagenases are able to cleave fibrillar collagen type I, II, III, IV and XI due to their ability to unwind triple helical collagen (Laronha & Caldeira, 2020).

Also involved with tissue remodelling are tissue inhibitors of metalloproteinases (TIMPs). TIMPs are inhibitors of the proteolytic activity of MMPs (Ries, 2014) the relative levels of MMPs and TIMPs determine if degradation or deposition of tissue is occurring. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a local regulator of MMPs known to be expressed in placental tissues including amniotic epithelial cells and chorionic trophoblast cells (Li et al., 2004). levels of glycosylated EMMPRIN were found to be increased in tissue from patients who had undergone labour (Li et al., 2004).

In term pregnancies MMP-1, -2, -3, -7 and –9 are known to be present in the amniotic fluid (Weiss et al., 2007). Decidual neutrophils and macrophages secrete MMP-9 which contributes to cervical remodelling and rupture of membranes at term (Gomez-Lopez et al., 2014). On the other hand, sPTL and PPROM have been associated with increased levels of MMP-2 and MMP-9 (aka gelatinase A and B), and decreased levels of TIMP-1 (Fortunato et al., 1997; Vadillo-Ortega et al., 1996). MMP-2 degrades collagen and MMP-9 can act like either a collagenase or a gelatinase (Laronha & Caldeira, 2020).

As well as being part of normal tissue processes, MMPs and TIMPs can be released by immune cells in response to the presence of bacteria. Gonzalez et al, (2011) found that macrophage depletion prevented LPS-induced preterm birth in mice. Additionally, macrophage-derived cytokines IL-1b and TNF-a increase the levels of MMP-1, MMP-3 and MMP-9 (Watari et al., 1999).

In summary, MMPs and TIMPs are present for several broad reasons. 1) normal tissue processes e.g. maintenance of tissue 2) inflammatory signalling during term and preterm labour 3) in response to bacteria and infection 4) some bacteria are able to produce MMPs which are useful for gaining access deeper into tissues (Schoonmaker et al., 1989).

1.1.3.3 Mechanism of PPROM

Historically, it was believed that the mechanical stretch of the membrane in late pregnancy was the cause of rupture. However, more current evidence shows that in parallel to term pregnancies, there is an area of membrane weakness overlying the cervix which has reduced strength when challenged (El Khwad et al., 2006; McLaren et al., 1999). In addition, it has been shown that acute repetitive stretch does not weaken but actually strengthens fetal membranes (Pandey et al., 2007). El Khwad et al., (2005) examined fetal membranes prior to labour in women undergoing caesarean sections. They observed that the rupture zone had 45% less strength than a distal site of the same membrane.

Weakness of the membrane is thought to be caused by morphological and biochemical changes in the region above the cervix (Figure 1.5). Major contributing factors are cell death (Rangaswamy et al., 2012; Fortunato et al., 2002), collagen remodelling (Dixon et al., 2018; Lannon et al., 2014), epithelial to mesenchymal transition (EMT) (Janzen et al., 2017; Assumpcao et al., 2016), and inflammation (Kumar et al., 2005; Menon & Fortunato, 2004). Some researchers question the localisation of membrane weakening. Harirah et al.,(2012) argue that apoptosis is not restricted to the membranes overlying the cervix but that this region does have higher incidence. Furthermore, Fortner et al., (2014) observed thinning of the chorion overlying the cervix but also found global thinning of the chorion in all PPROM patients, including those without infection. However, these studies are vastly outnumbered by studies that did not find or did not report weakening in other areas of the membranes and so requires further investigation.

Increased MMP-9 was observed in amniotic fluid from patients with membrane rupture at both term and preterm but the median MMP-9 concentration was 2-fold higher in patients with PPROM compared with PROM (Athayde et al., 1998).

1.2 Spontaneous preterm labour vs PPROM

Both sPTL and PPROM are thought to have the same aetiology e.g. infection and inflammation. However, it remains unclear if sPTL and PPROM have different mechanisms that induce either premature contractions or premature membrane rupture in response to infection and/or inflammation. Some researchers argue that they have different mechanisms (Menon & Fortunato, 2004; Fortunato & Menon, 2001) possibly due to higher levels of bacterial DNA (Jacobsson et al., 2009) and different biomarkers (Cobo et al., 2011). Others have found no difference between sPTL and PPROM when investigating MMPs (Tency et al., 2012) and fetal membrane extracellular vesicles (Monsivais et al., 2020). More research is necessary to be able to better understand the intricate mechanisms of sPTL and PPROM.

1.3 Infection-Associated Preterm Birth

Up to 40% of all PTBs can be linked to infection (Romero & Dey., 2014; Goldenberg et al., 2008). Within the spontaneous preterm labour category up to 50% are associated with infection (Klein & Gibbs, 2005). Chorioamnionitis or intraamniotic infection is a broad term defined as acute infection or inflammation of the amnion, chorion or placenta with intact or ruptured membranes (Tita & Andrews, 2010). Symptoms include fever and fundal tenderness. However, many cases are subclinical and are thought to contribute to sPTL and PPROM (Tita & Andrews, 2010; Goldenberg & Culhane, 2003). Positive microbial culture from amniotic fluid is found in around 25-40% of PPROM patients and is significantly higher at early gestations (21-24 weeks) (Kim et al., 2015; Štimac et al., 2014; Simhan & Canavan, 2005). This is likely an underestimate as PPROM patients frequently have oligohydramnios (insufficient amniotic fluid) so cannot have amniocentesis.

There are several ways organisms can gain access to uterine cavity, such as, via the placenta, introduction from amniocentesis, or via the fallopian tubes. However, it is widely accepted that infection likely ascends from the lower genital tract as organisms found in amniotic fluid are species which are commonly found in the vagina (Chung et al., 2009; DiGiulio et al., 2008). In a recent study, the same species were found in the vagina and amniotic fluid at the time of amniocentesis (Romero et al., 2019). Additionally, chorioamnionitis found in the membranes, diagnosed by histology, was found to be worse in the area overlying the cervix (Romero et al.,

1989). Supporting this, a twin study reported the amniotic sac of the twin overlying the cervix often had a higher prevalence of infection whilst the second amniotic sac did not (Mazor et al., 1996).

An infection speculated to be associated with PTB is maternal periodontitis (Baskaradoss et al., 2011; Sharma et al., 2007). This causes the gums to recede forming "pockets" that harbour infectious anaerobic bacteria (NIH, 2013; Horton & Boggess, 2012). Other infections associated with PTB, such as malaria, syphilis and human immunodeficiency virus (HIV) have a much higher prevalence in some countries which may contribute to higher PTB rates in those places (Chawanpaiboon et al., 2019).

This project is specifically focussed on vaginal infections that have been historically associated with PTB (Hill, 1998; Andrews et al., 1995; Joesoef et al., 1995; McGregor et al., 1988), but despite decades of research, we still lack sufficient information to accurately predict or prevent PTB associated with infection.

1.3.1 Bacterial Vaginosis (BV)

Prior to bacterial sequencing and identification, vaginal infections, specifically bacterial vaginosis (BV), were identified using Amsel criteria (Amsel et al., 1983). This required examination of discharge including a whiff test for an amine odour. This was replaced by a more reproducible Nugent score (Nugent et al., 1991), which is still used to diagnose BV today (NICE, 2018). This method uses Gram staining to identify *Lactobacillus* (large gram-positive rods), *Gardnerella* (small gram-negative rods), and *Mobiluncus* (curved gram-negative rods) morphotypes. Due to the availability of this technique, a significant proportion of infection associated PTB research is focussed on BV.

BV is a dysbiosis of the vaginal microbiome and is characterised by an overgrowth of anaerobic bacteria with depletion of *Lactobacillus* spp. (Bradshaw & Sobel, 2016). Women with BV often have high diversity communities with no single dominant species. Organisms commonly associated with BV include: *Mycoplasma*, *Gardnerella, Mobiluncus, Atopobium, Prevotella, Bacteroides, Peptostreptococcus, Sneathia, Leptotrichia,* and *Clostridiales* (Onderdonk et al., 2016).

BV diagnosed by Nugent score during pregnancy has repeatedly been found to increase the risk of PTB (Shimaoka et al., 2019; Donders et al., 2009; Lee et al., 2009; Leitich & Kiss, 2007; Guaschino et al., 2006; Hillier et al., 1995). However, presence of BV has not been successfully used to predict PTB (Figueroa et al., 2011). Additionally, while treatment of BV during pregnancy eradicates the infection, a Cochrane review of 21 high quality studies found that antibiotic therapy did not reduce the risk of PTB (Brocklehurst et al., 2013). Many speculate that inflammation from infection may cause irreversible damage before BV is treated however, Brocklehurst et al also found that treatment of BV before 20 weeks' gestation did not reduce the risk of PTB (5 studies, n=4088). This indicates that BV and the resulting immune response is not the sole aetiological factor for PTB.

Additionally, criteria for BV diagnosis are broad so multiple aetiologies can be termed BV. Gram staining is not able to differentiate between species of lactobacilli which are known to play different roles in vaginal health (Moosa et al., 2020). For example, *L. iners* has been found to produce a more robust immune response than *L. crispatus* (Walsh et al., 2020).

Because of this, we are not limiting our studies to BV associated bacteria but aim to investigate common vaginal organisms, both commensal bacteria and those that are associated with PTB. Importantly, we want to include bacteria representative of women from a range of settings including LMIC. Incidence of BV and dysbiosis is thought to be higher in populations from LMIC (Jespers et al., 2014), this could be a factor in global PTB disparity.

1.4 The vaginal microbiome

The human microbiome is a rapidly expanding area of research. In the last few decades due to the increasing affordability of community sequencing, we have discovered many links to disease which were unknown 30 years ago. For example, the gut microbiome has been associated with behavioural disorders, cardiovascular disease and type 2 diabetes (Durack & Lynch, 2019). Dysbiosis of the female reproductive tract microbiome has not only been associated with PTB (Callahan et al., 2017) but also with idiopathic infertility and lower pregnancy rate after *in vitro* fertilisation (IVF) (Campisciano et al., 2017; Haahr et al., 2016).

1.4.1 Normal Vaginal Microbiome

A "normal" vaginal microbiome (henceforth referred to as VMB) is considered to be dominated by *Lactobacillus* spp. Compared to other sites such as the colonic microbiome, the VMB is considered relatively simple (Anahtar et al., 2018). In a keystone study, Ravel et al, (2011) proposed 5 distinct community state types (CST) based on the dominant microbial species present in the vagina (Table 1.3). However, 581 species from 10 bacterial phyla have been identified from the vagina (Figure 1.7).

Table 1.3 The five community state types (CST) of vaginal microbial communities and their associations with term or preterm birth (PTB). Introduced by (Ravel et al., 2011).

CST	Bacterial composition	Associations with health & PTB
I	Dominated by *Lactobacillus crispatus*	• Associated with term birth (Stafford et al., 2017), • Exclues *Gardnerella* (Callahan et al., 2017)
II	Dominated by *Lactobacillus gasseri*	• Transitions into CSTI (DiGiulio et al., 2015), • Associated with term birth (Stafford et al., 2017)
III	Dominated by *Lactobacillus iners*	• Coexists with *Gardnerella* (Callahan et al., 2017) • Transition to diverse community (Verstraelen et al., 2009) • Associated with PTB (Petricevic et al., 2014)
IV	Dominated by diverse anaerobes (BV-associated bacteria)	• Associated with PTB (DiGiulio et al., 2015) & (Callahan et al., 2017)
V	Dominated by *Lactobacillus jensenii*	• Associated with PTB (Stafford et al., 2017) & (Hyman et al., 2014) • associated with inflammation (Mitchell & Marrazzo, 2014)

These CSTs have been used to group bacterial communities in many studies involving European and American women (Freitas et al., 2017; Stafford et al., 2017; Romero, Hassan, et al., 2014). **CSTI** (dominated by *L. crispatus*) is considered the most health promoting community. Other *Lactobacillus* dominated communities such as *L. iners*-dominated (**CSTIII**) are considered intermediate as these have been shown to enable transition from protective to diverse communities (Verstraelen et al., 2009) and more recently have been associated with PTB (Petricevic et al., 2014). **CSTIV** (dominated by diverse anaerobes) is considered to be the least health promoting, colonisation with anaerobes is associated with inflammation (Mitchell & Marrazzo, 2014) and increased risk of PTB (Hyman et al., 2014).

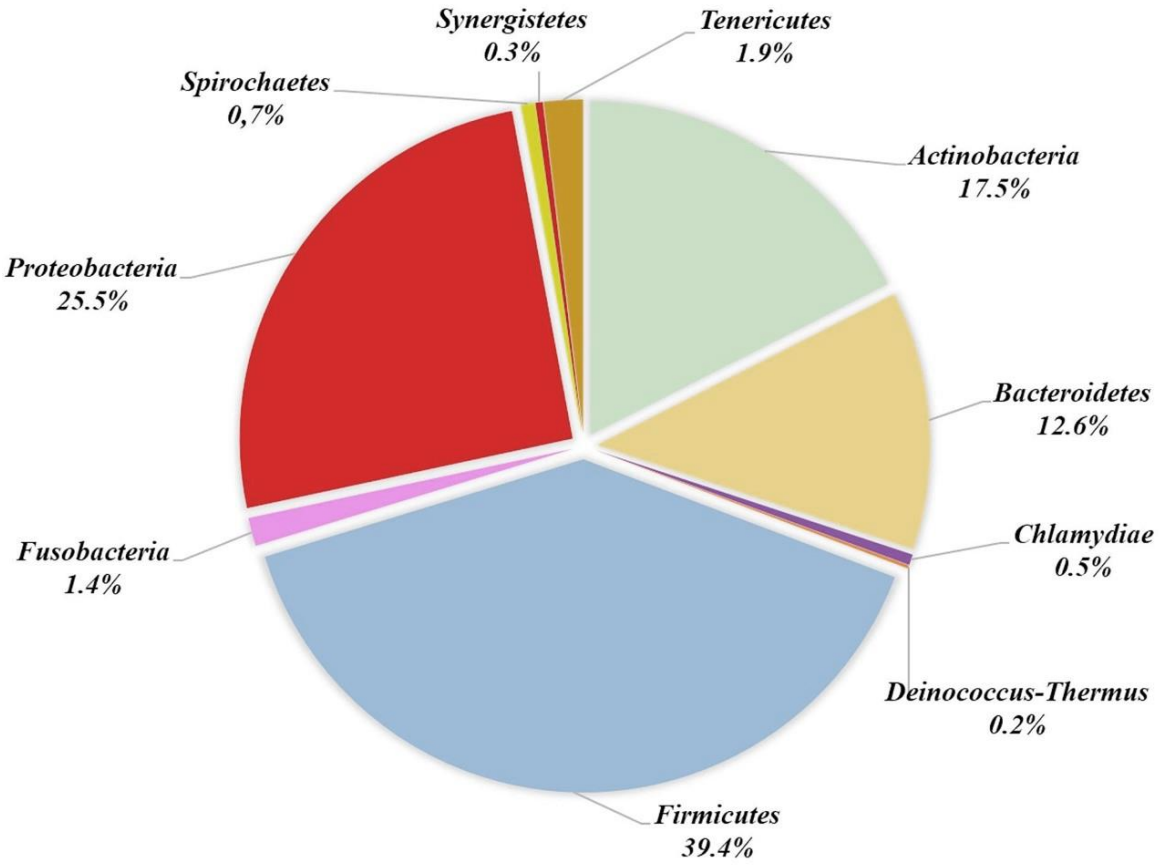


Figure 1.8 Proportion of bacterial phyla found from vaginal samples (n=581 bacterial species). Data produced by an exhaustive literature search of vaginal microbiome studies. Reproduced with permission from (Diop et al., 2019).

During pregnancy the VMB becomes more stable and less diverse due to the presence of high levels of oestrogen which increases levels of glycogen that promotes *Lactobacillus* colonisation (MacIntyre et al., 2015).

Lactobacillus spp. are thought to be health promoting for a number of reasons:

1) *Lactobacilli* produce lactic acid as part of their metabolism and this has been shown to maintain a low pH and have an anti-inflammatory effect within the vagina (Hearps et al., 2017). Lactic acid also appears to have an antimicrobial effect on BV-associated organisms (O'Hanlon et al., 2011, 2013). This is significant because it was initially thought that hydrogen peroxide was the antimicrobial agent. However, *in vivo* when mixed with CVF it appears hydrogen peroxide is not present in sufficient concentrations (O'Hanlon et al., 2010, 2011).

2) *Lactobacilli* are able to exclude other pathogens via competition by inhabiting the microbial niche (Kovachev, 2018; Boris & Barbés, 2000).

3) *Lactobacilli* have been shown to alter the membrane lipid structure of epithelial cells, preventing other bacteria from attaching not only by adhering and blocking but also through structural changes (Calonghi et al., 2017). This more recent discovery about *Lactobacillus* spp. demonstrates that they play a greater role in maintaining health than originally assumed. Despite this, *Lactobacillus* spp. can still cause symptoms of infection at high densities. Cytolytic vaginosis is a condition with similar symptoms to BV but differs by having: high abundance of lactobacilli, lack of other causative agents, low pH and Nugent score of 0 (Xu et al., 2019; Bhat et al., 2009).

Healthy vaginal communities dominated by *Lactobacillus* spp. and dysbiotic communities dominated by anaerobes is well established and widely accepted. However, most of this research was conducted in higher income countries specifically, the US and Europe. Fewer studies have investigated VMB in LMIC.

1.4.2 Vaginal microbial communities in low and middle-income countries

Fewer studies have investigated VMB in LMIC, but women in these countries are widely thought to have a higher prevalence of BV compared to European women (Kenyon et al., 2013). The majority of studies agree that women from LMIC have a lower prevalence of *Lactobacillus* dominated communities and higher prevalence of mixed anaerobic communities with no dominant species (Lennard et al., 2018; Borgdorff et al., 2014). Organisms commonly associated with dysbiosis were: *Prevotella*, *Sneathia, Megasphaera, Leptotrichia, Atopobium,* Bacterial vaginosis associated bacterium 1 *(BVAB1)* and *Mobiluncus.* Because of the lack of *Lactobacillus* dominance in these studies, authors have proposed many different ways to group women based on VMB (Lennard et al., 2018; Gosmann et al., 2017; Borgdorff et al., 2014; Kacerovsky et al., 2015; Anahtar et al., 2015), however, none are as reproducible or as widely accepted as Ravel et al, (2011) CST.

It is noteworthy that some of the above studies in LMIC recruited from STI clinics or were also investigating HIV, and so despite having a control group of healthy women attending the same clinics, may have selected a cohort of women with a higher proportion of dysbiosis compared to the general population.

However, lack of colonisation with *Lactobacillus* not specified by Ravel does not mean that women in LMIC necessarily have dysbiosis. Other organisms such as *Lactobacillus vaginalis* have a high prevalence in women from Rwanda, South Africa and Kenya and are thought to be important to vaginal eubiosis in these women (Jespers et al., 2015, 2017).

Anahtar et al (2015) investigated the vaginal microbial communities of young South African women and found that *Lactobacillus* was dominant in only 37% of the women. Of these women, 77% harboured predominantly *L. iners* which is associated with intermediate communities and PTB (Petricevic et al., 2014; Verstraelen et al., 2009). None of these women reported symptoms of BV and only half of those with diverse communities had a Nugent score indicative of BV. Supporting this finding, colonisation of *L. iners* was also found in 64% of samples from healthy women in Nigeria (n=241) (Anukam et al., 2006). This suggests that healthy vaginal communities are commonly dominated by *L. iners* and other non-*Lactobacillus* species in some settings.

More recent studies have challenged the accepted theory that women in LMIC have naturally more dysbiotic vaginal communities. Anukam et al., (2019) demonstrated that Nigerian women without BV had VMB comparable to the CSTs outlined by Ravel et al., (2011) with *Lactobacillus* making up to 95% of sequence reads.

Additionally, a study in India sequenced the VMB of 40 pregnant women and found communities could be classified using the accepted groups from Ravel et al, (2011). Samples were mainly dominated by *Lactobacillus* specifically, *L. crispatus* and *L. iners* (Mehta et al., 2020). Furthermore, a study of pregnant Nigerian women (n=68) again observed the major CST: CSTI (*L. crispatus)* had a 23.5% prevalence, CSTII (*L. gasseri*) had a 4.4% prevalence, CSTIII (*L. iners*) had a 39.7% prevalence, and CSTIV (non-Lactobacillus dominated) had a prevalence of 27.9%) (Odogwu, Onebunne, et al., 2021).

However, a recent study recruiting from routine antenatal appointments in Uganda found communities dominated by non-*L. iners Lactobacillus* (6%), *L. iners* (13%) and the most prevalent *G. vaginalis* (49%). *Gardnerella* species were present in over 90% of the samples (Bayigga et al., 2020).

Considering all of these reports, it is clear that vaginal microbiome cannot simply be classified as more diverse in LMIC but appears to vary between locations and populations. Some studies have comparable results to those seen in large US studies while others support the more diverse and dysbiotic vaginal communities seen in other studies. Many factors will affect the vaginal microbiome such as pregnancy, oestrogen levels, HIV status and hygiene practises such as douching (Lewis et al., 2017). However, one probable cause of variable results not from the variation in VMB is the different methods used in these studies.

1.4.3 Variation in microbiome methods

Variation in methods at each experimental step may bias results. Varying bacterial lysis techniques has been shown to cause small but significant differences in DNA yield and diversity (Gill et al., 2016). Different commercial kits commonly used in VMB research have been found to produce varying DNA extraction, DNA yield, DNA quality, microbial sequence count and microbial diversity (Mattei et al., 2019). Additionally, choice of polymerase chain reaction (PCR) primers/target regions (Sirichoat et al., 2021), database and sample sequence clustering parameters have also been shown to impact results (Van Der Pol et al., 2019).

Finally, varying sequencing methods produce varying results. The Illumina platform was shown to give a greater number of reads and similar ability to distinguish bacteria to the species level when compared to 454 pyrosequencing and Sanger sequencing (Smith et al., 2012).

The commonly used 27F primer has 3 mismatches to *Gardnerella* species which can lead to reduced amplification and skewed ratios of pathogens to the more easily amplifiable *Lactobacillus* species (Zhang et al., 2019; Frank et al., 2008). Many studies investigating the VMB have used this primer including (Stafford et al., 2017; Shiozaki et al., 2014; Romero, Hassan, et al., 2014). Primer biases need to be considered when interpreting results.

Advantageously, storage at -20°C or -80°C and self-collection vs clinician collected swabs do not appear to have any effect on microbiome sequencing results (Bai et al., 2012; Forney et al., 2010). This allows us to collect swabs from women who are not comfortable with speculums, which are used during clinician collected swabs. It also allows storage of swabs in low resource settings that do not have access to -80°C freezers.

1.4.4 The effect of race on vaginal microbiome

The other probable cause for variation is population-specific factors such as socioeconomic status, access to healthcare and race. Callahan et al, (2017) identified the need to investigate different populations with consistent methods. The group first studied a predominantly Caucasian population (n=49) (DiGiulio et al., 2015), then repeated the study with a comparable population of Caucasian women (n=39) and an additional population of predominantly African American women, with a prior history of PTB, receiving progesterone treatment (n=96). The association between reduced *Lactobacillus* and increased *Gardnerella* with PTB that was observed in the first study, was replicated in the comparable population but not in the African American population. Additionally, the African American population were found to have a higher frequency of *Gardnerella* compared to the Caucasian (Callahan et al., 2017). Despite the clear differences between populations, it is not certain that this is due to race, as the African American population also had a higher risk of PTB and received progesterone treatment. In order to determine differences in microbiota and the effect on PTB risk, treatments and risk of PTB need to be consistent for study participants.

Another study that investigated race is (Hyman et al., 2014), they observed different *Lactobacillus* species abundance. Specifically, African American and Hispanic participants had a higher *L. iners* content compared to Caucasian participants who had a higher *L. crispatus* and *L. gasseri* content. Importantly, they were able to predict of PTB by using a combination of race, PTB history and SDI, which is a measure of abundance of different species within a community. York et al, (2010) observed an increased variance of gestational age in the African American group compared to European American. They concluded that environmental factors, particularly factors that differ between pregnancies such as unreliable access to healthcare during pregnancies, were largely responsible for the increased variability in gestational age.

In summary, previous studies have found significant differences in the bacterial species present in the VMB during pregnancy when comparing women from different races. In some studies, these differences in bacterial species were able to predict PTB. However, studies do not often investigate the causes of the differences which may be challenging to measure and interpret. Many of these studies were conducted in the US. Racial discrimination is likely to contribute to these differences in race via chronic maternal stress levels. One study found 36.9% of black women reported chronic worry about racial discrimination compared to 5.5% of white women. Both groups were U.S born, postpartum women with singleton live births during 2011–2014 (Braveman et al., 2017). This may be a significant contributing factor in PTB disparities.

The issue is, compared to other factors investigated in PTB research such as gestation or maternal age which are both measured in days, race is a subjective category. Race typically contains limited options, for example in US studies participants are commonly grouped into Black, White, Black Hispanic, White Hispanic or Asian. These categories do not allow for participants with parents from different races and group many different populations of people together. Additionally, some studies comment on a potential genetic aspect of PTB by investigating race without genetic data. This is may result in poor predictive values as self-reported ancestry has been shown to differ from genomic data (Mersha & Abebe, 2015). Furthermore, genetic diversity was found to be higher within races than between races and for that reason other data such as geographical information or genetic analysis are recommended (Long & Kittles, 2009; Olson et al., 2005; Goodman, 2000).

One benefit is that race is available on most health records and may indicate social factors which are not routinely collected in healthcare. However, race cannot provide insight into genetic ancestry, one study found that self-reported race and genetic markers are often discordant (Mersha & Abebe, 2015). Race is a promising factor in PTB prediction, so where possible we should aim to collect more detailed data on more specific factors that reflect the individuals environmental and genetic factors.

Burris et al, (2020) explored how race may lead to different omic biomarkers in PTB between racial groups, to name a few: Social stressors such as discrimination and poverty, physical stressors such as air pollution and access to healthcare and healthy foods. If we were better able to measure these factors, disparities in PTB prediction and prevention could be improved. Since these environmental and social factors differ between settings and populations, it cannot be assumed that the vaginal microbiome of African women will be similar to that of African American women. This is another reason we should strive for research inclusive of races and settings whenever possible.

1.5 Vaginal microbiome and preterm birth

Vaginal microorganisms have long been implicated in PTB (Goldenberg et al., 1998; Hillier et al., 1995). These studies historically used culture dependent techniques and so were only able to identify a limited number of species, specifically those able to grow in lab conditions. Since the use of ever improving molecular techniques, we can better identify the diversity and relative abundance of bacteria within the vagina, and therefore better understand which species are clinically significant to PTB.

Although significant research has investigated the VMB during pregnancy, few studies focussed on the association between PTB and the VMB, without treatment such as antibiotics in the study design (Peelen et al., 2019). Within this specific group, five studies found some association (Callahan et al., 2017; Stout et al., 2017; Stafford et al., 2017; Nelson et al., 2016; DiGiulio et al., 2015).

DiGiulio's case control study (2015) analysed vaginal bacterial communities from 49 women. Samples were collected weekly, which allows for more detailed insight into temporal community changes. It was observed that CSTIV (dominated by anaerobes) was associated with preterm delivery (PTD). Furthermore, an increased number of samples classified as CSTIV increased the risk of PTD, particularly in the presence of *Gardnerella vaginalis* or *Ureaplasma urealyticum.* Interestingly, CSTII *(L. gasseri*) was seen to transition only to CSTI (*L. crispatus*), all other CSTs had multiple links to other CSTs (DiGiulio et al., 2015).

In a follow up study, (Callahan et al., 2017) used the same methods as (DiGiulio et al., 2015) and confirmed the association between *Gardnerella* (specifically a sub species *Gardnerella vaginalis* G2) and PTB within a comparable population. They observed that *Lactobacillus crispatus* and *Gardnerella* strongly exclude each other, in contrast to *Lactobacillus iners* which often coexisted at near equal frequencies. Because *L. iners* and *G. vaginalis* were often found at near equal frequencies, Callahan argued that there is a lack of distinction between CSTIII (*L. iners*) and CSTIV (anaerobes), instead they recommended classifying CSTs based on three key taxa: *G. vaginalis, L. crispatus,* and *L. iners*.

A previous study from our group, including 133 patients, (Stafford et al., 2017) observed an association between CSTI (*L. crispatus*), CSTII (*L. gasseri*) and term birth. In contrast, CSTV (*L. jensenii*) were associated with preterm delivery.

Additionally, (Nelson et al., 2016) investigated the VMB in nulliparous African American women. They observed higher interindividual community diversity at 9-16 weeks gestation in the preterm group. Contrary to other studies, no significant difference in levels of *Lactobacillus* or *Gardnerella* were found between term and preterm groups. However, significantly lower levels of the family *Coriobacteriaceae, Sneathia, Prevotella* and *Aerococcus* were observed at 9 to 16 weeks among women delivering preterm. This finding does not fit with the current hypothesis that presence of diverse anaerobes (anything other than a *Lactobacillus* dominated community) may cause cervical inflammation and increased rates of PTB.

It is important to note that 83% (32/40) of patients in Nelson's study were Nugent-defined BV positive women. This is a much higher prevalence than would be expected in the average pregnant population. Peebles et al, (2019) reported prevalence of BV in pregnant women from all global regions: in Sub-Saharan Africa this was 25%. A BV prevalence of 83% would impair the ability of the studies to demonstrate associations with *Lactobacillus* species. Additionally, as discussed in section 1.3, Nugent score is a very broadly specific diagnostic tool and could classify a healthy vaginal community as BV in some cases.

Consistent with (Nelson et al., 2016), (Stout et al., 2017) investigated a predominantly African-American cohort and found no difference in *Lactobacillus* or *Gardnerella* levels between term and preterm, in their investigation including 77 patients. Not consistent with the studies previously discussed, Stout observed stable diversity throughout pregnancy in the term group but a significantly decreasing richness and diversity in the preterm group.

Four studies with participant numbers ranging from 40-90 found no significant association between the VMB and PTB (Subramaniam et al., 2016; Shiozaki et al., 2014; Hyman et al., 2014; Romero, Hassan, et al., 2014). However, based on their findings, (Hyman et al., 2014) recommend that *Bifidobacterium* and *Ureaplasma* should be investigated further. Additionally, (Romero, Hassan, et al., 2014) reported the composition of the VMB during normal pregnancy changed with increasing gestation. They observed an increase in *Lactobacillus* spp. and decreased anaerobe relative abundance despite no difference in VMB in relation to PTB (Romero, Hassan, et al., 2014).

A meta-analysis published this year containing 17 studies found that participants with "low *Lactobacilli"* communities were at highest risk of PTB (Gudnadottir et al., 2022). This broader category works well as a prediction tool as it encompasses dominated by a range of bacteria associated with PTB such as *Gardnerella* and *Prevotella* without having to identify them.

1.5.1 Vaginal microbiome and preterm prelabour rupture of membranes (PPROM)

More specifically, VMB has also been associated with the sub type of PTB, PPROM. Brown et al, (2018) observed that vaginal dysbiosis was present in a third of cases prior to membrane rupture. Women with more diverse variable communities appear to be at higher risk of PPROM (Brown et al., 2018; Jayaprakash et al., 2016; Kacerovsky et al., 2015; Baldwin et al., 2015; Silva et al., 2003). Additionally, women with PPROM were found to have lower abundance of *Lactobacillus* spp. and more diverse, variable communities (Brown et al., 2018; Jayaprakash et al., 2016; Kacerovsky et al., 2015; Baldwin et al., 2015; Silva et al., 2003). The most abundant organisms found in these studies, in women with PPROM were: *Prevotella* spp. Specifically *Prevotella timonensis, Sneathia sanguinegens, Ureaplasma urealyticum* and *Peptoniphilus* spp.

Despite the well-accepted theory that diverse anaerobes cause dysbiosis, leading to cervical inflammation and increased levels of PTB, the evidence is inconsistent.

Table 1.4. Summary of cervico-vaginal microbiome studies and their findings in relation to preterm birth.

Study	Patient Number	Main Findings
(Baldwin et al., 2015)	15	• Decreased lactobacilli species in PPROM • *Prevotella* and *Peptoniphilus* prevalent in PPROM • Above observations were unchanged with antibiotic treatment
(Brown et al., 2018)	250	• Decreased lactobacilli species prior to PPROM • Lactobacillus dominant communities shifted to dysbiotic after erythromycin treatment
(Callahan et al., 2017)	135	• *Lactobacillus crispatus* associated with low risk of PTB • *Gardnerella* and *L. iners* often coexisted
(DiGiulio et al., 2015)	40	• Diverse CSTIV had strongest PTB association • *Ureaplasma* and *Gardnerella* associated with PTB
(Jayaprakash et al., 2016)	36	• *Mycoplasma* and *Ureaplasma* associated with lower GA at delivery • PPROM patients had highly variable communities
(Kacerovsky et al., 2015)	61	• *Gardnerella vaginalis* and *Sneathia sanguinegens* dominated communities in PPROM patients • PPROM patients with *L. crispatus* dominated communities had lower rates of microbial invasion of the amniotic cavity
(Nelson et al., 2016)	40	• women delivering preterm had lower diversity than term
(Romero, Hassan, et al., 2014)	90	• No association with PTB • increasing levels of Lactobacillus spp. and decreasing anaerobic bacteria throughout the progression of normal pregnancies
(Silva et al., 2003)	64	• Low rate of *Lactobacilli* in PPROM group • *Staphylococcus aureus* associated with membrane inflammation
(Stout et al., 2017)	77	• Term group had stable diversity • Preterm group had decreasing diversity

PTB= preterm birth, PPROM= premature prelabour rupture of fetal membranes, CST= community state type

In summary, most studies found some association between VMB and preterm birth, although evidence of associations with specific bacteria is mixed. Participants in two studies (Stout et al., 2017) (Nelson et al., 2016) were predominantly African American which may strongly influence the composition of the vaginal microbiota (see section 1.4.4). It is also worth noting that whilst the more recent papers found an association, the older papers did not.

Associated with term birth:

Lactobacillus gasseri (Stafford et al., 2017; DiGiulio et al., 2015)

Lactobacillus crispatus (Callahan et al., 2017; Stafford et al., 2017; DiGiulio et al., 2015)

Associated with PTB:

Gardnerella species (Callahan et al., 2017; DiGiulio et al., 2015),

Lactobacillus iners (Callahan et al., 2017)

Lactobacillus jenseni (Stafford et al., 2017)

1.5.2 VMB and PTB in low- and middle-income countries

There are significantly fewer studies investigating VMB and PTB in LMIC. No studies to date have included women from different continents using the same protocols and analysis. This comparison would greatly contribute to evidence on whether normal vaginal microbiome during pregnancy significantly differs between settings and if this contributes to PTB.

Studies from LMIC have reported varying correlations between the vaginal microbiome and PTB:

- A study in Nigeria supports the idea that *L. iners* is part of a healthy microbiota as many women had CVF communities dominated by *L. iners* and the majority delivered at term. Interestingly, *Atopobium* remained persistent throughout pregnancy in women who delivered preterm (Odogwu, Onebunne, et al., 2021).
- A study conducted in India observed similar abundance of *Lactobacillus* at term (41.6%) and preterm (37.6%), although *L. gasseri* was significantly higher in women who delivered term. *L. iners*, *Sneathia sanguinegens*, *Gardnerella vaginalis*, *Megasphaera* spp. were all found to be significantly higher in preterm samples (Kumar et al., 2021).

1.6 How does infection or dysbiosis cause preterm birth?

Microbes have extensively been associated with PTB by identifying clinical infection during pregnancy or detecting the presence of microbes in gestational tissues after birth. Infection associated PTB is typically either sPTL or PPROM.

In PPROM patients, Fortner et al, (2014) observed significantly thinned chorion and significantly higher bacterial presence regardless of labour, gestational age, or presence of histologic chorioamnionitis compared to term and preterm without PPROM. There is still much to be discovered regarding the mechanisms of PPROM, however it is thought that microorganisms can induce mechanisms required for both sPTL and PPROM in a similar variety of ways. As previously discussed (Figure 1.3, Figure 1.4), the three main physiological conditions needed for birth are: cervical ripening, myometrial contractions and rupture of the fetal membranes. Microorganisms have been shown to be able to influence these mainly via bacterial presence and products which induce inflammation and collagen degradation.

1.6.1 Bacterial metabolites

Bacterial metabolites can influence a range of cell functions, produce inflammation, and alter disease susceptibility (Ansari et al., 2021). For example, the ratio of L- and D-lactate produced by *Lactobacillus* spp. have been found to influence MMP-8 production (Witkin et al., 2013). If D-Lactate is low because of low lactobacilli abundance, higher L-lactate concentrations induces MMP-8 (Witkin et al., 2013) which increases the risk of PTB and PPROM (Figure 1.9).

Additionally, short chain fatty acids (SCFA) including formate, acetate, succinate, and lactate are produced from carbohydrates and dietary fibres by bacteria (Cselovszky et al., 1992). SCFA (excluding lactate which is largely produced by *Lactobacilli)* are associated with BV (Aldunate et al., 2015) and BV is associated with PTB (as discussed in 1.3.1).

Broadly, dysbiotic bacterial communities produce inflammatory metabolites which further contribute to dysbiosis and it is this inflammation that is thought to contribute to PTB (Ansari et al., 2021).

1.6.2 Inflammation

Microbial presence can be detected by the vaginal epithelium (Fichorova et al., 2011), chorioamniotic membranes (Kim et al., 2004) and decidua (Krikun et al., 2007) via toll like receptors (TLR). These pattern recognition receptors detect microbes via their products such as lipopolysaccharide (LPS) and short chain fatty acids (SCFA) (Mirmonsef et al., 2012) or conserved characteristics shared by a range of bacteria e.g. flagellin (Hargreaves & Medzhitov, 2005). When TLR (specifically TLR-2, TLR-4 and TLR-7 which have been identified in the reproductive tract) detect a pathogen associated molecular pattern (PAMP), they initiate the NF-κB signalling pathway leading to translocation of NF-κB subunit p65 and induction of cytokine expression leading to cytokine release (Padron et al., 2020; Peltier, 2003) (Figure 1.8). Species associated with dysbiosis have been shown to induce an inflammatory response via cytokines (Mitchell & Marrazzo, 2014; Bastek et al., 2011) (Figure 1.9).

Cytokines are small signalling molecules which communicate within and between cells in a range of tissues (Tadamitsu et al., 1994). Cytokines such as IL-6 and TNF-α are known to play a role in sPTL by both inducing the production of additional cytokines and prostaglandins, therefore initiating a cytokine cascade (Simpson et al., 1999). Prostaglandins in turn contribute to cervical ripening, myometrial contractions and membrane activation (Simpson et al., 1999). If levels of pro-inflammatory molecules reach a significant level, sPTL will be initiated. However, the exact threshold is yet to be determined and likely varies between individuals. In addition to direct effects of inflammation, cytokines produced in response to pathogens can induce increased apoptosis in the membranes (Uchide et al., 2012), which would contribute to weakening and potentially PPROM.

Despite this, bacterial presence alone is not thought to result in PTB, as organisms have been found in fetal membranes in non-complicated term pregnancies (Steel et al., 2005). Most likely, it is the individual's immune response (i.e. cytokine production) to the presence of bacteria that can induce the mechanisms of labour and membrane rupture prematurely as these are also inflammatory processes (Alvares, Cruz, Brandão, 2010).

Inflammation and activation of TLR could be a factor in the racial disparity of PTB. African women have been found to have higher baseline levels of inflammation (Anahtar et al., 2015). While this could be in response to a more diverse VMB, genetic studies have shown that single

nucleotide polymorphisms (SNP) in the TLR-4 and IL-6 gene increased the risk of PTB. Black women with BV were more likely to have these polymorphisms (Genc et al., 2004) (Goepfert et al., 2005). Small changes like these could influence the balance of pro- and anti-inflammatory signals which in combination with other factors could lead to an increased risk of PTB.

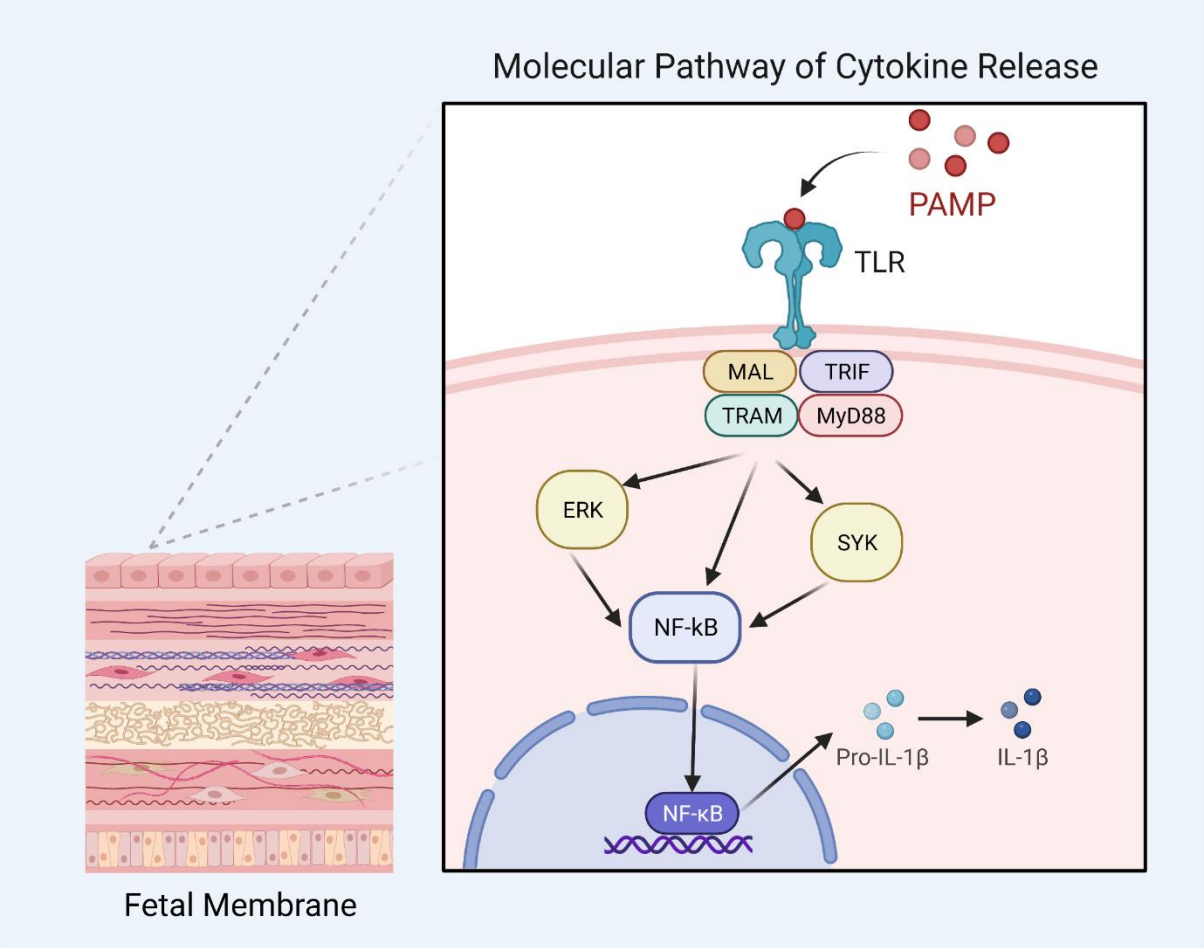


Figure 1.9. Infection associated cytokine release pathways leading to inflammation and increased risk of preterm birth. Pathogen associated molecular pattern (PAMP) (e.g., LPS, flagellin, bacterial metabolites) stimulate cytokine release via toll like receptors (TLR) on immune cells and fetal membrane tissue. Activation of TLR leads to signalling downstream pathways involving receptor adaptors: Toll/Interleukin-1 receptor domain containing adaptor protein (MAL), TIR Domain-containing Adaptor-inducing Interferon-B (TRIF), TRIF-related adaptor molecule (TRAM), myeloid differentiation primary response 88 (MyD88) and modulatory proteins: extracellular signal-regulated kinase (ERK), spleen tyrosine kinase (SYK). This activates NF-kB in the nucleus, that leads to induction of pro-inflammatory cytokines e.g Interleukin-1-beta (IL-1b), (NF-kB), (Pro-IL-1b), Adapted from (Padron et al., 2020). Created with BioRender.com

1.6.3 Collagen degradation

In addition to inflammation, microbes can also contribute to PTB initiation by producing MMPs or, stimulate an immune response leading to the host to produce increased levels of MMPs which degrade collagen found in fetal membranes (Weiss et al., 2007). PPROM is associated with increased levels of MMP-9 in the amniotic fluid and low levels of TIMP-1 which in combination leads to significant collagen degradation (Weiss et al., 2007).

In vitro infection studies of fetal membranes have consistently shown that collagenase producing bacteria significantly weaken membranes (Flores-Herrera et al., 2012; French, 2005; Schoonmaker et al., 1989; Sbarra et al., 1987). These studies investigated Group B *Streptococcus* (*Streptococcus agalactiae*), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. However, less research has investigated commensal vaginal bacteria which may still play a role. In order to properly understand the interaction between the fetal membranes and the vaginal microbiome, we aim to test a range of commensal vaginal organisms rather than well studied pathogens.

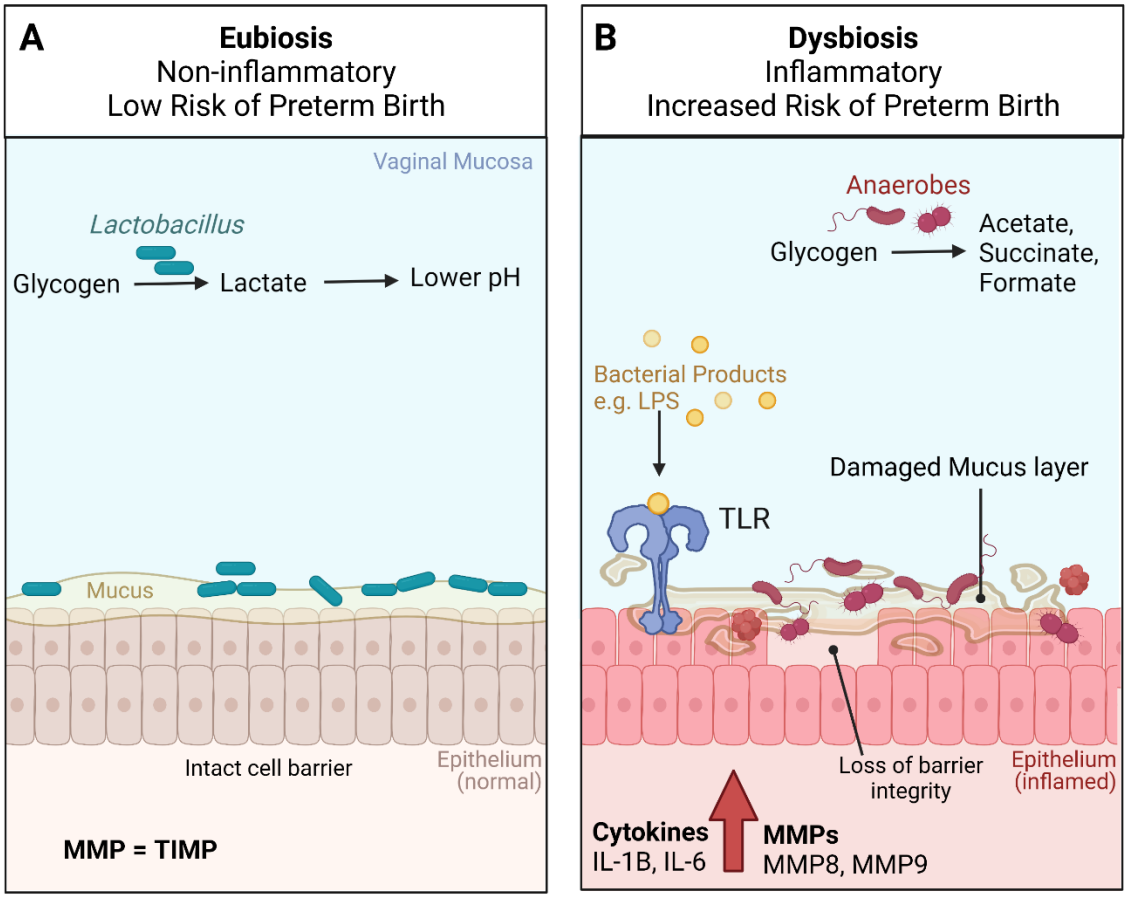


Figure 1.10. Impact of bacterial dysbiosis on vaginal ecosystem. **A)** Eubiotic community dominated by *Lactobacillus* spp which metabolise glycogen into lactate lowering pH. **B)** Dysbiotic vaginal community dominated by anaerobes e.g. *Gardnerella, Sneathia, Streptococcus*. These bacteria metabolise glycogen into short chain fatty acids e.g. acetate which do not lower pH. Bacterial products e.g. lipopolysaccharide (LPS) initiate TLR4 signalling which increases levels of inflammatory cytokines e.g. IL-6 and matrix metalloproteases (MMPs). Created with BioRender.com

1.6.5 Treatment of vaginal infection and PTB

Antibiotics are useful in treating intra-amniotic infection during pregnancy (Yoon et al., 2019) and effectively eradicate BV but, do not decrease the risk of PTB unless given before 20 weeks' gestation (Lamont, 2015; Brocklehurst et al., 2013). This suggests that inflammation from infection, which is often sub-clinical, can irrevocably affect pregnancy outcome if not detected and treated early.

At later gestations antibiotics are not effective in preventing PTB, one study observed a high prevalence of anaerobes *Prevotella* and *Peptoniphilus*, in PPROM patients that was not eradicated with antibiotic treatment (Baldwin et al., 2015). In addition to their limited effectiveness there is evidence to suggest that antibiotics may have a negative impact on pregnancy outcome. Brown et al, (2018) observed a shift from Lactobacillus dominant communities to dysbiotic communities after erythromycin treatment. Additionally, a recent study suggested that antibiotic treatment for pregnant women without infection may contribute to PTB via cytokine release (Hantoushzadeh et al., 2020). Therefore, it is important that pregnant women are not given treatment without specific need.

Some speculate that antibiotics may be more effective in preventing PTB in LMIC where infections are not routinely screened. However, a randomised placebo-controlled clinical trial in India does not support this. Bellad et al, (2018) observed no difference in PTB rates, before 37w or 34w, between treated and untreated women.

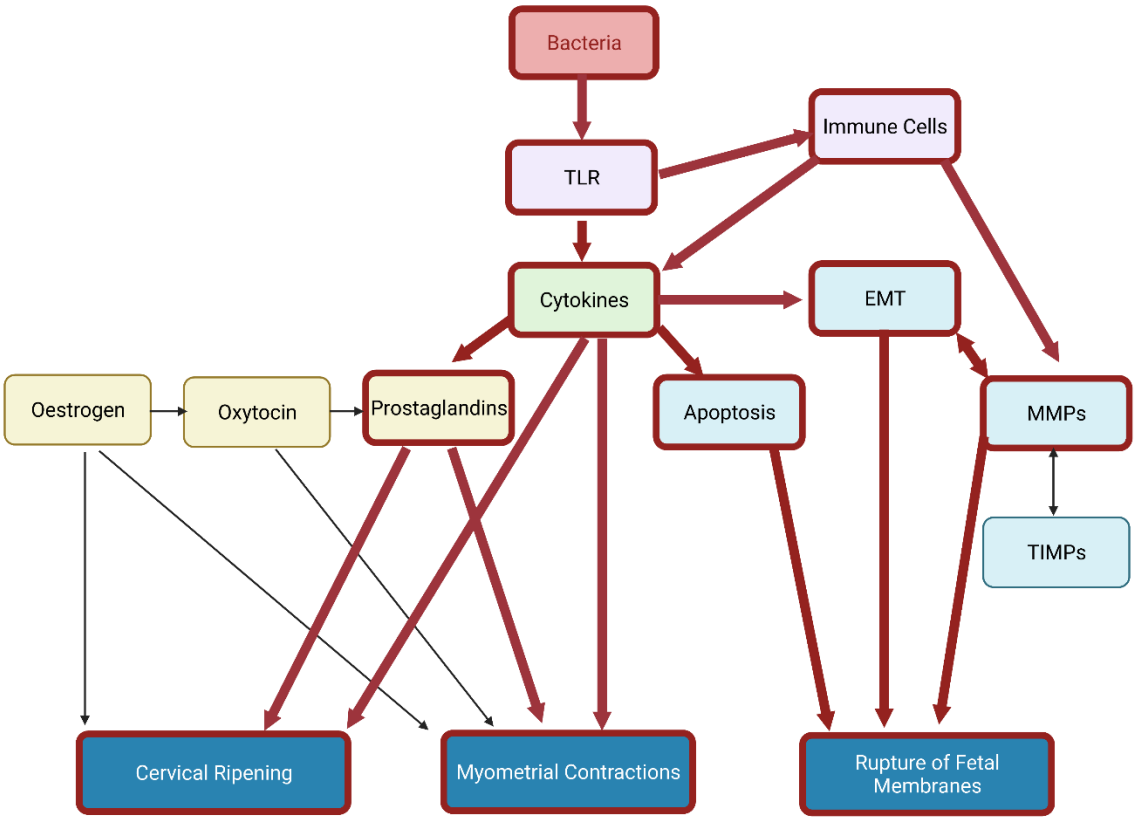


Figure 1.11. Initiation of infection-associated preterm labour

Boxes along the bottom contain physiological events needed for term labour. Boxes at the top represent factors which influence term labour. Arrows indicate upregulation/ recruitment/ induction/ stimulation. Arrows highlighted in red indicate pathways turned on by the presence of bacteria. TLR = toll-like receptors, immune cells e.g. neutrophils and macrophages, MMP = matrix metalloproteinases, TIMP = tissue inhibitor of metalloproteases, EMT = Epithelial to Mesenchymal Transition. Created with BioRender.com.

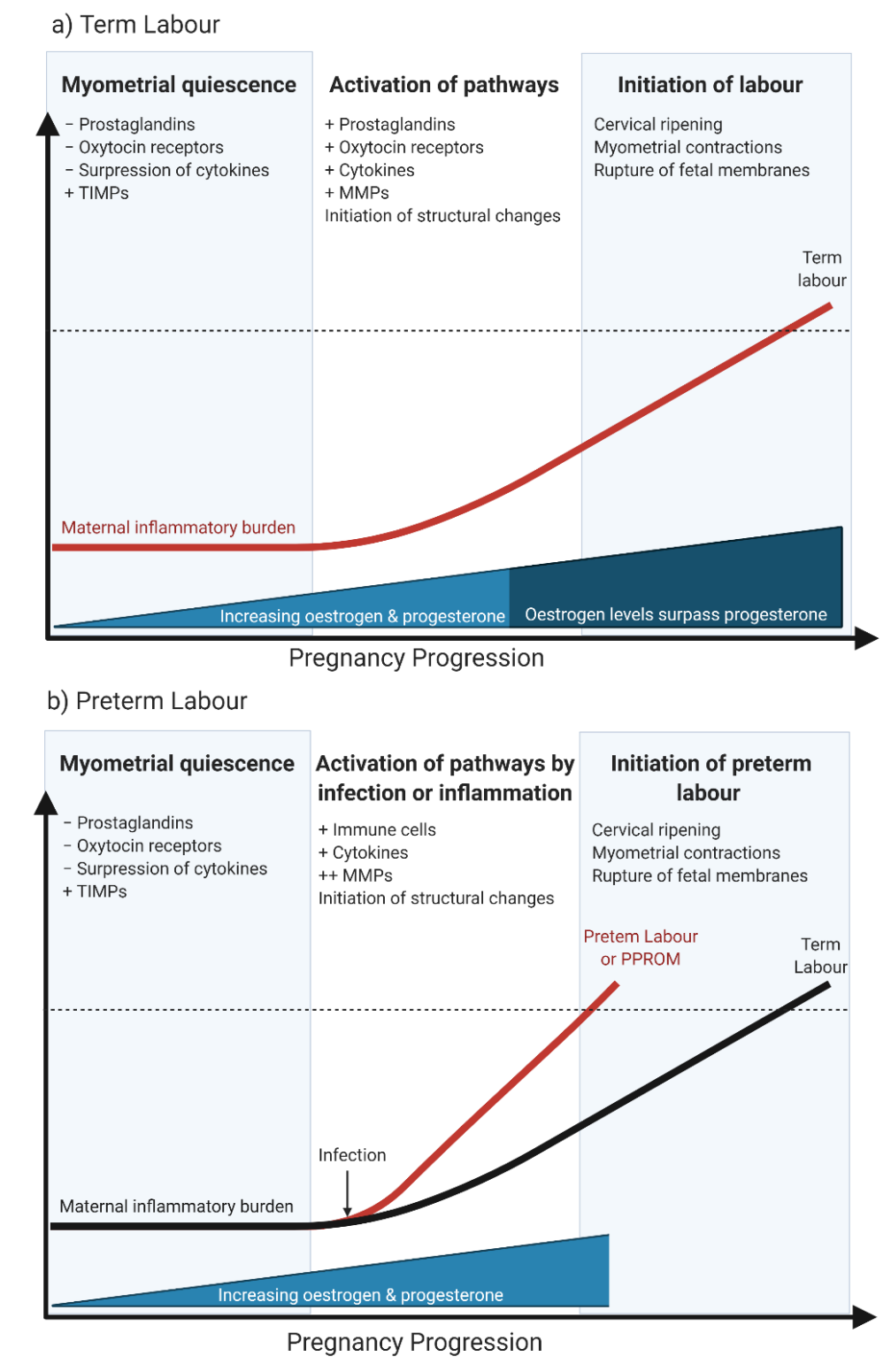


Figure 1.12 Initiation of term vs preterm birth. a) Graph illustrating the change in labour inducing factors throughout term pregnancy. b) Graph illustrating the activation of inflammatory pathways in preterm birth or preterm prelabour rupture of fetal membranes (PPROM). MMP = matrix metalloproteinases, TIMP = tissue inhibitor of metalloproteases. Dotted line represents a theoretical threshold of inflammatory signals needed for labour. Created with BioRender.com.

1.7 Preterm Birth Prediction

Currently, when mothers present with symptoms of PTB, we are unable to determine who will give birth prematurely. This leads to some mothers being discharged when observation and treatment is necessary. Others are kept in for observation unnecessarily which uses resources and causes distress and disruption to the family. If we are able to determine which women were going to deliver preterm, those women who need observation and treatments such as progesterone (Jarde et al., 2019), cerclage (Jarde et al., 2019), tocolytics (Hanley et al., 2019), magnesium sulphate (Burhouse et al., 2017) and corticosteroids (Schmitz, 2016) can receive them and women who have low risk of delivery can be discharged. Additionally, if we could predict infection associated PTB at an early gestation, before the initiation of sPTL, this would allow more time to prevent possible PTB with treatments such as antibiotics or probiotics before irrevocable processes such as inflammation, degradation of membranes or shortening/remodelling of the cervix will occur.

1.8 Current prediction methods

Currently, in the UK, there are several methods available to predict PTB. Unfortunately, these techniques are unable to predict PTB with high sensitivity and have high rates of false positive or negative. Due to the differing aetiologies, only PTB prediction methods for singleton pregnancies will be discussed in this thesis.

1.8.1 Clinical history

The strongest predictor of PTB is a previous PTB, for this reason most studies group women based on risk. High risk women are generally classified by previous PTB/miscarriage or a short cervical length. Low risk women are generally classified by no history of PTB or miscarriage and a cervical length within the normal range (Figure 1.12a). The sensitivity of many prediction methods decreases when applied to all women but can still be useful within the high-risk group.

1.8.2 Ultrasound assessment

Ultrasound is a cost-effective technique for developed countries such as the UK but, rural areas in LMIC are unlikely to have access to ultrasound equipment. This poses a challenge for dating the pregnancy which may lead to an inaccurate gestational age.

1.8.2.1 Cervical Length Measurements

One technique using ultrasound is cervical length (CL). This screening is a cost-effective predictive tool for PTB when available. Transvaginal ultrasound is generally used for CL measurements, however, in some cases e.g. in PPROM patients', trans-perineal ultrasound can be used to reduce the risk of infection. Cervical length is measured in a straight line from the internal os to the external os (Kagan & Sonek, 2015) (Figure 1.12a). In addition to cervical length, features such as funnelling of the internal os and cervical sludge (Figure 1.12b) are measured. Funnelling was able to predict birth before 37 weeks in a study from Maia et al, (2020) with a small sample size (PTB n=13).

The current NICE guidelines recommend using cervical length measurement to assess the risk of PTB.A CL of >25mm is considered low risk of PTB. Treatments vary depending on gestation. Between 16^{+0} and 24^{+0} weeks women with a CL of <25mm should be offered vaginal progesterone and prophylactic cervical cerclage to delay delivery. At 30^{+0} weeks or more in women with suspected sPTL, a CL of <15mm is used to diagnose sPTL, after which tocolytics and corticosteroids can be offered (NICE, 2019).

Research supports the use of CL to predict PTB. Kunzier et al, (2016) found a CL of ≤15 mm was the most clinically optimal cut-off with the lowest false positive rate and highest positive predictive value. Additionally, Sotiriadis et al, (2010) conducted a metanalysis which concluded that cervical length was effective at predicting PTB within one week.

However, some studies concluded that CL is not clinically useful. Conde-Agudelo & Romero, (2015) observed that a change in cervical length over time is not a clinically useful test but, a single measurement between 18–24 weeks of gestation is better able to predict PTB. Furthermore, Berghella et al, (2017) confirmed that additional research is needed before screening can result in better clinical management and outcomes. It is because of these drawbacks that we are still searching for effective PTB prediction methods.

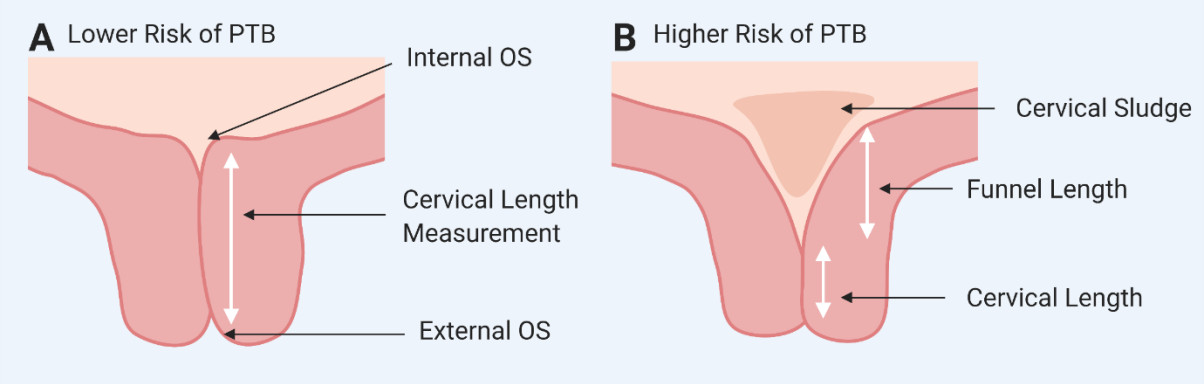


Figure 1.13 Cervical length measurement, funnelling and cervical sludge. Used to predict preterm birth. Created with BioRender.com

1.8.2.2 Amniotic/cervical sludge

Another assessment tool that can be performed using ultrasound is observation of amniotic sludge or cervical sludge, described as particulate matter in the amniotic fluid close to the internal cervical os (Figure 1.12b). Many studies have concluded that the presence of amniotic sludge is an independent risk factor of preterm delivery, histological chorioamnionitis and microbial invasion of the amniotic cavity in patients with spontaneous preterm labour and intact membranes (Yasuda et al., 2020; Hatanaka et al., 2019; Kusanovic et al., 2007; Romero et al., 2007). However, despite the reliable prediction of PTB in women with amniotic sludge, not all patients experiencing PTB have amniotic sludge.

1.8.3 Fetal fibronectin

In addition to ultrasound methods, rapid bedside tests have also been developed for detecting markers of sPTL. One of the most effective is fetal fibronectin (FFN), an extracellular matrix glycoprotein that is produced by fetal cells and can be detected in cervicovaginal fluid (CVF). Low levels of FFN in the second trimester indicate a low risk for PTB (Ruma et al., 2017). Additionally, high levels of FFN are associated with intraamniotic infection (Oh et al., 2019).

1.8.4 Placental alpha microglobulin-1 (PAMG-1)

Placental alpha microglobulin-1 (PAMG-1) is a protein found in the amniotic fluid. It is used as a biomarker in the CVF as its presence indicates PPROM or PROM (Petrunin et al., 1976). A recent metanalysis showed cervical PAMG-1 can be used to accurately predict PTB within 7-

14 days (Pirjani et al., 2021). However, studies investigating PAMG-1 have prioritised symptomatic women leading to insufficient research in asymptomatic high-risk women. Additionally, this test is less effective in women with sPTL and intact membranes.

1.8.5 Phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1)

Phosphorylated insulin-like growth factor binding protein-1 (phIGFBP-1), is produced by decidua and is present in the CVF when the decidua and chorion detach, indicating tissue damage (Brik et al., 2010). This test, also known as Actim Partus, is currently used in Jessop wing Maternity branch of the Royal Hallamshire Hospital, Sheffield, UK, where our study was conducted. However, this test has some drawbacks. The Actim Partus test can only be used when membranes are intact and only has negative predictive value. In other words, a negative result indicates a low risk of PTB, however, a positive result does not provide an accurate risk assessment.

1.8.6 Interleukins

Interleukin-6 (IL-6) is a cytokine which is the most associated with PTB. Recent studies suggest that IL-6 can be used to predict PTB within 7 days (Raba & Tabarkiewicz, 2018). IL-1β has also been found to predict PTB with a 79% negative predictive value (Amabebe et al., 2018).

1.8.7 Multiple prediction factors

By far the most accurate approach to predict PTB is by combining the most effective methods of predition available. A recent study (Radan et al., 2020) demonstrated that a combination of CL measurements and PAMG-1 can accurately predict PTB in symptomatic women better than either factor alone.

Cervical length and CVF cytokines IL-6 and IL-8 were found to predict delivery within 7 days. The prediction model had a specificity of 92.8% but the sensitivity was only 56.4% (Jung et al., 2016).

A previous study from our group found that RANTES, IL-6 and Acetate/Glutamate ratio sampled from CVF were associated with delivery within 14 days. However, in combination L/D-lactate ratio + Acetate/Glutamate ratio + IL-6 was found to be a better predictor of delivery within 14 days (Amabebe et al., 2019).

1.8.8 Potential prediction methods

Ideally, prediction techniques should utilise non-invasive samples such as urine, CVF, saliva or blood as collection poses no risk to the pregnancy. More invasively obtained samples such as amniotic fluid, or cervical or placental tissue present a risk to the pregnancy and should be avoided unless absolutely necessary.

There is a range of prediction techniques which are currently under investigation including: cervical collagen alignment (W. Li et al., 2019), cervical impedance spectroscopy (Anumba et al., 2021), protein markers in CVF and amniotic fluid (Holst et al., 2011), cytokines markers in serum, CVF and amniotic fluid (Short et al., 2018; Bogavac et al., 2012), cervical microRNA (Elovitz et al., 2014) and metabolite markers (Liang et al., 2019; Barberini et al., 2019). All these prediction methods are in the early stages of development and will require additional studies on a larger scale before clinical efficacy can be determined.

We are focussing on investigating CVF because its proximity to reproductive tissues which enable smaller changes within the CVF to be detected before a systemic change in serum or saliva is detectable. It is accepted that during PPROM amniotic fluid can leak into the vagina and change the pH and metabolome of the CVF. However, the vaginal metabolome is not currently used to predict PTB due to lack of evidence/research. Microbiology swabs are taken to identify BV or pathogenic organisms such as GBS which has an established link to preterm birth but does not yet have sufficient evidence to use as a predictor (Tano et al., 2021).

Our aim is to identify potential biomarkers for PTB by investigating the CVF microbiome and metabolome along with clinical factors.

1.8.9 Vaginal microbiome for Preterm Birth Prediction

Despite the numerous studies which investigate the relationship between the vaginal microbiome and PTB using molecular sequencing techniques (Elovitz et al., 2019; Stout et al., 2017; Ghartey et al., 2017; Hyman et al., 2014; Romero, Hassan, et al., 2014; Choi et al., 2012; DiGiulio et al., 2008; Wilks et al., 2004; Oyarzun et al., 1998) (as discussed in 1.5), detection of microorganisms is not used for prediction of PTB. This is predominantly because no causal relationships have been established, due to the unique and changing nature of the human microbiota and the high number of organisms that have been detected.

If causal relationships were established in the future, DNA sequencing of microorganisms could present a good PTB prediction method as this technique is becoming cheaper and more portable e.g., Oxford Nanopore Sequencing, so could be used in LMIC.

A broader approach to predicting PTB using VMB is to look at the metabolism of PTB associated bacteria. Bacterial metabolism differs between species, however, bacteria which occupy the same niche often have similar metabolisms due to their energy source and environment (Amarasekare, 2003; Whittaker, 1965). This could allow detection of a pathogenic bacterial group such as vaginal anaerobes without being species specific. This is beneficial as the VMB differs between individuals and as discussed in section 1.5.2, may differ depending on setting. Once established, predictive metabolite tests are likely to be more accessible to LMIC as they do not require expensive equipment such as ultrasound or sequencing platforms.

1.8.10 Cervico-vaginal Fluid Metabolites for Preterm Birth Prediction

The CVF metabolome is a snapshot of the host-pathogen interaction at the time of sampling, as both host cells and microorganisms contribute to the metabolites present. Looking at metabolites allows a broader view of the microbial community metabolism as well as interaction with the host. Metabolites are not only produced as waste products from metabolism but, have been shown to be used as signalling molecules by human cells (Wang & Lei, 2018) and bacteria (Bassler & Losick, 2006). In addition, bacterial metabolites have been implicated in the regulation of the host immune system (Levy et al., 2016).

Molecular techniques e.g. PCR are able to detect targeted organisms, similarly, microbiology techniques are able to isolate targeted organisms from clinical samples via specialised culture plates. Both of these techniques could identify the presence of bacteria associated with PTB. However, by identifying the metabolites present, we are able to identify related bacterial species as well as bacteria which may not be closely related but inhabit the same niche.

Few studies have investigated the predictive value of metabolites in relation to PTB. A 2019 systematic review of untargeted metabolites as PTB biomarkers found only 3 studies investigating CVF, 9 investigating amniotic fluid, 1 investigating amniotic fluid and blood and 1 investigating blood and urine (Carter et al., 2019). The authors concluded that significant

metabolites and changes in metabolites are not consistent within the 14 studies they analysed. This was likely due to the variations in sample collection methods and the limited number of studies investigating each sample type.

Focusing on CVF, most studies that looked at untargeted metabolomics to predict PTB found significant differences between the term and preterm groups. A nested case-control study of asymptomatic high risk women (Ghartey et al., 2015) observed a significant change in amino acid metabolism in preterm patients between the 2nd and 3rd trimester. Additionally, no significant change in carbohydrate metabolism between 2nd and 3rd trimester was observed in the preterm group despite glucose, glucose-6-phosphate, xylulose, maltohexose, N-acetylglucosamine, and N-acetyl galactosamine being significantly decreased in the term group.

In a follow up study investigating symptomatic women from a larger prospective cohort used in the study previously mentioned, (Ghartey et al., 2017) observed a significant upregulation of mannitol and methyl phosphate in addition to a significant down regulation of medium chain-fatty acids and collagen degradation markers in preterm patients compared to term. In relation to PTB, Ghartey et al., (2017) has shown that women who give birth prematurely have a different vaginal metabolite signature compared to women who give birth at term. Because of this, CVF metabolites can be used as a PTB predictor.

In contrast, (Thomas et al., 2015) did not find any significantly different metabolites in preterm compared to the term group. However, this study investigated low risk patients at 20 weeks gestation. The significant findings from (Ghartey et al., 2015, 2017) were changes in metabolism between 20 – 28 weeks gestation in patients deemed to have a high risk of PTB.

Previous studies from our group have repeatedly shown that acetate, which is thought to be a product of vaginal dysbiosis, was predictive of PTB within 2 weeks of assessment in women presenting with symptoms suggestive of preterm labour (Amabebe et al., 2019; Amabebe, Reynolds, V. L. Stern, et al., 2016). Additionally, the ratio of acetate and glutamate was found to be predictive of PTB within 2 weeks (Amabebe et al., 2019)

Begum et al., (2017) also found metabolites to aid in PTB prediction. Vaginal fluid Urea concentration was found to have 100% positive predictive value with a sensitivity of 98% and specificity of 100%. Concentration of creatine in vaginal fluid was found to have a 97.83%

positive predictive value with 90% sensitivity and 93.83% specificity. This study highlighted the cost-effectiveness of using metabolites as a predictor of PTB in low resource settings. If further research is able to identify reliable metabolite prediction methods this would allow accessible prediction of PTB in high and low resource settings.

However, much research is needed before metabolites can be used as a reliable prediction technique as the vagina is a complex ecosystem. For example, we could target communities with low levels of *Lactobacillus* spp. for metabolomic prediction of PTB as these communities are thought have the highest risk of PTB (Gudnadottir et al., 2022). Low *lactobacilli* communities will have lower levels of lactate in CVF. Additionally, communities dominated by *L. iners* were found to have lower levels of lactate (Witkin et al., 2013). Therefore, measuring lactate can provide insight into the community rather than performing more specific microbiology assessments that may not apply to all women.

However, *Lactobacillus* are not the only lactate producing bacteria, other genera such as *Leuconostoc, Pediococcus,* and *Streptococcus* are also known to produce lactic acid (Quinto et al., 2014). Therefore, it is useful to study not only metabolome profiles but also the microbial source to ensure a robust predictive test that can be used in a range of women with varying vaginal microbiota.

1.9 Summary & Aims

PTB is a complex multifactorial problem. Some aspects are relatively well known such as multiple births, pre-eclampsia, intrauterine growth restriction (IUGR). sPTL is less well understood, however, it is established that infection and inflammation play a big role in many sPTB.

LMIC have disparately high rates of PTB and yet most of the research is conducted in high income countries. Research should not assume that predictive and preventive methods developed in places such as the UK will be applicable in LMIC. Despite an increasing number of microbiome sequencing studies in LMIC, no studies to date have compared the vaginal microbiome of pregnant women from multiple continents using the same methods to reduce potential areas of variation.

The mechanisms of sPTL and PPROM have been studied but are still not completely understood. Most studies focus on pathogens and infections that are historically associated with PTB. However, recent research utilising microbiome and metabolome techniques allows us to study microbial induced mechanisms more comprehensively than previous approaches.

The role of commensal vaginal bacteria in the aetiology of PPROM is an understudied area. Considering the far-reaching recent developments in the study of the gut microbiome, it is likely that the VMB could have similar immune regulation functions in relation to parturition at term or preterm.

1.9.1 Aims

One aim of PRIME NIHR Global Health Research Group was to gather samples with partner countries around the world to ensure PTB research is reflective of women from a range of settings. Unfortunately, due to the SARS-CoV-2 pandemic only the Sheffield samples are included in this thesis. Considering this, the aims of this thesis are:

1. To optimise a standardised CVF microbiome protocol that can be used consistently for partner country samples
2. To Investigate prediction methods of PTB and PPROM utilising CVF metabolites and microbiome
3. To investigate how microorganisms in the VMB may contribute to PPROM

To improve current prediction methods and develop preventive treatments, we need to better understand how microorganisms, both commensal bacteria and pathogens, contribute to PTB and PPROM. We want to better understand the nuances of the VMB and PTB or specifically PPROM, in order to develop prediction methods which are applicable not only in the UK but around the world.

PRIME aims to collect vaginal swab samples from pregnant women in Sheffield UK, Cape Town South Africa, and Bangladesh. This research will be the first to use consistent methods across 3 continents. Research in this thesis will lay the foundations for a continuing global study.

Chapters 2 & 3 investigate clinical samples to identify microbes and microbial products and metabolites that could be used to predict PTB in the future.

Chapter 4 details *in vitro* studies investigating the mechanisms of PPROM and how vaginal bacteria may contribute to PPROM.

2 Chapter 2 – Oxford Nanopore Sequencing of the Vaginal microbiome

2.1 Introduction

The VMB has been associated with PTB and PPROM, however evidence is mixed. Some studies found no association (Stout et al., 2017; Nelson et al., 2016) whilst others found that abundance of specific organisms e.g *Gardnerella vaginalis* and *Lactobacillus crispatus* were associated with outcome (Hyman et al., 2014). *Lactobacillus gasseri* and *Lactobacillus crispatus* are thought to promote term birth. Whilst *Gardnerella*, *Lactobacillus iners* and *Lactobacillus jenseni* are thought to contribute to PTB (Callahan et al., 2017; Stafford et al., 2017; DiGiulio et al., 2015).

The participant population and sequencing methods can greatly affect results (see section 1.4.3). Because of this, the PRIME study aimed to investigate the VMB of pregnant women from different continents using the same methods from sample collection to data analysis. Oxford Nanopore sequencing lends itself well to this aim as it is portable and can be used in a range of settings without the need for large equipment and laboratories. This work contains results from Sheffield participants only.

During the first year of this PhD investigation, a literature review was undertaken and at that time in October 2018 – January 2019 there were no published studies on vaginal microbiome using Oxford Nanopore sequencing technologies. Most of the lab work in this chapter was undertaken in June – September 2021. During this time, (Komiya et al., 2021) published a study investigating vaginal microbiota analysis in a clinical setting using Nanopore sequencing, focussing on non-pregnant infertility patients with no BV symptoms. Methods published by Komiya et al were tested during the primer optimisation work (see section 2.3.4). After the completion of the laboratory work, several studies have now investigated the VMB using Nanopore Sequencing (Marquet et al., 2022; Kerry-Barnard et al., 2022) in non-pregnant women.

2.1.1 16S Sequencing

16S sequencing which targets the 16S rRNA gene, discovered by Fox et al., 1977, is commonly used for identification of bacterial species. The 16S rRNA gene encodes a ribosomal RNA molecule present in all bacteria on the 30S subunit of the ribosome (Figure 2.1) (Fukuda et al., 2016). The ribosome is integral to translating mRNA into proteins and so the genes for the ribosome contain many conserved regions which are present throughout the bacterial kingdom. PCR is typically performed prior to sequencing to increase the concentration of DNA. Universal primers target conserved regions of the 16S gene so they are able to amplify all bacterial species present however, amplicons need to include one or more variable regions for species identification (Martinez-Porchas et al., 2017). Universal primer performance can vary depending on which V region they target, amplicons spanning V1-3 or V3-4 have been shown to perform best for species identification (Hugerth et al., 2020).

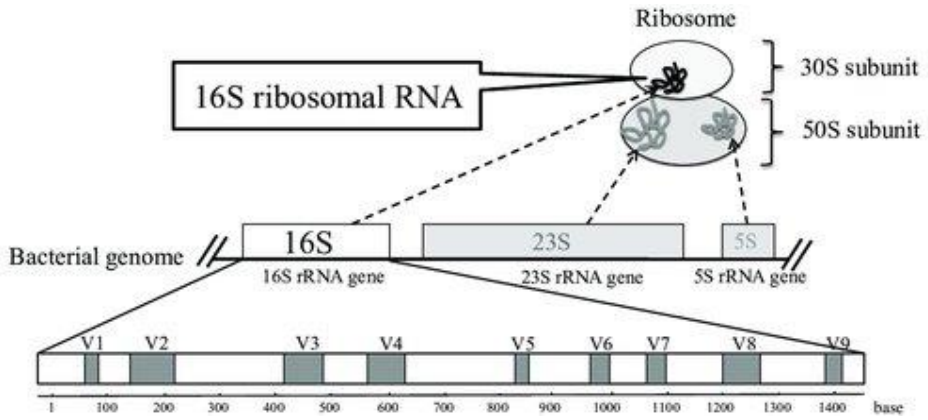


Figure 2.1 Graphical representation of the ribosome complex and 16S rRNA gene. The white and grey sections of the 16S rRNA gene indicate conserved regions and hypervariable regions (V1-V9) respectively. Reproduced with permission (Fukuda et al., 2016).

2.1.2 Oxford Nanopore sequencing

Nanopore sequencing is considered to be the 3rd generation of sequencing techniques, the 1st being sanger sequencing, which is unable to sequence bacterial communities, 2nd being Illumina which is able to read short fragments of up to 300 bp. This method is able to read much longer fragments of DNA compared to older methods, allowing for more accurate identification of bacterial species via the 16s gene which is roughly 1600 bp long.

The basis of this technique is an electrically resistant polymer membrane containing protein pores, each positioned above an electrode. When voltage is applied across the membrane ionic current is directed through the nanopore. During sample preparation a sequence adaptor is added to the start of each DNA strand. The sequence adaptor is made up of a motor protein which controls the speed of translocation as it unzips the double stranded DNA, whilst feeding the single strand through the pore one base at a time. As each base pair passes through the nanopore, the electrical current is disrupted. Each base produces a different disruption, this signal is captured by an Application Specific Integrated Circuit (ASIC) chip connected to the electrode under each nanopore and is converted into base pair calls. The identified base pairs on a DNA strand are recorded and can be analysed to identify the bacterial species present. Pores on the membrane act independently and concurrently. Data is transferred into a readable format as soon as the strand has passed through the nanopore, this allows results to be seen in real time (Figure 2.2 & Figure 2.3).

Our study aims to sequence samples from a range of settings in order to produce a preterm birth prediction strategy that can be utilised in both high and low resource settings. Nanopore sequencing lends itself to our aims due to its portability and lack of large equipment needed. The device itself (minion) can be held in your hand and requires only a laptop to read the real time data. This will allow clinical facilities which do not have access to large and expensive laboratory equipment to utilise this PTB prediction method. Additionally, as the equipment is portable samples could be taken into a rural community setting to combat barriers to healthcare that some mothers face.

The limitations of Nanopore sequencing include a potentially high error rate of base calling however, many new methods for improving read accuracy are being developed all the time. Including NanoCLUST which improves bacterial identification and abundance profile estimation at species-level resolution (Rodríguez-Pérez et al., 2021) and NanoReviser which is a post-processing tool to reduce error rate (Wang et al., 2020). As this method is still very new, a standardised analysis pipeline has not yet been established. As this technology evolves and improves our ability to rapidly identify species will also improve. Currently species level resolution may not be achieved consistently but likely will in the near future.

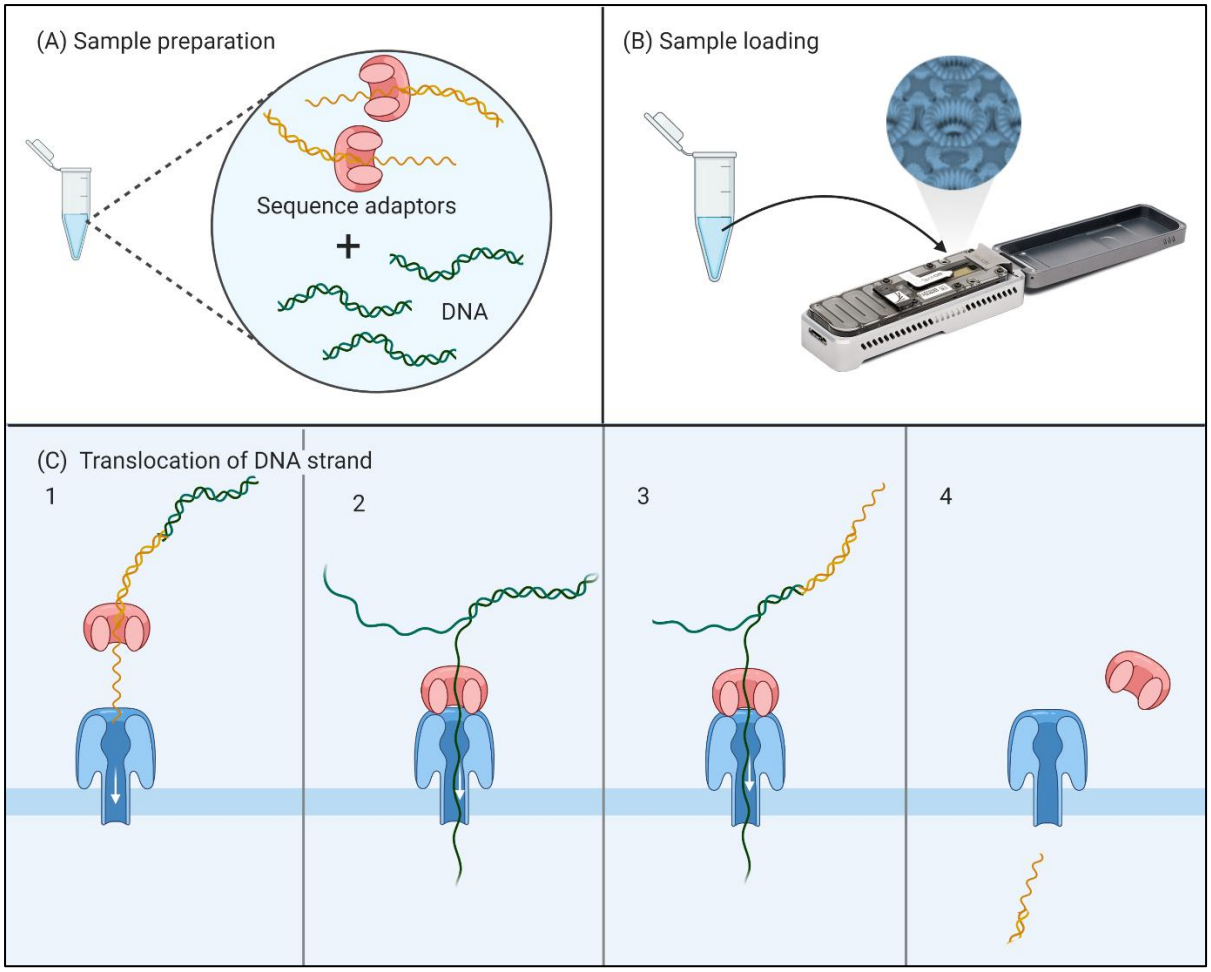


Figure 2.2 Nanopore sequencing methods. (A) During sample preparation sequence adaptors containing a motor protein are added to the end of each DNA molecule. **(B)** Sample is loaded into the minion device and sits above the nanopore membrane. **(C)** Ionic current is applied across the membrane and DNA strands begin to translocate through the nanopores. C1 – Sequence adaptors are drawn toward the nanopore by tethers on the membrane and the motor protein sits on top of the nanopore. C2 – The motor protein unzips the double stranded DNA and feeds a single strand through the nanopore reader where the bases are identified (see Figure 2.3). C3&4 – Once the DNA strand has passed through the pore the motor protein detaches ready for the next strand. Created with BioRender.com

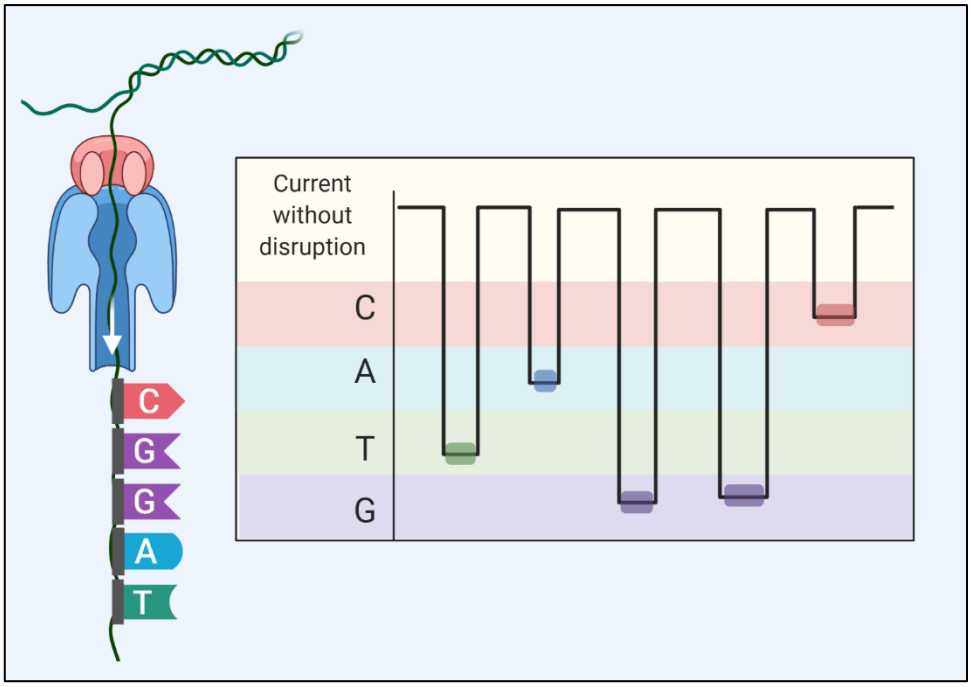


Figure 2.3 Nanopore sequencing base calling. Ionic current flows through the nanopore, as each base pair passes through, the ionic current is disrupted. Each base produces a distinct disruption, this signal is captured by an Application Specific Integrated Circuit (ASIC) chip connected to the electrode under each nanopore and is then converted into base pair calls. Created with BioRender.com

2.2 Hypothesis, Aims & Objectives

As future work will be conducted on clinical samples from LMIC, we chose to use novel Oxford Nanopore Sequencing for 16s microbiome sequencing as this method is the only portable sequencing technology that could be used in a range of settings.

Objectives:

1. To optimise the DNA extraction method and universal primer pair selection.
2. To extract DNA from clinical samples and perform 16s sequencing to identify bacterial species present in the samples.
3. To compare results from term, preterm and PPROM delivered patients to identify potential biomarkers and vaginal organisms associated with PTB.

We hypothesise that women who give birth prematurely will have a significantly different VMB that can be used to identify vaginal bacterial communities that result in a higher risk of PTB.

We hope to better understand which microorganisms put women at most risk of PTB and PPROM in order to develop predictive models inclusive of women not just from the UK but around the world.

Unfortunately, the SARS-CoV-2 pandemic delayed sample collection from all research sites and so this work includes only samples from the UK. However, based on the methods optimised in this study, the PRIME research team continue to work with clinical samples from all partner countries (discussed in future work).

2.3 Methods

2.3.1 Recruitment and eligibility

Studies were undertaken at the Jessop Wing Maternity Unit of the Royal Hallamshire Hospital, a tertiary referral unit with a birth rate of nearly 7,000 deliveries per year. Two cohorts of women were recruited: symptomatic and asymptomatic high risk. Sample size calculations were conducted using MedCalc® software based on local birth statistics and published studies which reported an AuROC of around 0.80-0.84 for sPTB prediction using CVF metabolite profiling (Amabebe, Reynolds, V. L. Stern, et al., 2016; Amabebe, Reynolds, V. Stern, et al., 2016). Given an estimated prevalence of sPTB in the range of 25% for the proposed cohorts, the expected ratio of negative (term-delivered) to positive cases (preterm delivered) was set at 4:1. Assuming a power of 80%, a type I error of 5% and a null hypothesis of AuROC=0.5, a sample size of 35-45 women was determined for each arm (Narice., 2019).

Women with signs of cervical infection, previous cervical surgery, abnormal cervical smear within the previous 3 years, multiple pregnancy and known fetal anomaly were excluded from the study as these conditions are known to be independent risk factors for PTB.

Participants and samples within this thesis were part of the ECCLIPPx II study (**E**le**C**tri**C**al **I**mpedance **P**rediction of **P**reterm Birth by Spectroscopy of the **C**ervix II), or the PRIME study (**Pr**eterm B**i**rth Prevention and **M**anag**e**ment).

The ECCLIPPx II study was conducted with Human Research Authority approval from the Yorkshire & the Humber Research Ethics Committee (17/YH0179) in alignment with Sheffield Teaching Hospitals NHS Trust regulations, registration number STH19385. Between May 15th 2018 and August 1st 2019

PRIME study was conducted with Human Research Authority approval from Health and Care Research Wales (HCRW) Approval, IRAS project ID: 256135, REC reference: 18/LO/2044 in alignment with Sheffield Teaching Hospitals NHS Trust regulations, protocol number: STH20635. Between 1st March 2018 and 25th March 2021

2.3.1.1 *Asymptomatic-high risk patient cohort*

A cohort of asymptomatic high risk (AHR) women were judged to be at high risk of PTB based on their previous obstetric history (one or more premature births <37 weeks or one or more late miscarriages). Or alternatively, they were included if they had an incidental short cervix (<25 mm) before 22 weeks or had undergone LLETZ with an excised tissue greater than 1.2 cm thick regardless of their previous obstetric history (Khalid et al., 2012).

AHR participants were approached at their booking antenatal visit at Jessop Wing. The study was explained, and study materials were provided including the Patient information leaflet. If they wished to participate in the study, women were scheduled to attend Feto-maternal Unit at gestational time point 1 (GTP) 20-22 weeks and later at GTP2 26-28 weeks. As these study appointments were performed on the same day patients were already scheduled to attend their PTB clinic, participating in the study did not increase their number of clinical appointments and so resulted in higher compliance. However, the appointment for sample collection at 30-32 weeks (GTP3) did not align with a pre-scheduled appointment therefore there was a higher rate of drop-out at GTP3. Additionally, a small proportion of preterm births occurred prior to GTP2 and many preterm births occurred prior to GTP3 resulting in fewer samples in these groups.

2.3.2 Data collection

After securing written consent, a full history was taken, and all clinical and demographic data was retrieved and recorded. Ethnicity data was grouped into White, Black, Asian or Hispanic due to insufficient group numbers. White including White British, White Irish & White Other or Black including Black British, Black Caribbean, Black African & Black Other or Asian including Asian British, Asian Bangladeshi, Asian Indian, Asian Pakistani, Asian Chinese & Asian Other or Hispanic including Hispanic Cuban, Hispanic Mexican, Hispanic Puerto Rican, Hispanic South American & Hispanic Other.

2.3.2.1 *Swabs*

For the microbiome and metabolome studies, swabs were taken at GTP1 (20-22 weeks), GTP2 (26-28 weeks) and subsequently at GTP3 (30-32 weeks).

A speculum (Robinson Healthcare®) lubricated with sterile water was inserted into the vagina to visualise the cervix and facilitate retrieval of cervicovaginal fluid from the posterior vaginal fornix for fFN measurement and metabolites quantification. Three swabs were taken from the posterior vaginal wall: one for fFN analysis, two Dacron swabs for metabolome and microbiome analysis (Delta lab Eurotubo300263, Fisher Scientific). Swabs for metabolome and microbiome analysis were immediately stored at -20°C in Jessop Wing for a maximum of 3 days before transfer for long-term storage at -80°C at the University of Sheffield.

2.3.2.2 Cervical length

Cervical length measurements including dynamic changes as well as the presence of cervical sludge or funnelling were recorded as added independent risk factors of PTB (Kagan and Sonek, 2015). Cervical length measurement was taken using a transvaginal probe (Hitachi Aloka ProSound Alpha 7). An image of the cervix was obtained, and CL was measured in a straight line. The procedure was repeated 3 times, and the shortest CL measurement was recorded.

2.3.2.3 Fetal fibronectin measurement

As an additional method of PTB prediction, a fFN swab was collected. As per manufacturers' instructions, the fFN was the first swab to be collected to avoid sample contamination. After retrieving the sample from the posterior vaginal fornix, the tip of the swab was immersed in buffer, before being checked with the bedside Rapid fFN Hologic® Perilynx system which yielded quantitative results measured in ng/mL.

2.3.2.4 pH readings

Vaginal pH which indicates infection when above normal range was measured with Fisherbrand™ narrow range pH indicator paper strips (3.6- 6.1) placed on the tip of the speculum after being withdrawn from the vagina.

2.3.2.5 Disclosure of results and clinical management

Participants were advised on their CL and fFN results at the time of the visit. Their risk of PTB was also computed using the QUiPP® app (Watson et al., 2017).

Patients with a short CL, a positive fFN result or deemed at high risk of delivery within 7 days were offered appropriate management as per local and national guidelines including admission, referral to the Jessop Wing specialised Preterm Birth Clinic, consideration of

cerclage or progesterone and/or administration of antenatal steroids and magnesium sulphate, and neonatal counselling (NICE, 2014).

2.3.3 DNA Extraction

2.3.3.1 Optimisation of DNA extraction

To identify the best DNA isolation method for cervico-vaginal swabs stored at -80 in PBS that can be reproduced easily in a variety of settings we tested 3 DNA isolation protocols from 2 commercially available kits. **Protocol A** utilised QIAmp DNA mini kit (Qiagen, 51304), protocol adapted from Stafford et al., 2017. **Protocol B** also utilised the QIAmp DNA mini kit but follows the protocol from manufacturer including appendix D from handbook which is specific for gram positive bacteria. **Protocol C** utilised QIAamp DNA Microbiome Kit (Qiagen, 51704) and included a host DNA degradation step prior to the breakdown of bacterial cell walls. See Figure 2.4 for a comparison of protocols A, B &C.

Vaginal Microbiome Whole Cell Mix (ATCC® MSA-2007™) was used to compare DNA extraction methods. Two concentrations of bacterial cells were used for optimisation: 500,000 bacterial cells/ isolation which represents number of bacteria on an average CVF swab, assuming a swab holds 500µl of CVF which contains 10^6 cells/ml. A second concentration of 1 million bacterial cells/isolation was used to test protocols with a larger number of bacteria in addition to a negative control which consists of a clean swab that has undergone DNA isolation and PCR with the samples.

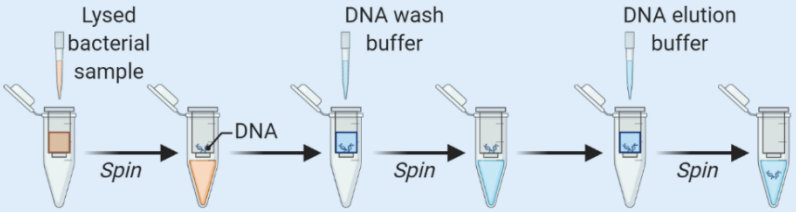
Protocol step	Protocol A	Protocol B	Protocol C
Processing	- Swab sample collected & stored at -80°C - Thawed, 400µl PBS added & vortexed for 5 min		
		Elution from swab centrifuged for bacterial pellet & supernatant removed	
Removal of host DNA			Benzonase & Proteinase K 20µl 24mg/ml 56°C 30 min degradation of DNA & RNA not protected within bacteria
Bacterial lysis	Lysozyme 75µl, 10mg/ml 37°C 1hr Proteinase K 20µl 24mg/ml & RNAse A 4 µl 100mg/ml 56°C 10 min	Lysozyme 180µl, 20mg/ml 37°C 1hr Proteinase K 20µl 24mg/ml & RNAse A 4 µl 100mg/ml 56°C 30 min 95°C 10 min	Mechanical lysis Proteinase K 40µl 24mg/ml 56°C 30 min
Spin column & Wash			
Elution	100µl Elution buffer	200µl Elution buffer	50µl Elution buffer x2

Figure 2.4 Comparison of DNA kits for isolation of bacterial DNA from cervico-vaginal swabs. **Protocol A** = QIAmp DNA mini kit (Qiagen, 51304), protocol adapted from (Stafford et al., 2017), **Protocol B** = QIAmp DNA mini kit following the protocol from manufacturer including appendix D from handbook which is specific for gram positive bacteria. **Protocol C =** QIAamp DNA Microbiome Kit (Qiagen, 51704). Created with BioRender.com

2.3.3.2 DNA extraction from CVF swabs protocol

For microbiome analysis, DNA was extracted from the clinical samples. After sample collection, swabs were immediately stored at -20°C in the clinic. Swabs were then transferred to the laboratory where the swab tip was cut off, placed into an Eppendorf and stored at -80 °C until DNA extraction. Swabs from the ECCLIPPx study were stored in PBS at -80 °C but, swabs collected for PRIME were stored without PBS and were eluted into PBS after thawing shortly before DNA extraction in line with other studies (Callahan et al., 2017; Stout et al., 2017; Subramaniam et al., 2016; DiGiulio et al., 2015).

All tips, tubes, tweezers and scissors were UV sterilised for 15 minutes to reduce bacterial DNA contamination. To elute CVF for DNA extraction, the swab was thawed and 500 µl PBS was added then vortexed for 5 minutes. The swab was placed into a new tube and centrifuged at 10,000 xg for 1 minute to draw out any remaining fluid from the swab.

QIAmp DNA mini kit (QIAGEN, UK, 51304) was used to extract DNA from CVF using the following protocol: 75 µl of lysozyme 20 mg/ml (Fisher Scientific) was added to 500 µl of CVF, Vaginal Microbiome Whole Cell Mix (ATCC® MSA-2007™) or PBS from a blank control swab and was incubated at 37°C for 1 hour in order to degrade bacterial cell walls. Proteins and RNA were digested by adding 20 µl proteinase K, 24 mg/mL (Qiagen, UK), 4 µl RNase A, 100 mg/mL (QIAGEN) and 500 µl of lysis buffer AL (1:1 ratio) to each tube. Samples were inverted 2–3 times to mix and incubated for 10 minutes at 56°C. 500 µl of 100% ethanol was added and vortexed for 15s to precipitate the DNA out of solution. Each sample was added to a spin column and centrifuged at 6,000 x g for 1 minute to allow DNA to bind to the silicone membrane and for the supernatant to pass through. The filtrate was discarded and 500 µl of wash buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. Filtrate was again discarded and 500 µl of wash buffer AW2 was added and centrifuged at 20,000 x g (14,000 rpm) for 3 min. The spin column was added to a new collection tube and was centrifuged at 20,000 x g (14,000 rpm) for 1 min to dry the membrane and eliminate buffer AW2 carryover. Each column was then transferred to a clean 1.5 ml microfuge tube and 50 µl of buffer AE water was added and incubated for 1 minute at room temperature before finally being centrifuged at 6,000 x g for 1 minute to remove isolated DNA from the column. DNA sample was placed on ice prior to PCR amplification. In addition to the CVF samples, a blank

swab also underwent DNA extraction in order to control for bacterial contaminants in reagents.

2.3.4 Optimisation of universal bacterial primers for vaginal organisms

In order to identify suitable universal primers that can amplify the majority of species within the vaginal microbiome for Nanopore sequencing, a literature search was performed to identify universal primers previously used in vaginal microbiome sequencing studies. Additionally, two reverse primers were designed. Subsequently, 1) *in silico* and 2) laboratory experiments were performed to assess the primers identified from the literature in addition to the universal primer pair recommended by Oxford Nanopore (27F/1492R).

2.3.4.1 Primer design

Two novel universal reverse primers named MCRevA & MCRevB, optimised for vaginal species, were created using Bioedit. Sequences of the 16s gene of each organism from the DNA standard were downloaded from the ATCC website (https://genomes.atcc.org/genomes). This includes *Gardnerella vaginalis* (ATCC 14019), *Lactobacillus gasseri* (ATCC 33323), *Mycoplasma hominis* (ATCC 23114), *Prevotella bivia* (ATCC 29303), *Streptococcus agalactiae* (ATCC BAA-611), *Lactobacillus jensenii* (ATCC 25258).

Sequences were aligned using the Clustal W function (https://www.ebi.ac.uk/Tools/msa/clustalw2/) in Bioedit to identify regions where species share a common sequence. Annealing temperature and location on the V region were taken into account when designing these primers.

2.3.4.2 In silico analysis

To assess the suitability of universal primers from the literature for amplification of all vaginal taxa *in silico* analysis was performed. A total of 28 primers were identified from studies that have previously sequenced the VMB. Using a combination of the 28 literature primers and 2 designed primers (Table 2.2), 30 different primer pairs were tested (Table 2.3). All primers were custom DNA oligos (IDT, UK).

Using the downloaded 16s gene sequences, location of the forward and reverse primer was identified on each sequence and number of base pairs between were counted to calculate amplicon size. Optimal annealing temperature, CG content and potential for heterodimers were assessed using IDT OligoAnalyzer™ Tool.

Larger scale analysis was performed using an online tool TestPrime (https://www.arb-silva.de/search/testprime) (Klindworth et al., 2013) which runs "*in silico* PCR" using SILVA database. We provided TestPrime with the primer sequences, selected the small sub-unit database (SSU r138.1) and the non-redundant reference dataset (RefNR). Each primer pair was then analysed by the online tool which includes assessing each sequence against the SILVA database, merging the results for the forward and reverse primer and sorting into to three groups. Match = both primers matched at the intended match position, mismatch = only one primer matched, nodata = sequences for which no clear decision could be made. Values for % amplification for each taxonomic unit were calculated by dividing the number of matched sequences by the number of matched or mismatched sequences. Full details of the TestPrime tool and its script can be found in (Klindworth et al., 2013).

2.3.4.3 Laboratory analysis of universal primer pairs

To assess the performance of promising primer pairs identified during *in silico* analysis, PCR was performed using a DNA standard (Vaginal Microbiome Genomic Mix ATCC, MSA-1007) containing 16.7% Gardnerella vaginalis (ATCC 14019), 16.7% Lactobacillus gasseri (ATCC 33323), 16.7% Mycoplasma hominis (ATCC 23114), 16.7% Prevotella bivia (ATCC 29303), 16.7% Streptococcus agalactiae (ATCC BAA-611), 16.7% Lactobacillus jensenii (ATCC 25258)

PCR conditions: Taq PCR master mix kit (QIAGEN, UK) with final concentrations: $MgCl_2$ 1.5mM, 2.5 units Taq DNA polymerase, 200µM of each dNTP, 0.2µM each primer and 5pg of bacterial DNA in TE buffer as recommended by QIAGEN. A negative control containing the reaction mix but no template control (NTC) was included for each pair in every PCR reaction.

Due to the varying annealing temperatures of the forward and reverse primers (see results section Table 2.2) each pair was tested with an annealing temperature of 55°C and 60°C, some pairs were also tested at 50°C and 52°C because of their lower recommended annealing temperature.

Once a shortlist of suitable primer candidates was identified PCR was repeated with 4 pairs with an additional barcode sequence on the 5' end (see supplementary data) for multiplexing.

Table 2.1 PCR reaction conditions

Step	Temp	Duration	Cycles
1) Initial denaturing	94	3 min	
2) Denaturing	94	45s	
3) Annealing	Variable	1 min	
4) Extending	72	1 min 30s	Return to step 2, repeat 35 times
5) Final Extending	72	10 min	
6) Storage	4	Overnight	

After PCR, gel electrophoresis was performed to identify if an amplicon had been produced at the expected size. Initially a 0.8% gel at 100v for 1 hr using TAE buffer stained with SYBR green DNA dye, marked with Invitrogen 100bp ladder was used. After optimisation, electrophoresis gels were performed using 1.2% agarose, containing GelRed nucleic acid stain (1:10,000) and was run at 80v for 1hr 30 min. TAE buffer stored at 4°C was used to prevent overheating. FullRanger 100bp DNA Ladder (geneflow L3-0014) was used to identify size of amplicons on the gel. Gels were imaged using Syngene G box.

2.3.5 PCR of CVF samples

After extraction of total DNA from the clinical samples, PCR was performed using optimised bacterial universal primers in order to amplify bacterial DNA. Half of the extracted DNA was used per PCR reaction; PCR was performed on 2 separate occasions for each sample to ensure maximum output of amplified DNA and to reduce the chance of contamination resulting in an unusable sample. Details of PCR reagents and conditions are listed in the optimisation section (2.3.4.3). Barcoded primers were used in order to match bacterial DNA strands to the clinical sample ID. Each PCR experiment included clinical samples, a positive control containing 5pg of template DNA from the bacterial standard used in the optimisation, a negative control containing DNA extracted from a blank swab which indicates microbial contamination from reagents and finally, a negative control containing the PCR mix but no DNA template (NTC).

2.3.5.1 Sample Clean-up & Size Selection

Samples sent for sequencing were cleaned up using AMPure XP clean up beads (Beckman, A63880). Clean up removes short fragments of DNA such as primer dimers and works by adding magnetic beads to the sample, DNA binds to the beads with longer strands preferentially bound. This allows selection of DNA size to remove from the sample using different ratios of beads: sample. We used a ratio of 1:1.2 to remove DNA <150 bp long. The sample was placed over a magnet to form a pellet of beads and DNA bound to them. Any DNA not bound was removed along with the supernatant, beads were then washed with 70% ethanol. Samples were then removed from the magnet and elution buffer was added to the beads which resuspends the DNA. The sample was then placed back onto the magnet and the beads, now with no DNA bound, formed a pellet at the bottom of the tube and the sample was moved to a new tube ready for sequencing.

2.3.6 Library preparation

In order to sequence multiple samples simultaneously, samples were normalised to the same concentration and pooled into a sequencing library. For each sample, DNA concentration was measured using Qubit dsDNA HS Assay Kit (Invitrogen, 10606433). To ensure maximum reads from samples with low DNA concentration, samples were sequenced in 2 batches. Samples in the lower concentration batch were all diluted to 1.5 ng/sample in order to get a representative number of reads from each sample. Samples in the higher concentration batch were diluted to 52 ng/sample.

2.3.7 Nanopore sequencing

To identify which bacterial species were present in the clinical samples, Oxford Nanopore MinION Sequencing was performed at the Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, UK.

2.3.7.1 Demultiplexing

After pooling barcoded samples during library preparation, demultiplexing was carried out using Porechop v0.2.0. In this process, the barcode sequence in the primer is used to match sequences with the samples they originated from.

2.3.7.2 Quality check & Adaptor trimming

Adaptors were removed using Porechop v0.2.3, these allow DNA strands to attach to the nanopore but must be removed as they interfere with the downstream analysis.

Quality check was performed using PycoQC v2.5.2. A commonly used quality score for sequencing is PHRED score which indicates the probability of sequencing error. The median PHRED score for the reads was 10.33. This indicates a 10% error rate whereas a score of 20 would indicate a 1% error rate (Delahaye & Nicolas, 2021). A PHRED score of <8 was not considered for subsequent analysis.

2.3.7.3 OTU Picking

Processed reads then underwent dereplication. In this process, reads were compared against each other, and similar reads were grouped and assigned a representative sequence.

Sequences were then clustered into operational taxonomic units (OTUs) using vsearch within qiime2. Sequences are clustered against a reference database using closed-reference clustering performed at 85% identity against the SILVA database to identify a species name for each OTU.

2.3.7.4 Abundance

In order to visualise abundance for each sample, qiime2. was used to produce an interactive plot that can be viewed on https://view.qiime2.org/ (interactive file available in supplementary materials).

2.3.7.5 Prevalence

To identify taxa common across groups prevalence was calculated. After OTU picking and identification using the SILVA database, read counts were exported to excel (see supplementary materials). The prevalence of each Taxonomy ID was determined by assessing the presence or absence within each sample and calculating a percentage (number of samples Taxonomy ID present in/ total number of samples ×100). This was calculated for 4 groups: all samples, term, preterm and PPROM.

2.3.7.6 Richness

As a measure of diversity, the richness of each sample was assessed by counting the total number of Taxonomy ID present. This was performed in excel and inputted into GraphPad

Prism 9.2.0 for statistical analysis and graphical representation. A Kruskal-Wallis test was used for analysis of 3 conditions (term vs preterm vs PPROM) and Mann-Whitney test was used for analysis of 2 conditions (term vs preterm).

2.3.7.7 Alpha Diversity

An additional method of diversity assessment that considers both richness and evenness of a community was also used. Alpha diversity was calculated using the Shannon diversity index (SDI) (Shannon, 1948) using the following equation. ln= Natural log, pi = The proportion of the community made up of species

$$\text{Shannon Diversity Index (H)} = -\Sigma pi * \ln(pi)$$

H values for each sample were inputted into GraphPad Prism 9.2.0 for statistical analysis and graphical representation. A Kruskal-Wallis test was used for analysis of 3 conditions (term vs preterm vs PPROM) and Mann-Whitney test was used for analysis of 2 conditions (term vs preterm).

2.3.7.8 Relative Abundance

In order to assess community composition, relative abundance of each Taxonomy ID was calculated by dividing number of reads per Taxonomy ID by the total reads in the sample. As highlighted by (Callahan et al., 2017) the presence and proportion of *G. vaginalis, L. crispatus* and *L. iners* is clinically important. Relative abundance of these organism was calculated from abundance values using the following formula: Organism 1/(Organism 1 + Organism 2). In this case 1 represents the presence of only Organism 1, 0.5 represents even abundance of Organisms 1 & 2 and 0 represents the presence of only Organism 2. All statistical analysis was performed using GraphPad Prism 9.2.0 using Mann-Whitney test. Correlation of the relative abundance was calculated using Pearson r correlation function on GraphPad Prism 9.2.0.

2.3.7.9 Classification of Community State Types (CST)

We attempted to group samples according to community state type (CST) depending on the most abundant bacterial species. These groups are based on the widely accepted CST from (Ravel et al., 2011) (See Table 1.3). The taxonomy ID with the greatest relative abundance was considered the dominant species (see supplementary materials).

2.3.8 Predictive Analysis

In order to assess the predictive capacity of bacterial species a Receiver-operating characteristic (ROC) curve was used. Area under the curve was assessed, a value of 0.5 was considered to have no predictive value. A value of 1 was considered to perfectly predict PTB and a value of 0 was considered to perfectly predict a term birth. Sensitivity, specificity and likelihood ratio were calculated using criteria outlined in (Shreffler & Huecker, 2020). All analysis was performed using GraphPad Prism 9.2.0.

Sensitivity represents the % of true positives out of all subjects who will have a PTB. In other words, it is the ability of the test to give a true positive result.

$$\frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

Specificity represents the % of true negatives out of all subjects who will not have a PTB. In other words, it is the ability of the test to give a true negative result.

$$\frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}$$

Positive Predictive Value (PPV) represents the proportion of positive results that are correct out of all positive results

$$\frac{\text{True Positives}}{\text{True Positives} + \text{False Positives}}$$

Negative Predictive Value (NPV) represents the proportion of Negative results that are correct out of all negative results

$$\frac{\text{True Negatives}}{\text{True Negatives} + \text{False Negatives}}$$

2.4 Results

2.4.1 Optimisation of DNA extraction

DNA extraction method has been shown to effect microbiome sequencing results. We optimised the DNA extraction method of cryopreserved CVF swabs using 2 commercial kits and 3 different protocols see Figure 2.4 for a comparison of protocols A, B & C. We found that pre-PCR, protocol A appeared to isolate the highest concentration of total DNA from a CVF swab. No bands were present for the bacterial whole cell standard at 5×10^5 or 10×10^6 cells for any extraction protocol (Figure 2.5).

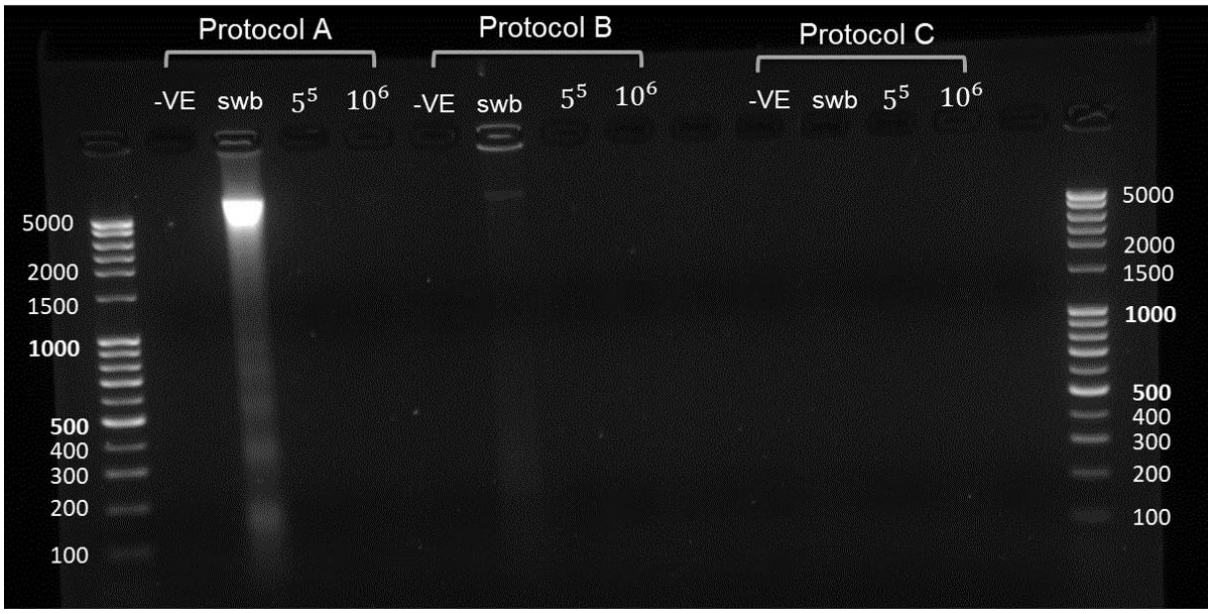


Figure 2.5 Electrophoresis gel image pre-PCR comparing 3 different DNA extraction methods. Protocol **A =** QIAmp DNA mini kit protocol detailed in (Stafford et al., 2017), protocol **B =** QIAmp DNA mini kit protocol from manufacturer including appendix D from handbook. **C =** QIAamp DNA Microbiome Kit. -VE = DNA extraction from a blank swab, swb= positive control from a CVF swab, 5^5 & 10^6 = number of bacterial cells from Vaginal Microbiome Whole Cell Mix (ATCC, MSA-2007). Assuming a swab holds 500µl of CVF which contains 10^6 cells/ml, 5^5 represents number of bacteria on an average CVF swab.

Post-PCR, protocol A produced amplicons visible on electrophoresis gel for the swab and the higher concentration of bacterial standard. Protocol B did not produce any amplicons visible on a gel, protocol C produced an amplicon for 5×10^5 bacterial cells and a larger amplicon for 10×10^6 bacterial cells (Figure 2.6).

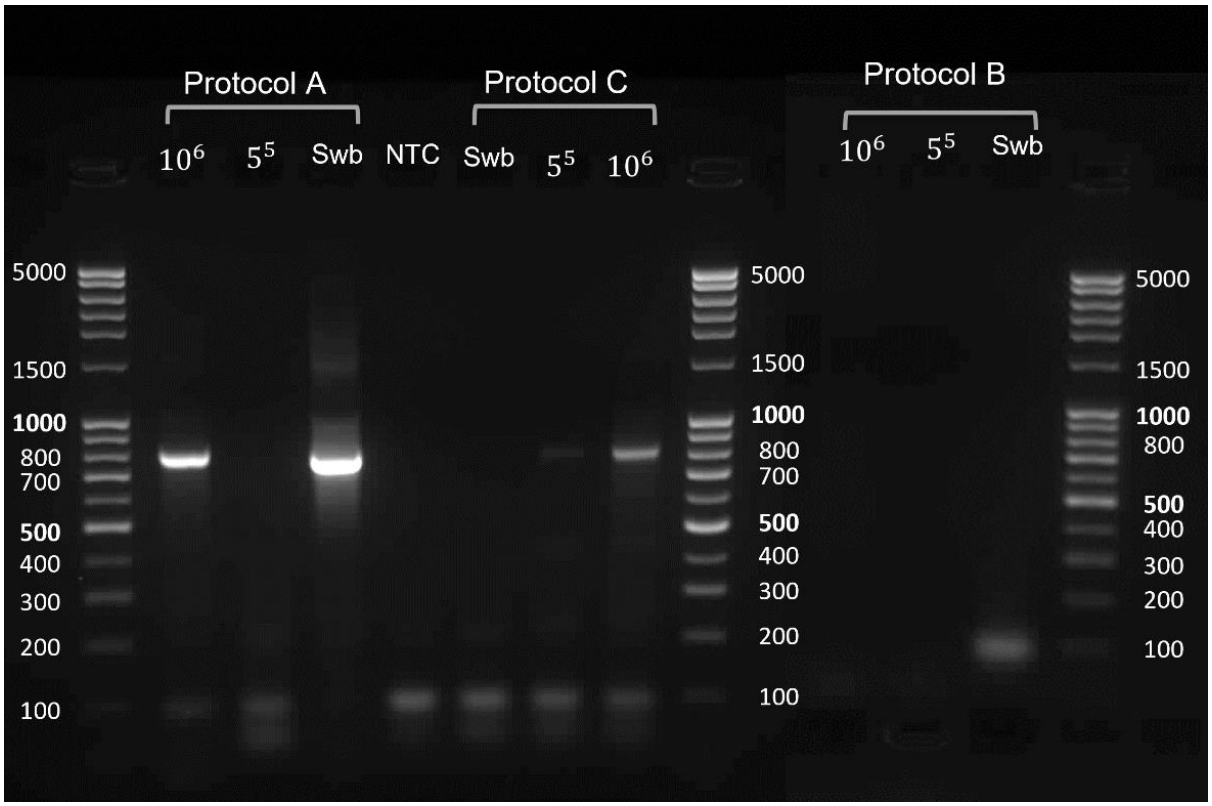


Figure 2.6 Electrophoresis gel image post PCR comparing 3 different DNA extraction methods. Protocol **A** = QIAmp DNA mini kit protocol detailed in (Stafford et al., 2017), **B =** QIAmp DNA mini kit protocol from manufacturer including appendix D from handbook. **C** = QIAamp DNA Microbiome Kit. swb= positive control from a CVF swab, 5^5 & 10^6 = number of bacterial cells from Vaginal Microbiome Whole Cell Mix (ATCC, MSA-2007). Assuming a swab holds 500µl of CVF which contains 10^6 cells/ml, 5^5 represents number of bacteria on an average CVF swab.

2.4.2 Optimisation of universal primers

In order to assess each universal primer pair's ability to amplify key vaginal organisms we performed *in silico* and laboratory analysis on primers identified from the literature. We identified 30 primers including 2 novel universal reverse primers, optimised for vaginal species, created using Bioedit (MCRevA & MCRevB) (Table 2.2).

Table 2.2 Universal primers identified from previous vaginal microbiome sequencing studies. Melt temperature, homo-dimer analysis and hairpin formation were determined using IDT online OligoAnalyzer® Tool.

Name	Sequence	Length (bp)	MeltTemp (1.5mM Mg)	Gc Content (%)	Homo-Dimer Analysis (bp)	Hairpin Tm (°C)
28F	GAGTTTGATCNTGGCTCAG	19	58.7	50	6	30.2
515F	GTGCCAGCMGCCGCGGTAA	19	70.1	71.1	6	15-26
338F	ACTCCTACGGGAGGCAGCA	19	66.5	63.2	4	55
8F	AGAGTTTGATCCTGGCTCAG	20	61.1	50	4	35
27Fm	AGAGTTTGATCMTGGCTCAG	20	60.3	47.5	6	35
27F	AGAGTTTGATYMTGGCTCAG	20	59.3	45	6	35.4
Fwd-P1	GTTYGATYMTGGCTYAG	17	54.3	47.1	6	-17
Fwd-P2	RTTTGATCYTGGTTCAG	17	52.9	41.2	4	41974
357F	CCTACGGGNGGCWGCAG	17	64.8	73.5	6	16
319F	CTCCTACGGGAGGCAGCAGT	20	66.8	65	4	50
U968F	AACGCGAAGAACCTTAC	17	56.7	47.1	4	21
388R	TGCTGCCTCCCGTAGGAGT	19	66.5	63.2	4	52 & 63
806R	GGACTACHVHHHTWTCTAAT	20	54	35	10	-2
906R	CCGTCAATTCCTTTGAGTTT	20	58.6	40	4	41 & 36
1492Rm	TACGGYTACCTTGTTAYGACTT	22	61.2	40.9	3	26 & 7.5
1492R	CGGTTACCTTGTTACGACTT	20	59	45	3	21 & 23 &26
534R/ Rev1B	ATTACCGCGGCTGCTGG	17	64.3	64.7	6	34
519R	GTNTCACNGCGGCKKGCTG	19	67.1	68.4	6	19 & -8
785R	GACTACHVGGGTWTCTAAT	19	55.3	42.1	10	25 & 7.6
926R	CCGTCAATTCMTTTRAGT	18	54.9	38.9	4	-25
MCRevA	CTCACGACACGAGCTGACGAC	21	65.6	61.9	4	45
MCRevB	GTATCTAATCCTGTTYGCTMC	21	57.7	42.9	3	10 & -15
FWD S-D-Bact-0008-c-S-20 (+anchor sequence)	TTTCTGTTGGTGCTGATATTGCAG RGTTYGATYMTGGCTCAG	42	73.5	45.2	6	21-34
FWD S-D-Bact-0008-c-S-20 (no anchor sequence)	AGRGTTYGATYMTGGCTCAG	20	61.3	50	6	-
341F (+anchor sequence)	TTTCTGTTGGTGCTGATATTGCCC TACGGGNGGCWGCAG	39	76	55.1	5	34-45
341F (no anchor sequence)	CCTACGGGNGGCWGCAG	17	64.8	73.5	6	16
REV S-D-Bact-0008-c-S-20 (+anchor sequence)	ACTTGCCTGTCGCTCTATCTTCCG GYTACCTTGTTACGACTT	42	74.6	48.8	4	32 & 35 &40
REV S-D-Bact-0008-c-S-20 (no anchor sequence)	CGGYTACCTTGTTACGACTT	20	60.5	47.5	3	21-26
806R (+anchor sequence)	ACTTGCCTGTCGCTCTATCTTCGG ACTACHVGGGTWTCTAAT	42	73.7	47.6	10	31 & 32 &35
806R (no anchor sequence)	GGACTACHVGGGTWTCTAAT	20	57.9	45	10	32

2.4.2.1 *Small scale universal primer pair analysis*

In order to calculate the amplicon size for gel electrophoresis imaging we aligned each primer pair with sequences from the vaginal microbiome DNA standard (ATCC, MSA-1007) (Table 2.3).

Table 2.3 Analysis of universal primer pairs ability to amplify *Gardnerella vaginalis* (ATCC 14019), *Lactobacillus gasseri* (ATCC 33323), *Mycoplasma hominis* (ATCC 23114), *Prevotella bivia* (ATCC 29303), *Streptococcus agalactiae* (ATCC BAA-611), *Lactobacillus jensenii* (ATCC 25258). Genomes downloaded from https://genomes.atcc.org/genomes . Green number indicates amplicon size, orange fwd or rev indicates only the forward or reverse primer were present in the sequence, red indicates both the forward and reverse primer were not present in the sequence.

Name	Tm difference	GC difference	Hetero Dimer (bp)	*Gardnerella vaginalis*	*Lactobacillus gasseri*	*Mycoplasma hominis*	*Prevotella bivia*	*Streptococcus agalactiae*	*Lactobacillus jensenii*
28F/519R	-8.4	-18.4	4	-	fwd	fwd	fwd	fwd	fwd
515F /806R	16.1	36.1	6	292	292	291	292	291	292
319F/806R	12.8	30	6	451	468	467	463	468	468
338F/906R	7.9	23.2	4	fwd	590	fwd	580	590	590
8F/1492Rm	-0.1	9.1	3	rev	1537	fwd	1493	1513	1527
27Fm/1492Rm	-0.9	6.6	4	rev	1537	fwd	1493	1513	1530
27F/1492R	0.3	0	4	-	fwd	fwd	fwd	fwd	fwd
U968F/1492Rm	-4.5	6.2	4	549	547	-	rev	546	544
27Fm/Rev1B	-4	-17.2	3	rev	556	520	527	534	549
Fwd-P1/Rev1B	-10	-17.6	2	505	553	517	524	531	546
27Fm/519R	-6.8	-20.9	4	-	fwd	fwd	fwd	fwd	fwd
357F/785R	9.5	31.4	5	448	465	464	460	465	465
319F/926R	11.9	26.1	4	fwd	589	578	579	589	589
357F/926R	9.9	34.6	4	fwd	578	576	577	587	587
Fwd-P1+Fwd-P2 /Rev1B	56.7	47.1	2	505	553	517	524	531	546
515F/Rev1B	5.8	6.4	16	NA	NA	NA	NA	NA	NA
338F/Rev1B	2.2	-1.5	5	180	197	197	192	198	197
357F/Rev1B	0.5	8.8	6	177	194	194	189	195	194
319F/Rev1B	2.5	0.3	6	179	196	196	191	197	196
515F/MCRevA	4.5	9.2	4	568	568	553	fwd	567	568
338F/MCRevA	0.9	1.3	4	728	745	730	fwd	745	745
357F/MCRevA	-0.8	11.6	4	725	742	727	fwd	742	742
319F/MCRevA	1.2	3.1	4	727	744	729	fwd	744	744
515F/MCRevB	12.4	28.2	4	281	281	280	fwd	281	281
338F/MCRevB	8.8	20.3	4	441	458	457	fwd	458	458
357F/MCRevB	7.1	30.6	4	438	455	454	fwd	455	455
319F/MCRevB	9.1	22.1	4	440	457	456	fwd	457	457
338F/806R	12.5	28.2	6	452	469	468	464	469	469
Fwd-P1/806R	0.3	12.1	7	777	825	788	796	802	818
Fwd-P1/785R	-1	5	5	776	824	787	795	801	817

Out of a total of 30 primer pairs, 10 pairs were expected to amplify all 6 species in the standard. However, 3 of these pairs were expected to produce amplicons <300 bp long and so would not give species level resolution after sequencing. From the remaining 7, 5 pairs had a Tm difference >5°C which could result in non-specific binding of the primers. After analysis 2 pairs appeared to fill all the criteria, Fwd-p1/806R and Fwd-p1/785R however, these pairs repeatedly failed to produce an amplicon when tested experimentally (Table 2.7). Primers known to produce an amplicon were used as a positive control during PCR and gel electrophoresis but are not shown in the figure.

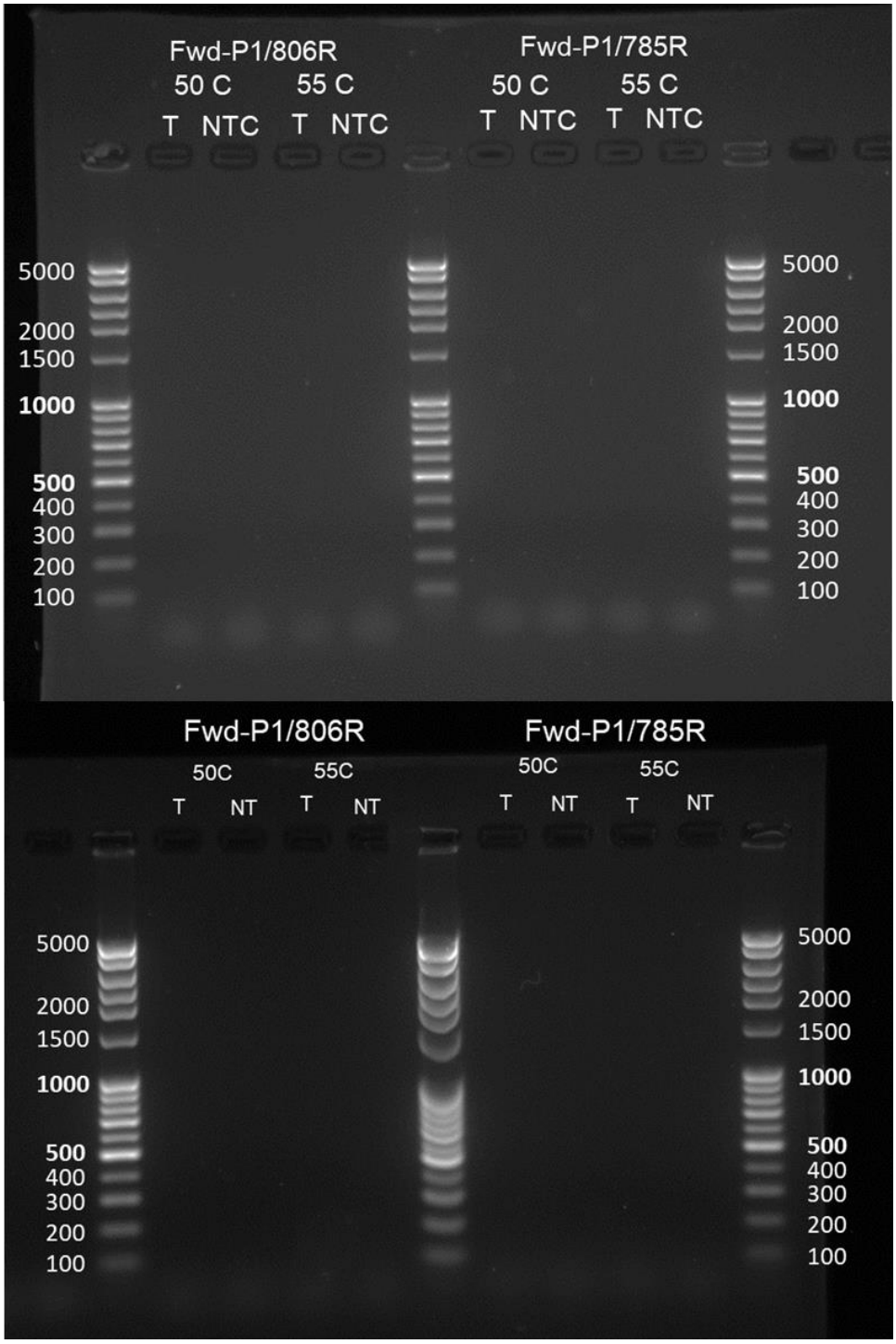


Figure 2.7 Gel electrophoresis image of the PCR products from Fwd-p1/806R and Fwd-p1/785R using vaginal microbiome DNA standard (ATCC, MSA-1007) as a template. T = template present, NTC = No Template Control. 50 °C & 55 °C indicates the annealing temperature during PCR. Primers known to produce an amplicon were used as a positive control during PCR and gel electrophoresis but are not shown in the figure. Amplicons run on the gels in figure A and B were produced in separate PCR reactions on different occasions.

Oxford Nanopore provide 27F/1492R primers for use with their kits. From the small-scale analysis, we would not expect this pair to produce any amplicons from the vaginal microbiome standard. However, other studies (Hyman et al., 2014; Romero, Hassan, et al., 2014) have used slightly modified versions of these primers which we termed 27Fm/1492Rm (details available in Table 2.2). Based off the small-scale analysis we would expect 27Fm/1492Rm to amplify more species compared to 27F/1492R (4/6 vs 0/6 species) (Table 2.3).

All 30 primer pairs were experimentally tested at a range of annealing temperatures and 4 pairs were found to consistently produce an amplicon. These were 319F/MCRevA, 319F/MCRevB, 27Fm/1492Rm and 27F/1492R (Figure 2.8).

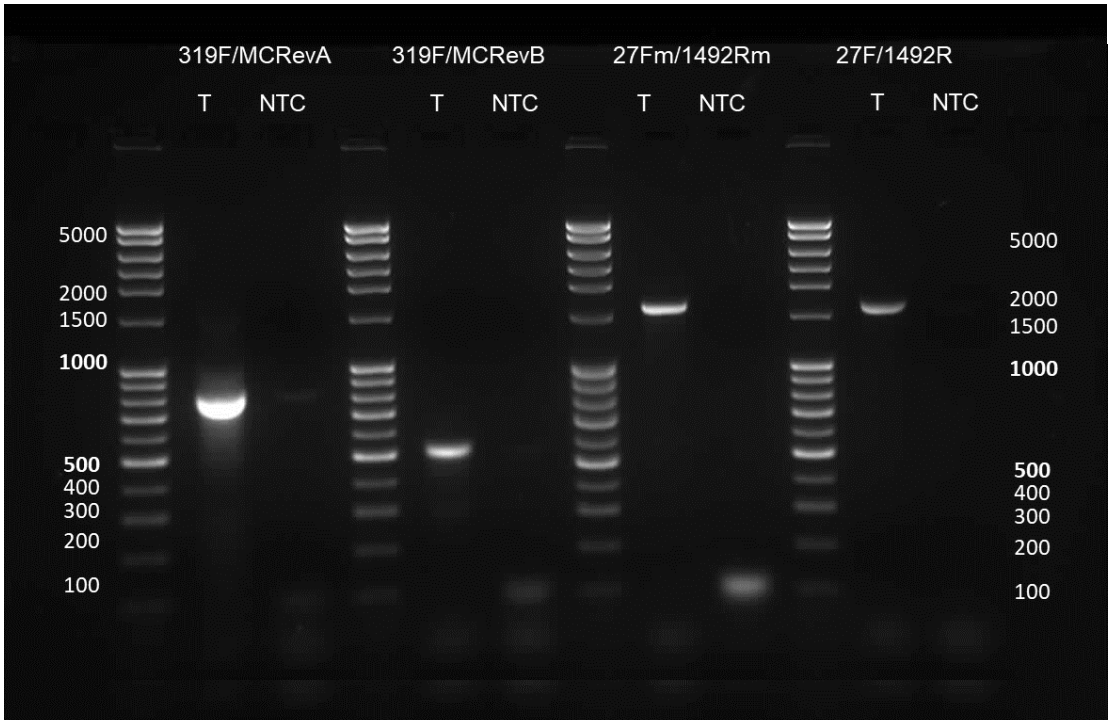


Figure 2.8 Gel electrophoresis image showing amplicons from 4 universal bacterial primer pairs. T= template, NTC= no template control. Template was vaginal microbiome DNA standard (ATCC, MSA-1007).

The amplicon producing primer pairs subsequently underwent clean-up which successfully removed short DNA fragments which can interfere in downstream measurements and sequencing (Figure 2.9). Concentration of DNA after clean-up can be found in Table 2.4.

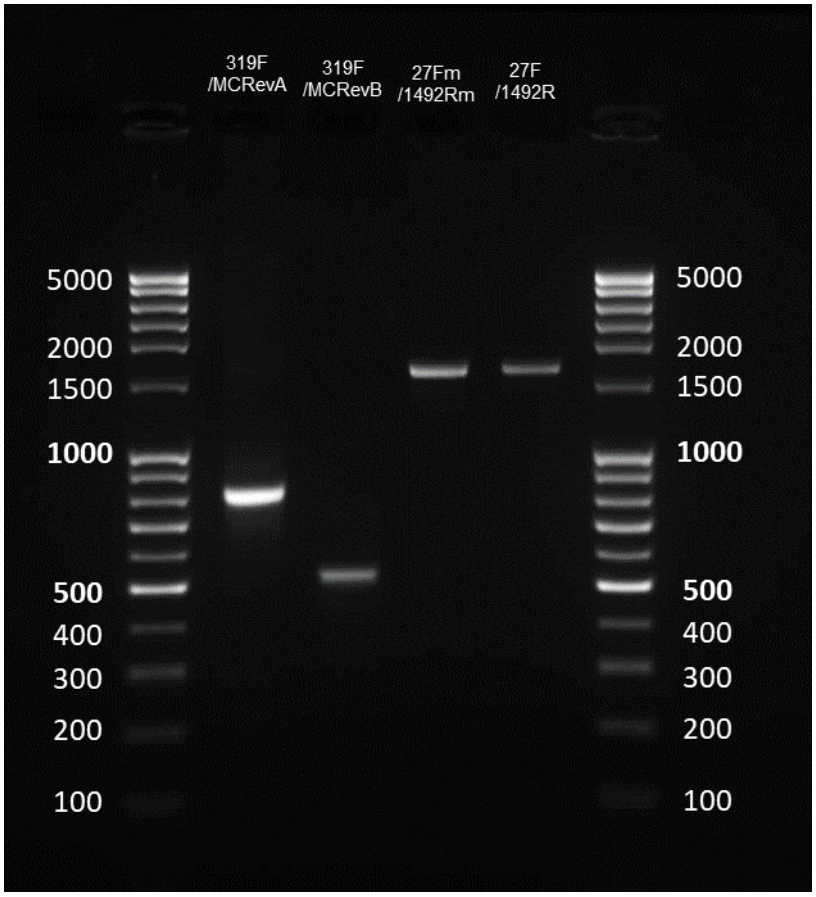


Figure 2.9 Post clean up gel electrophoresis image of amplicons from Figure 2.8.

Table 2.4 Concentrations of the most promising primers from optimisation, after PCR & clean-up, prior to sequencing.

Barcode	Primer names	DNA Concentration after PCR & clean-up (ng/ul)	Amplicon size
BC03	319F/MCRevA	21.4	730
BC04	319F/MCRevB	12.2	460
BC06	27m/1492Rm	31.5	1600
BC07	27F/1492R	14.7	1600

2.4.2.2 *Large scale Universal primer pair analyses*

Using the online tool TestPrime we assessed the 4 primers that performed best in laboratory experiments. We identified which organisms each pair should amplify from the SILVA non redundant reference database which includes 510,508 sequences (https://www.arb-silva.de/projects/ssu-ref-nr/) (Table 2.5)

Table 2.5 Assessment of universal primer pairs for amplification of key vaginal microorganisms. Analysis was performed using TestPrime online tool that utilises 510,508 sequences from the SILVA non redundant reference database.

Family	Genus	% amplification of species within genus			
		319F/MCRevA	319F/MCRevB	27Fm/1492Rm	27F/1492R
Actinomycetaceae	Mobiluncus	100%	100%	100%	0%
Atopobiaceae	Atopobium	88%	84%	29%	14%
Bifidobacteriaceae	Gardnerella	93%	87%	29%	29%
Corynebacteriaceae	Corynebacterium	82%	81%	76%	3%
Enterobacteriaceae	Escherichia-Shigella	2%	81%	86%	86%
Lactobacillaceae	Lactobacillus	85%	83%	56%	4%
Mycoplasmataceae	Mycoplasma	75%	55%	6%	6%
Mycoplasmataceae	Ureaplasma	94%	0%	0%	0%
Pseudomonadaceae	Pseudomonas	82%	81%	59%	7%
Staphylococcaceae	Staphylococcus	78%	1%	86%	2%
Streptococcaceae	Streptococcus	84%	82%	79%	1%
Veillonellaceae	Megasphaera	82%	3%	60%	10%
Veillonellaceae	Dialister	80%	76%	32%	4%

2.4.2.3 *Sequencing results from primer optimisation*

As the final step in laboratory assessment, we sequenced 4 barcoded amplicons (Figure 2.10) to assess how well each are able to amplify the 6 key vaginal bacteria species included in the DNA standard (*Gardnerella vaginalis, Lactobacillus gasseri, Mycoplasma hominis, Prevotella bivia, Streptococcus agalactiae, Lactobacillus jensenii*) compared to the small-scale *in silico* assessment we performed (Table 2.3). Table 2.6 is a condensed version of Table 2.3 and contains results from the 4 most promising primer pairs only. This correlates with the result from the Flongle sequencing (Figure 2.11) which shows a peak in reads at ~500, ~750 and ~1500 bp.

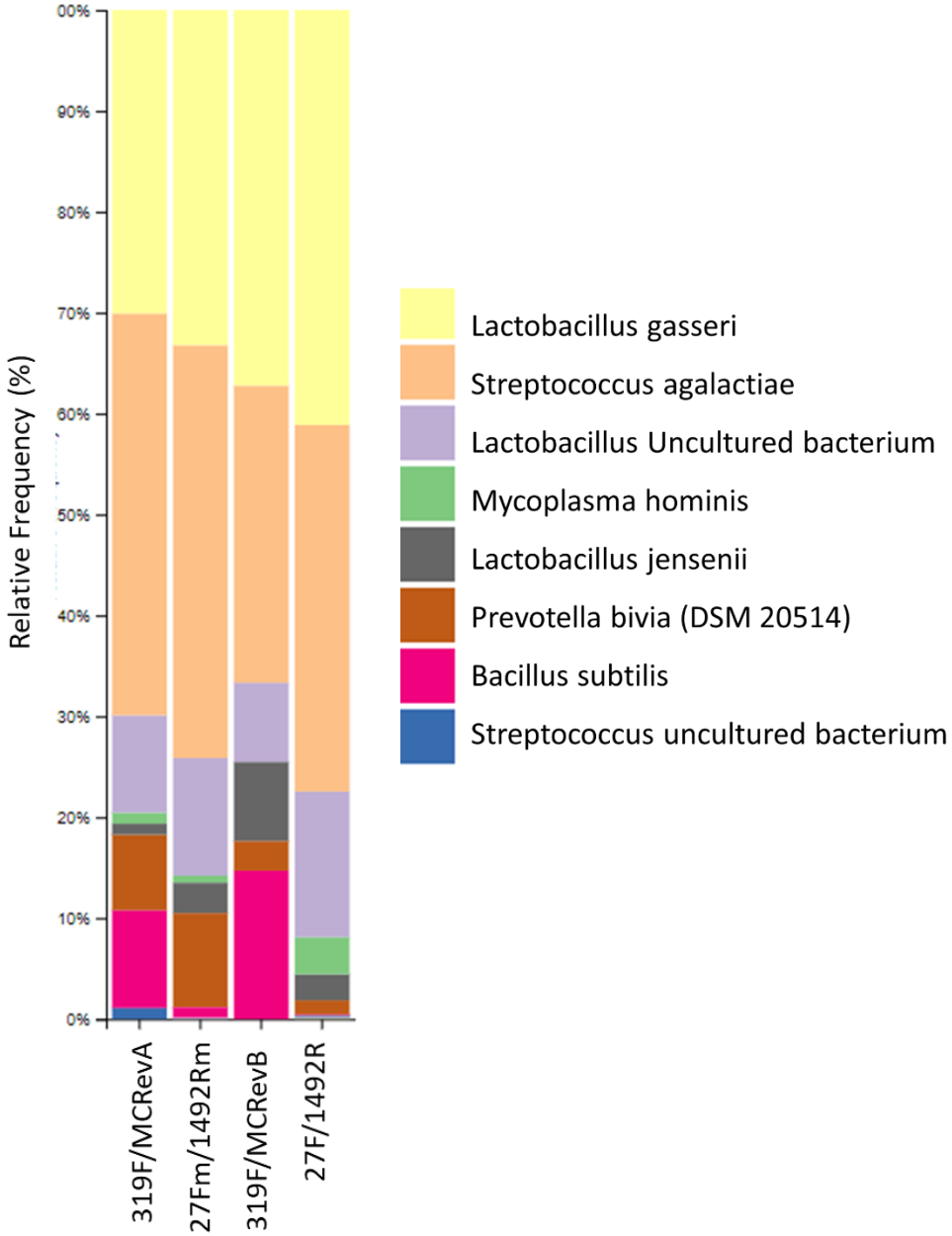


Figure 2.10 Sequencing results from Nanoprore Flongle to assess the performance of 4 primer pairs for use in vaginal microbiome sequencing. PCR template consisted of *Gardnerella vaginalis, Lactobacillus gasseri, Mycoplasma hominis, Prevotella bivia, Streptococcus agalactiae, Lactobacillus jensenii* from ATCC standard. Qiime2 analysis performed by Dr Neha Kulkarni.

Table 2.6 Expected amplicon lengths from small-scale Analysis of universal primer pair's ability to amplify Gardnerella vaginalis (ATCC 14019), Lactobacillus gasseri (ATCC 33323), Mycoplasma hominis (ATCC 23114), Prevotella bivia (ATCC 29303), Streptococcus agalactiae (ATCC BAA-611), Lactobacillus jensenii (ATCC 25258). Genomes downloaded from https://genomes.atcc.org/genomes . Green number indicates amplicon size, orange fwd or rev indicates only the forward or reverse primer were present in the sequence, red indicates both the forward and reverse primer were not present in the sequence.

Name	Tm difference	GC difference	Hetero Dimer (bp)	*Gardnerella vaginalis*	*Lactobacillus gasseri*	*Mycoplasma hominis*	*Prevotella bivia*	*Streptococcus agalactiae*	*Lactobacillus jensenii*
27Fm/1492Rm	-0.9	6.6	4	rev	1537	fwd	1493	1513	1530
27F/1492R	0.3	0	4	-	fwd	fwd	fwd	fwd	fwd
319F/MCRevA	1.2	3.1	4	727	744	729	fwd	744	744
319F/MCRevB	9.1	22.1	4	440	457	456	fwd	457	457

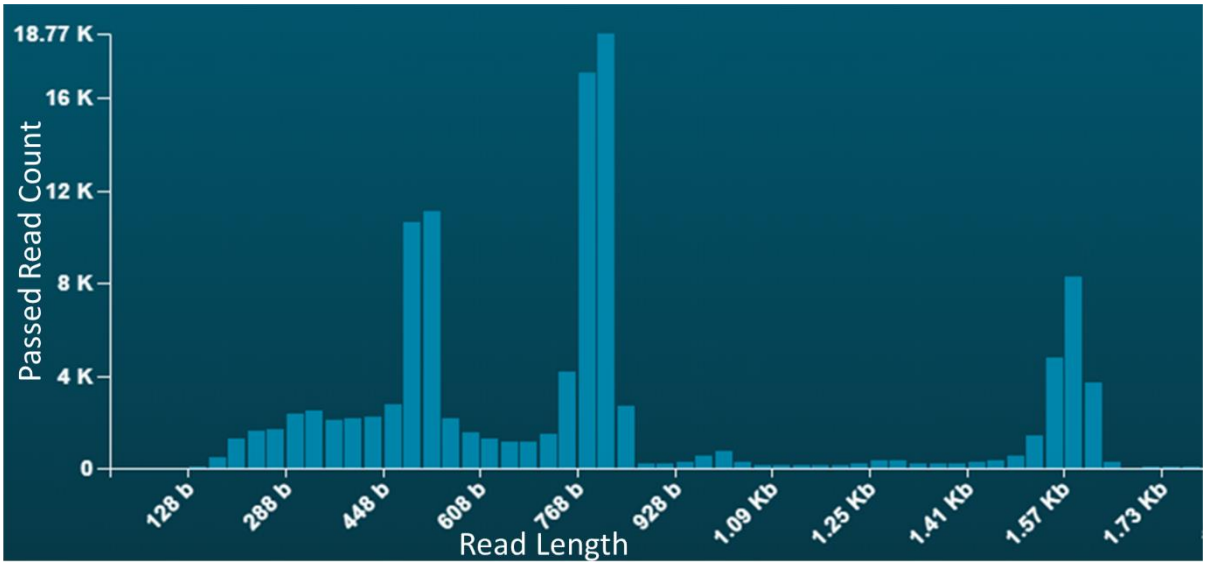


Figure 2.11 Passed read count and read length of DNA from Flongle nanopore sequencing of amplicons from 319F/MCRevA, 319F/MCRevB, 27m/1492Rm and 27F/1492R using vaginal microbiome DNA standard (ATCC, MSA-1007) as a PCR template.

2.4.3 Sample Processing & Library Preparation

Based on results from the previous section (Figure 2.10 & Table 2.5) the primer pair 319F/MCRevA was selected for use on the clinical samples. A total of 69 clinical samples underwent DNA extraction, PCR, clean-up, and quantification. 60 samples, 30 preterm and 30 term (Table 2.7) including a total of 34 patients (Table 2.8) were normalised during library preparation (Table 2.9) and were subsequently sequenced.

Table 2.7 Number of samples processed & sequenced at each gestational time point (GTP).

		Term	Preterm	Total
Samples	GTP1	15	13	28
	GTP2	14	14	28
	GTP3	1	3	4
	Total Samples	30	30	60
Patients	Patients with 1 GTPs	2	8	10
	Patients with 2 GTPs	14	8	22
	Patients with 3 GTPs	0	2	2
	Total Patients	16	18	34

Table 2.8 Patient demographic and clinical data

Age, BMI (body mass index), FFN (fetal fibronectin), GAP (gestation at presentation), GAD (gestation at delivery) are presented as Mean ±SD. * =*P*<0.05. PPROM (preterm prelabour rupture of membranes).

	GTP1		GTP2	
	Term (n=15)	Preterm (n=13)	Term (n=14)	Preterm (n=14)
Maternal factors				
Age (years)	31.28 ± 5.83	28.76 ± 6.09	31.65 ± 6.06	29.71 ± 5.54
BMI (Kg/m^2)	26.80 ± 5.84	26.18 ± 4.68	27.27 ± 6.28	26.94 ± 4.05
FFN (ng/mL)	4.0 ± 1.80	4.35 ± 0.93	3.65 ± 1.40	3.29 ± 1.64
GAP (days)	146.2 ± 7.86	140.3 ± 6.56	186.8 ± 6.69	182.9 ± 9.12
GAD (days)	273.2 ± 8.20	227.9 ± 28.28	273.2 ± 8.47	233.5 ± 21.49
Smoker n, (%)	2, (13.3)	2, (15.4)	2, (14.3)	2, (14.3)
PPROM	0	4	0	3
Ethnicity (n)				
White	11	11	11	12
Black	0	0	0	0
Asian	3	2	2	2
Hispanic	1	0	1	0
Treatment (n)				
Progesterone	1	1	3	2
Cerclage	0	0	0	0
Both	1	0	2	0

Table 2.9 Final DNA concentrations of clinical samples after clean-up

Low Concentration Batch. Normalised to 1.5 ng per sample						High Concentration Batch. Normalised to 52 ng per sample					
MIS Identifier	PRIME identifier	Barcode No	ng/ul	Volume	Total ng	MIS Identifier	PRIME identifier	Barcode No	ng/ul	Volume	Total ng
AHR 22.1		BC01	6.14	10	61.4	AHR 35.1		BC01	182	10	1820
AHR 22.2		BC02	13	10	130	AHR 44.2	SU0008 v2	BC02	32.8	10	328
AHR 23.1		BC03	22.4	10	224	AHR 35.2		BC03	422	10	4220
AHR 46.2	SU0003 v2	BC04	0.87	10	8.68	AHR 37.1		BC04	9.04	10	90.4
AHR 23.2		BC05	5.12	10	51.2	AHR 37.2		BC05	24.6	10	246
AHR 15.2		BC06	1.05	5	5.25	AHR 38.1		BC06	5.18	10	51.8
AHR 53.2	SU0031 v2	BC07	9.66	10	96.6	AHR 24.1		BC07	23.2	10	232
AHR 16.2		BC08	0.36	5	1.78	AHR 38.2		BC08	6.62	10	66.2
AHR 20.1		BC09	0.27	6	1.63	AHR 39.1		BC09	173	10	1730
AHR 20.2		BC10	0.6	5	3.01	AHR 53.3	SU0031 v3	BC10	24.8	10	248
AHR 24.2		BC11	22.2	10	222	AHR 55.1	SU0038 v1	BC11	30.6	10	306
AHR 32.2		BC12	216	10	2160		SU0039 v1	BC12	56.8	10	568
	SU87 V3	BC13	20.9	10	209	AHR 25.1		BC13	24.4	10	244
AHR 25.2		BC14	15.9	10	159	AHR 55.2	SU0038 v2	BC14	51.2	10	512
AHR 27.2		BC15	11.9	5	59.5	AHR 39.2		BC15	8.3	10	83
AHR 33.1		BC16	0.38	5	1.88	AHR 40.1		BC16	8.2	10	82
AHR 33.2		BC17	3.9	5	19.5	AHR 40.2		BC17	99.2	10	992
AHR 34.1		BC18	0.83	5	4.16		SU0055 v1	BC18	10.8	10	108
AHR 34.2		BC19	0.71	5	3.57	AHR 47.1	SU0009 v1	BC19	80.8	10	808
AHR 41.1		BC20	0.28	6	1.66	AHR 47.2	SU0009 v2	BC20	110	10	1100
AHR 41.2		BC21	4.8	5	24	AHR 48.1	SU0010 v1	BC21	43.4	10	434
AHR 44.1	SU0008 v1	BC22	2.12	5	10.6		SU0067 v1	BC22	76	10	760
AHR 46.1	SU0003 v1	BC23	2.2	5	11	AHR 31.1		BC23	19.5	10	195
AHR 31.2		BC24	39.4	10	394	AHR 65.1	SU0095 v1	BC24	111	10	1110
AHR 65.2	SU0095 v2	BC25	9.44	10	94.4	AHR 28.1		BC25	36.2	10	362
AHR 65.3	SU0095 v3	BC26	6.94	10	69.4	AHR 28.2		BC26	31.2	10	312
AHR 29.1		BC27	9.74	10	97.4		SU0097 v1	BC27	81.4	10	814
	SU0108 v1	BC28	10.1	10	101	AHR 29.2		BC28	562	10	5620
AHR 30.1		BC29	22.2	10	222		SU0108 v2	BC29	63.6	10	636
	SU0108 v3	BC30	16.92	10	169.2	AHR 30.2		BC30	318	10	3180

2.4.4 Nanopore Sequencing Run Summary

Samples were sequenced in 2 batches to maximise the number of reads from each sample. The median read length was 795.5 bp, additional details from the sequencing run can be found in Table 2.10

The median Phred score for both the low and high concentration batches was 10 however, as you can see in Figure 2.12 a&b the high concentration batch had a wider range of quality scores. We observed a visible difference in number of reads throughout the experiment (Figure 2.12 c&d), reflecting the different input concentrations of DNA in each batch. However, after excluding samples that did not meet the quality criteria, the difference in total number of reads was not significant between the low and high concentration batch (Figure 2.13a, p= 0.1462)

Table 2.10 Run summary from two batches of samples. High concentration batch normalised to 52ng/sample; Low concentration batch normalised to 1.5 ng/sample

Run Summary	High Concentration Batch	Low Concentration Batch
Reads Generated	2.89 M	42.86 K
Passed Bases	1.72 Gb	21.77 Mb
Failed Bases	1.09 Gb	17.76 Mb
Estimated Bases	3.3 Gb	35.47 Mb

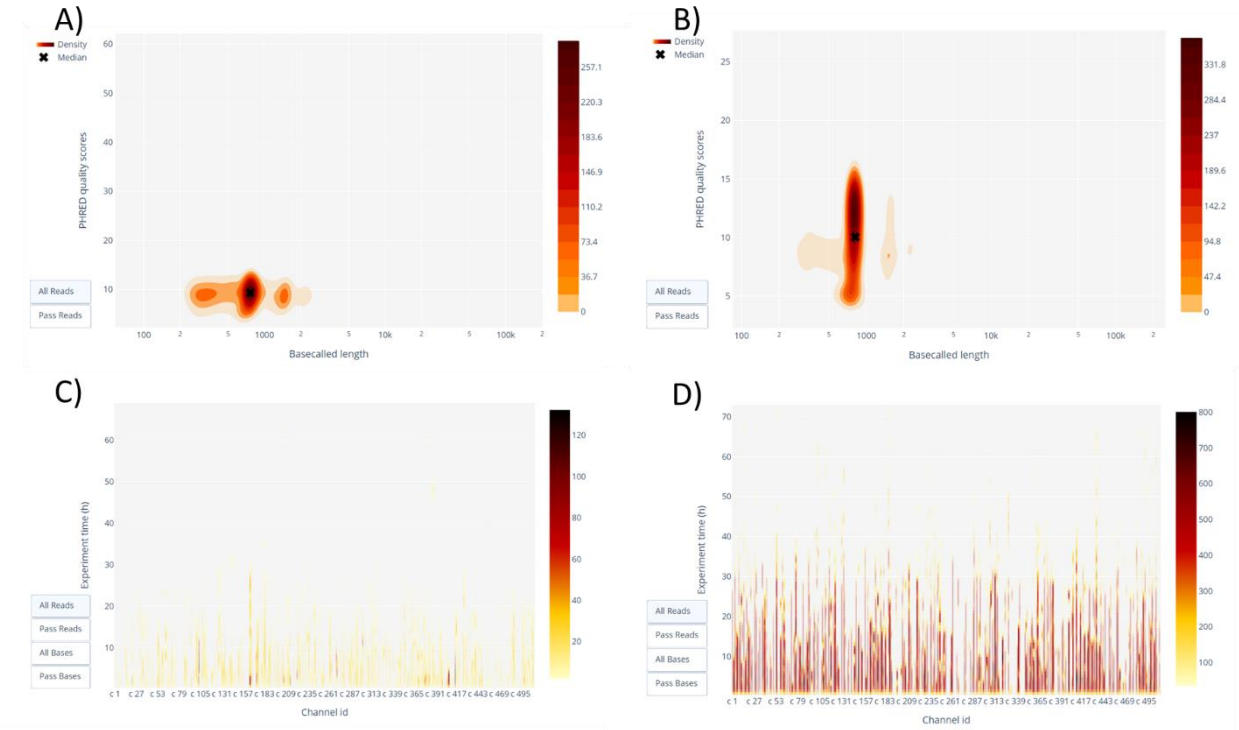


Figure 2.12 Quality score & read number results of Nanopore sequencing. Phred score for **A)** low concentration batch (n=30, samples normalised to 1.5 ng/µl) and **B)** high concentration batch (n=30, samples normalised to 52 ng/µl). Read number throughout experiment for **C)** Low concentration batch (n=30, samples normalised to 1.5 ng/µl) and **D)** High concentration Batch (n=30, samples normalised to 52 ng/µl). Quality assessment performed by Dr Neha Kulkarni

Number of reads in the preterm group was found to be significantly higher than the term group (Figure 2.13b, *p*= 0.0052). As a result of this, analysis was not performed on number of reads per bacteria or bacterial abundance as any significantly higher abundances in the preterm group would likely be due to this. Instead, relative abundance was analysed as this represents the proportion of each bacteria within a sample.

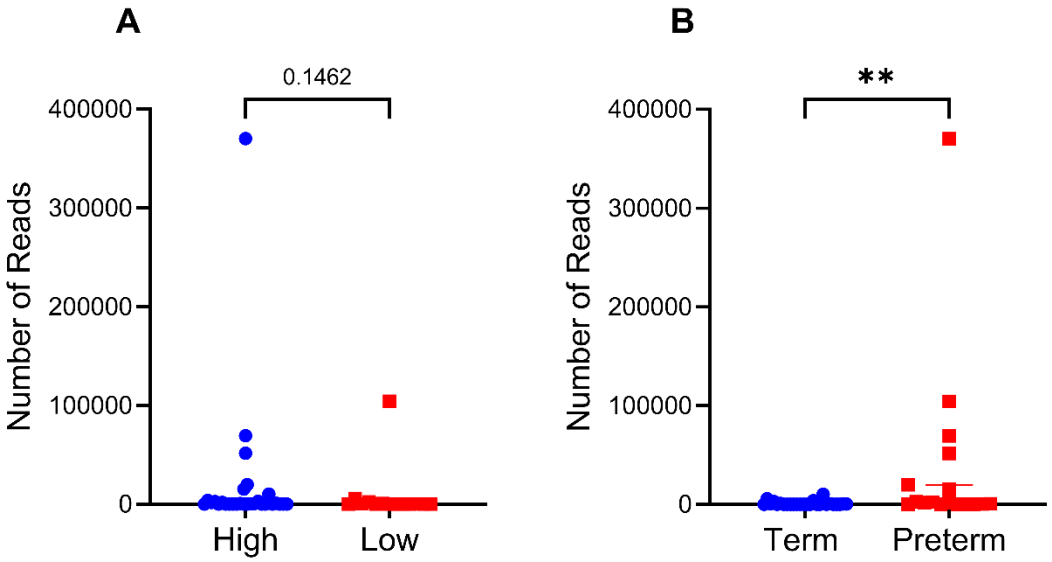


Figure 2.13. Number of sequencing reads from Oxford Nanopore Miniion in **A)** High concentration batch of samples (n=29, samples normalised to 52 ng/µl) vs low concentration batch (n=14, samples normalised to 1.5 ng/µl) *p*= 0.1462 **B)** Term (n=26) vs preterm (n=17) Term >37w gestation Preterm ⩽ 36+6 w gestation *p*= 0.0052. Analysis performed on GraphPad Prism.

2.4.5 Bacterial Community analysis

A total of 43 CVF samples were included in the analysis (GTP1 = 21, GTP2 = 19; GTP3 = 3, term = 26, preterm = 17 including 3 PPROM). Due to insufficient quality and read numbers, 16 samples from the low batch and 1 sample from the high batch were excluded from the analysis.

A total of 1212 sequences with unique Taxonomy ID's from the SILVA database matched to the clinical samples (see supplementary materials for complete list). The majority of sequences at 68% (821/ 1212) were named "uncultured" or "unidentified" at the species level. A total of 391 sequences were identified to a species level resolution. Within the identified species 53% (217/391) were present only once. Leaving a remaining 174 species present in more than one sample.

2.4.5.1 Prevalence

Prevalence of each bacterium was determined by assessing the presence or absence within each sample (Table 2.11). The most prevalent bacterium from all samples was *Streptococcus pneumoniae* (20/43, 45.5%), followed by *Lactococcus lactis subsp. Cremoris* and *Lactobacillus iners AB-1* both at (19/43, 44%). *Lactobacillus iners AB-1* was the most prevalent bacterium observed in the term samples (12/26, 27.3%). *Rhizobium rhizogenes* was the most prevalent in preterm samples (11/17, 65%) followed by *Streptococcus pneumoniae* (10/17, 59%) and *Gardnerella vaginalis* (9/17, 53%). Looking only at patients with PPROM (n=3), 19 bacteria were present at equal prevalence in the samples (see supplementary material).

Table 2.11. Most prevalent bacteria identified from cervicovaginal samples
All (n=43) Term (n=26) Preterm (n=17) PPROM (n=3). Term >37w gestation Preterm ≤36+6 w gestation, PPROM = Preterm Prelabour Rupture of fetal Membranes

Taxon	Taxonomy ID	Prevelance in ALL samples (%)	Prevelance in TERM samples (%)	Prevelance in PRETERM samples (%)	Prevelance in PPROM samples (%)
Atopobium vaginae	LFWE01000015.212.1732	25.6	11.5	47.1	33.3
Chlamydia trachomatis	CSTQ01000412.1.1397	32.6	26.9	41.2	33.3
Coriobacteriales bacterium DNF00809	LSCX01000005.274.1770	11.6	0.0	29.4	33.3
Gardnerella vaginalis 1400E	ADER01000013.440.1977	30.2	15.4	52.9	33.3
Idiomarina sp. P7-5-3	KF934477.1.1406	23.3	26.9	17.6	0.0
Lactobacillus acidophilus	KU324919.1.1228	27.9	19.2	41.2	0.0
Lactobacillus crispatus	DQ316397.1.1493	34.9	26.9	47.1	33.3
Lactobacillus helveticus	HM057881.1.1378	25.6	15.4	41.2	33.3
Lactobacillus iners AB-1	ADHG01000001.652476.654027	44.2	46.2	41.2	0.0
Lactobacillus jensenii	AF243161.1.1512	41.9	38.5	47.1	33.3
Lactobacillus plantarum	HG328252.1.1392	18.6	11.5	29.4	33.3
Lactobacillus sp. KC38	AF243160.1.1509	34.9	30.8	41.2	0.0
Lactococcus lactis subsp. cremoris	JQIC01000014.502742.504105	44.2	42.3	47.1	0.0
Rhizobium rhizogenes	CP019701.2614607.2616025	39.5	23.1	64.7	33.3
Streptococcus pneumoniae	CKQU01000078.7468.8814	46.5	38.5	58.8	0.0

2.4.5.2 *Species Richness*

Species richness (number of observed species) was significantly higher in the preterm group compared to term. Median number of species in the term group =23, in the preterm group =105.5 (p= 0.0134). PPROM patients had a median number of species of 5 (Figure 2.14.a) however, as there was only 3 samples this may be misleading (number of species per sample = 5, 44, 2). Species richness was not significantly different between gestational time points (Figure 2.14.b).

Species richness in the term group was similar for GTP1 and GTP2 (Figure 2.15.a). However, in the preterm group, samples from GTP1 had a larger range and high mean of species richness compared to GTP2 although this was not significant (Figure 2.15.b). Looking only at GTP1, the preterm samples again had a non-significant larger range and higher mean compared to term samples (Figure 2.15c). Whereas at GTP2, the mean species richness is comparable for term and preterm although the term samples have a larger range (Figure 2.15.d)

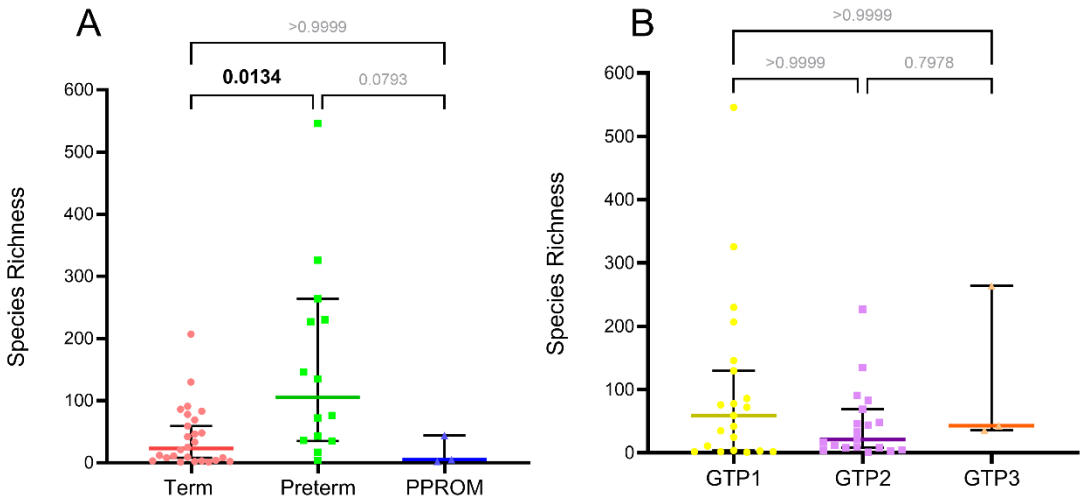


Figure 2.14. Species Richness (number of observed species) of cervico-vaginal samples collected from asymptomatic high risk pregnant women at 3 gestational time points (GTP) GTP1 = 20-22w, GTP2= 26-28w, GTP3= 30-32w. Term >37w gestation Preterm ≤36+6 w gestation, PPROM = Preterm Prelabour Rupture of fetal Membranes. Inner coloured line indicates median, black error bars indicate 95% confidence intervals. Kruskal Wallis test was performed on **A)** term (n=26) vs preterm (n=14) vs PPROM (n=3) **B)** GTP1 (n=21), GTP2 (n=19), GTP3 (n=3). Analysis performed on GraphPad Prism.

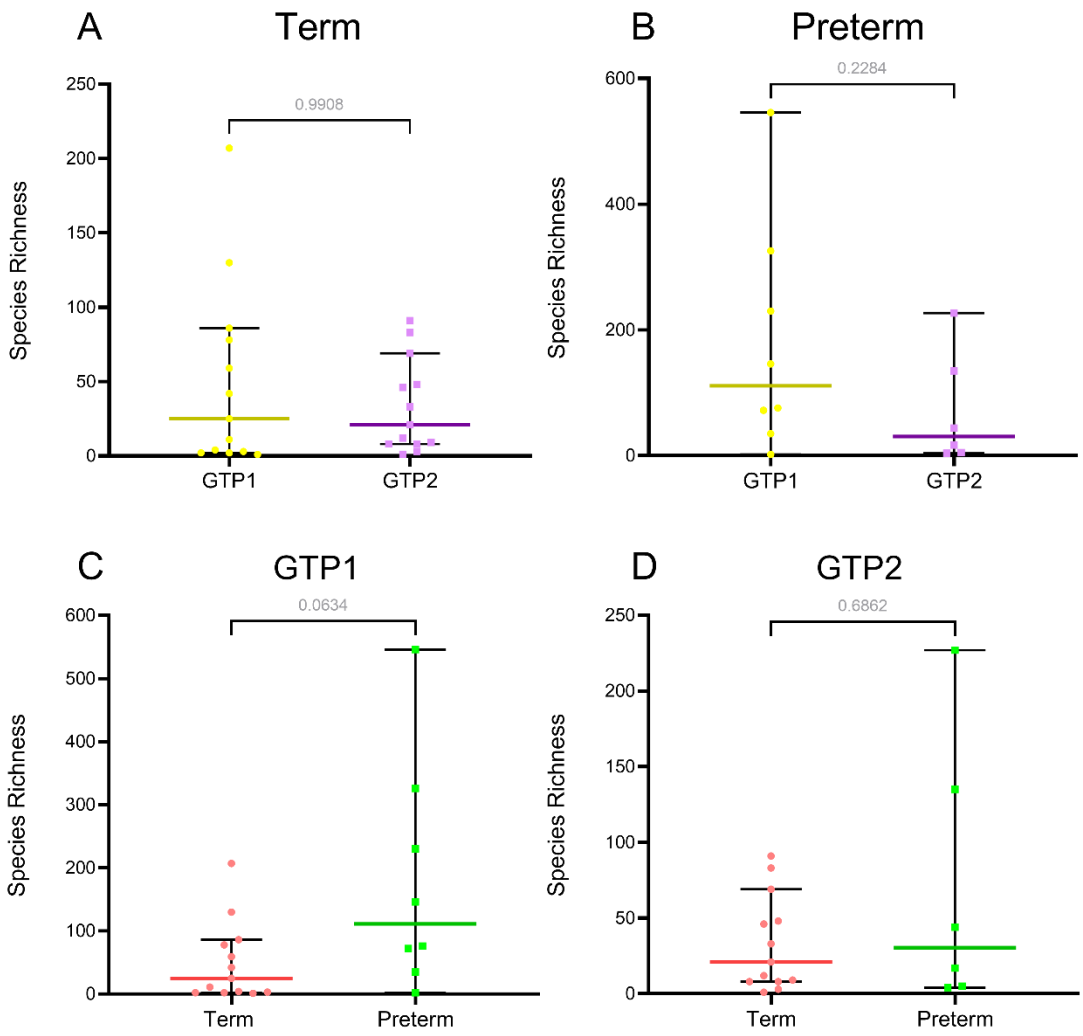


Figure 2.15 Species Richness (number of observed species) of cervico-vaginal samples collected at from asymptomatic high risk pregnant women at 2 gestational timepoints (GTP) GTP1 = 20-22w, GTP2= 26-28w. Term >37w gestation Preterm ⩽36+6 w gestation. Inner coloured line indicates median, black error bars indicate 95% confidence intervals. Mann-Whitney test was performed on **A)** GTP1 (n=13) vs GTP2 (n=13) at term **B)** GTP1 (n=8) vs GTP2 (n=6) at preterm **C)** term (n=13) vs preterm (n=8) at GTP1 **D)** term (n=13) vs preterm (n=6) at GTP2. Analysis for GTP3 was not performed due to small sample size (n=3). Analysis performed using GraphPad Prism.

2.4.5.3 Shannon Diversity Index

SDI was used to assess the diversity of each sample including richness (number of species) and evenness (relative abundance) of bacterial species. No results were found to be significant. Term (median= 2, n=26) and preterm (median= 2, n=14) patients were observed to have the same median diversity while PPROM patients were observed to have a lower diversity although had a small sample size (median= 1.6, n=3) compared to the other groups (Figure 2.16.a). All 3 gestational time points were found to have a similar diversity (median: GTP1= 1.9, n=21, GTP2=2, n= 19, GTP3 =1.6, n=3) (Figure 2.16.b).

When samples were further divided, diversity was found to increase in the term samples (median: GTP1= 1.4, n=13, GTP2=2.1, n=13) (Figure 2.17a) but decrease in the preterm samples (median: GTP1= 2.1, n= 8, GTP2= 1.6, n= 6) (Figure 2.17b). This is reflected when looking at gestational time points, at GTP1 preterm was found to have a higher mean diversity compared to term (GTP1 median: term= 1.4, n=13, preterm= 2.1, n= 8) (Figure 2.17c). But, at GTP2 the term samples had a higher mean diversity compared to preterm (GTP2 median: term= 2.1, n= 13, preterm= 1.6, n= 6) (Figure 2.17d).

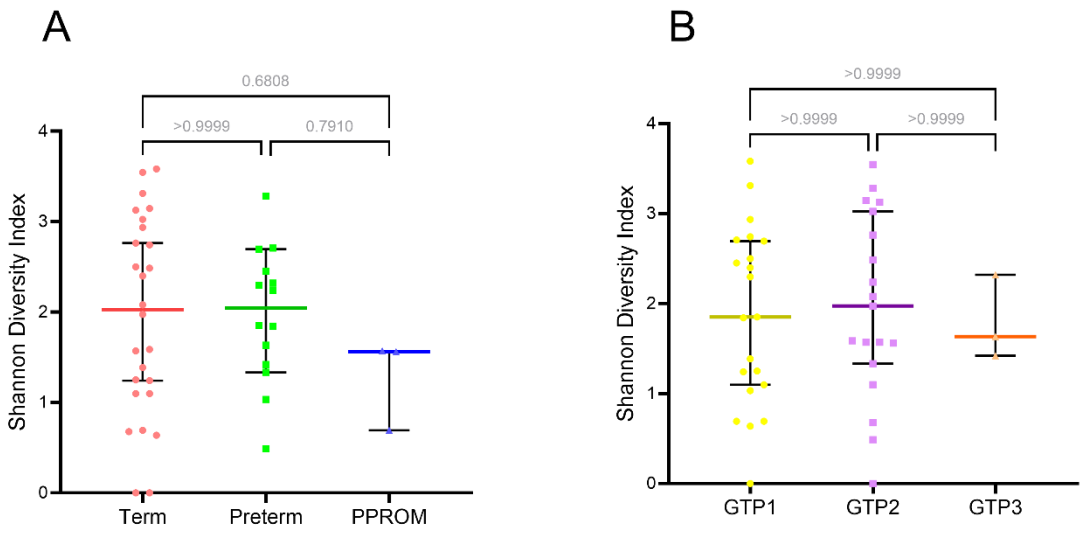


Figure 2.16. Shannon Diversity Index of cervico-vaginal samples collected at from asymptomatic high risk pregnant women at 3 gestational timepoints (GTP) GTP1 = 20-22w, GTP2= 26-28w, GTP3= 30-32w. Term >37w gestation Preterm ≤36+6 w gestation, PPROM = Preterm Prelabour Rupture of fetal Membranes. Inner coloured line indicates median, black error bars indicate 95% confidence intervals. Kruskal Wallis test was performed on **A)** term (n=26) vs preterm (n=14) vs PPROM (n=3) **B)** GTP1 (n=21), GTP2 (n=19), GTP3 (n=3). Analysis was performed with GraphPad Prism.

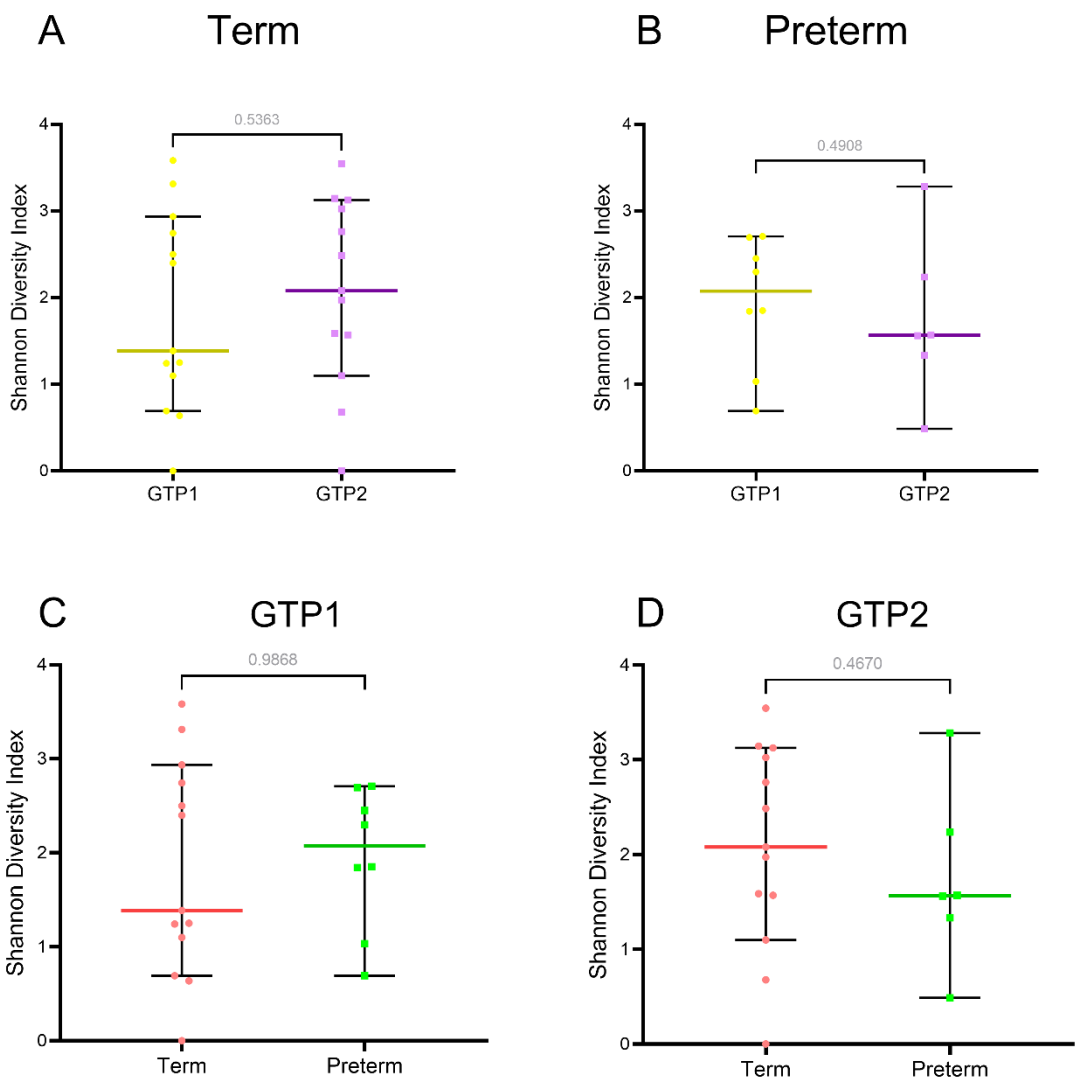


Figure 2.17 Shannon Diversity Index of cervico-vaginal samples collected at from asymptomatic high risk pregnant women at 2 gestational timepoints (GTP) GTP1 = 20-22w, GTP2= 26-28w. Term >37w gestation Preterm ⩽ 36+6 w gestation. Inner coloured line indicates median, black error bars indicate 95% confidence intervals. Mann-Whitney test was performed on **A)** GTP1 (n=13) vs GTP2 (n=13) at term **B)** GTP1 (n=8) vs GTP2 (n=6) at preterm **C)** term (n=13) vs preterm (n=8) at GTP1 **D)** term (n=13) vs preterm (n=6) at GTP2. Analysis for GTP3 was not performed due to small sample size (n=3). Analysis was performed with GraphPad Prism.

2.4.5.4 *Abundance*

The most abundant genus was *Lactobacillus* followed by *Lactococcus and Gardnerella* (Figure 2.18). The most abundant species were *Lactobacillus* uncultured*, Lactococcus lactis, Lactobacillus crispatus* and *Lactobacillus iners* AB-1 (Figure 2.19).

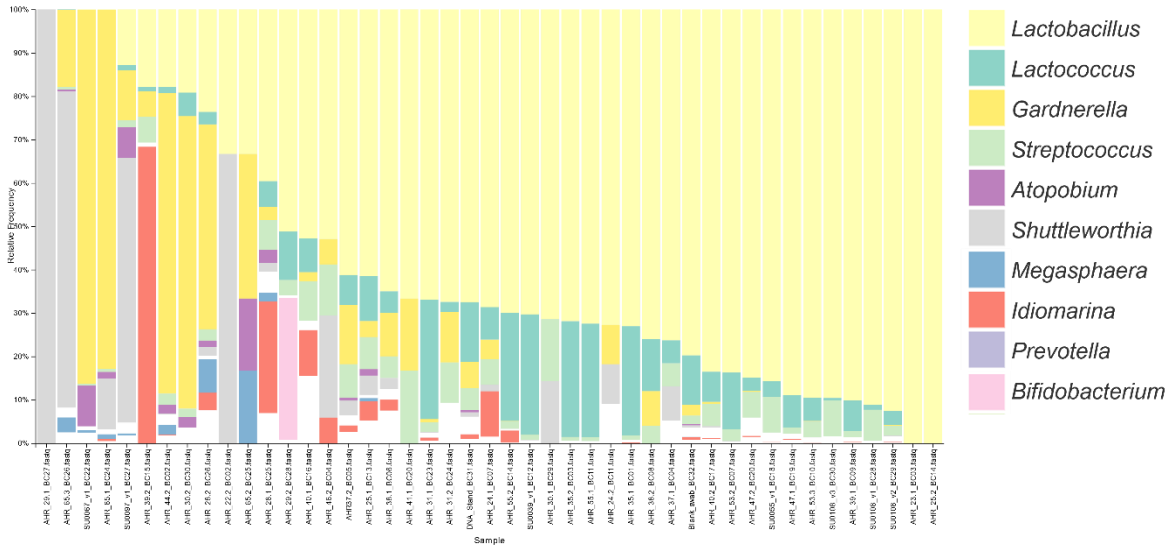


Figure 2.18. Genus level identification of bacteria from vaginal swabs of pregnant women (n=43) either >37w gestation (term) or ≤36+6w gestation (preterm). White areas of the graph contain organisms with <2.5% abundance and are not shown for clarity. A full version of this figure can be found in supplementary materials and contains 206 Genera. Qiime2 analysis performed by Neha Kulkarni (PDRA in bioinformatics, TUOS).

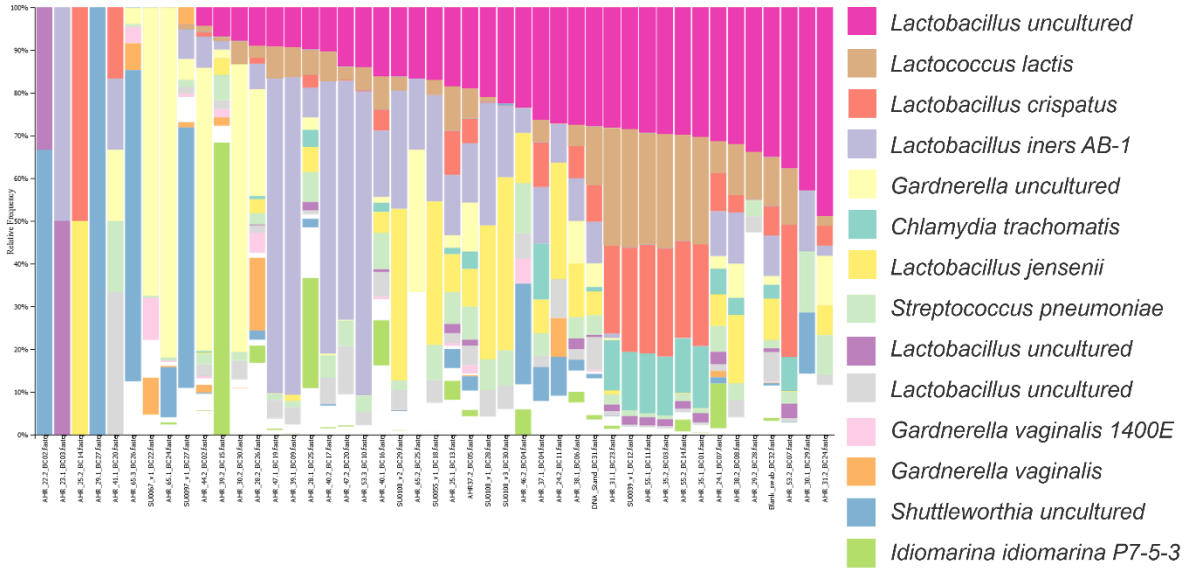


Figure 2.19. Species level identification of bacteria from vaginal swabs of pregnant women (n=43) either >37w gestation (term) or ≤36+6w gestation (preterm). White areas of the graph contain organisms with <4% abundance and are not shown for clarity. A full version of this figure can be found in supplementary materials and contains 450 species. Qiime2 analysis performed by Dr Neha Kulkarni.

2.4.5.5 Relative Abundance

In order to assess the microbial community composition, relative abundance of the vaginal community for each sample was calculated. Four species were found to significantly differ between term and preterm patients when grouping all GTP (Figure 2.20, Figure 2.21, Figure 2.22) *Coriobacteriales bacterium, Megasphaera unidentified, Rhizobium rhizogenes and Lactobacillus rhamnosus.*

When looking at GTP1 only the relative abundance of three species were significantly different in term compared to preterm patients *Atopobium vaginae, Coriobacteriales bacterium, Gardnerella vaginalis.* No organisms significantly differed in relative abundance at GTP2.

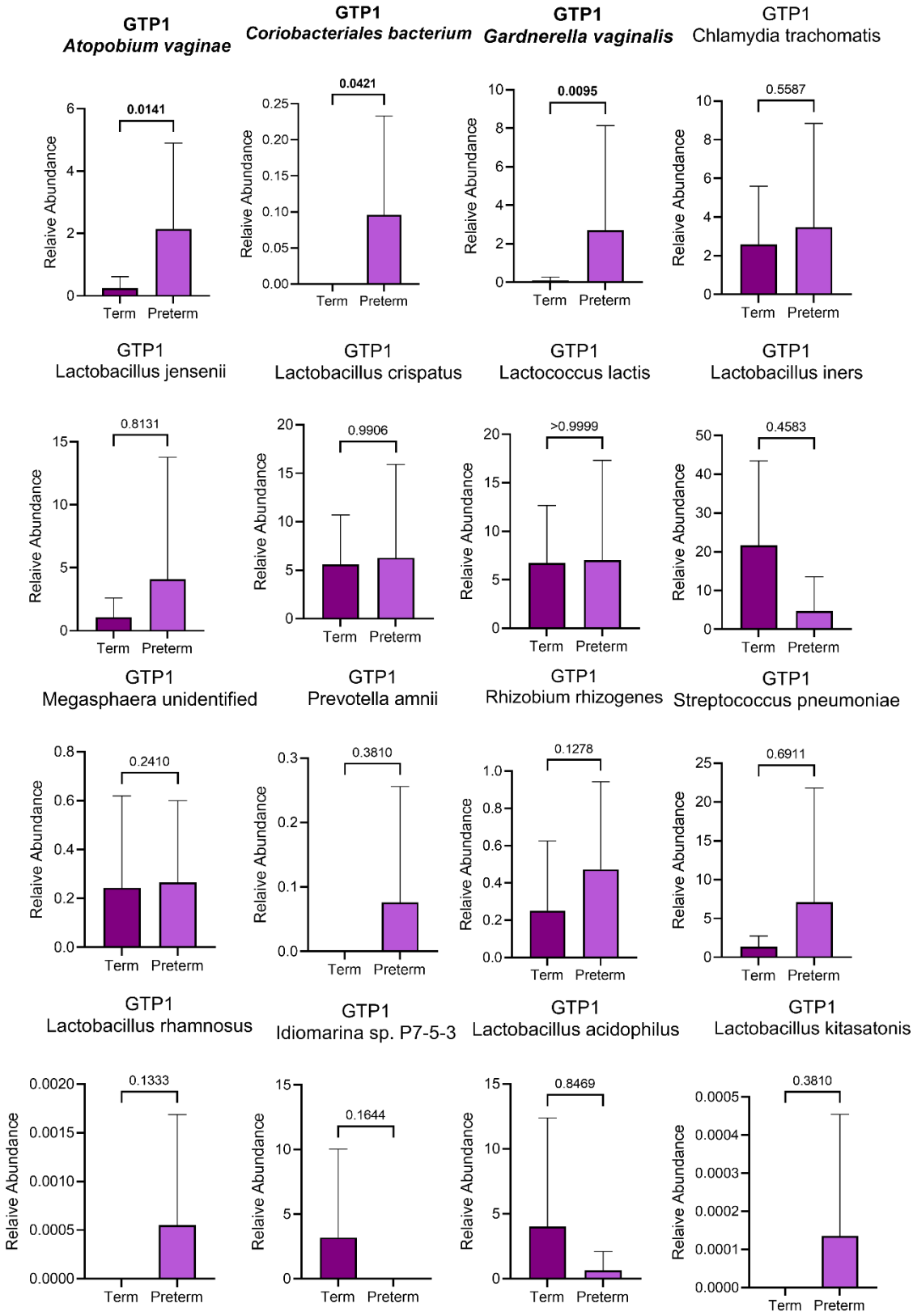


Figure 2.20 Relative Abundance of key vaginal species at GTP1 Term = birth >36+6 w (n=26) Preterm ≤36+6 w (n=17). Bars represent mean, error bars represent 95% confidence interval. Analysis performed with GraphPad Prism.

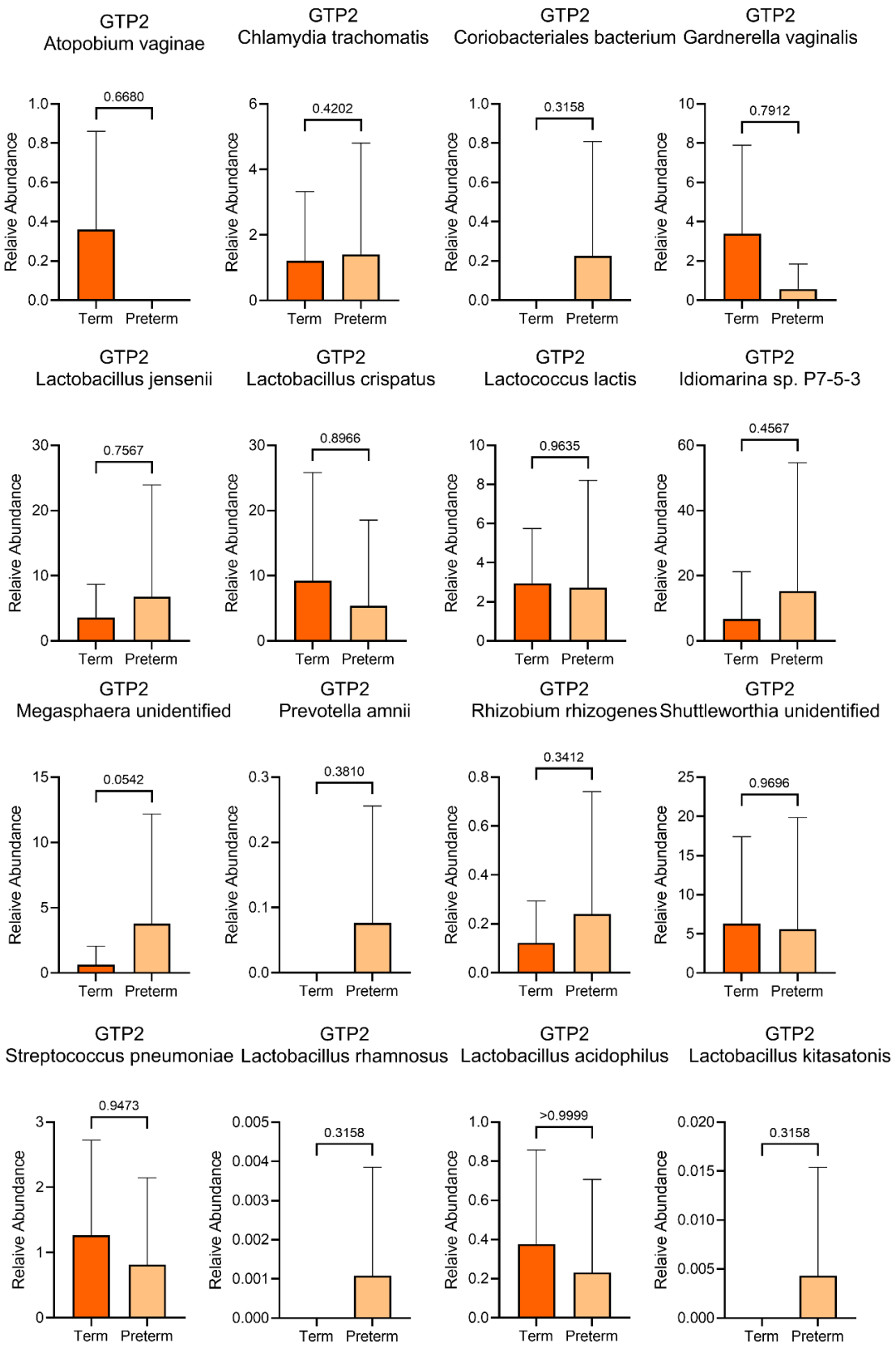


Figure 2.21. Relative Abundance of key vaginal species at GTP2 Term = birth >36+6 w (n=26) Preterm ≤36+6 w (n=17). Bars represent mean, error bars represent 95% confidence interval. Analysis performed with GraphPad Prism.

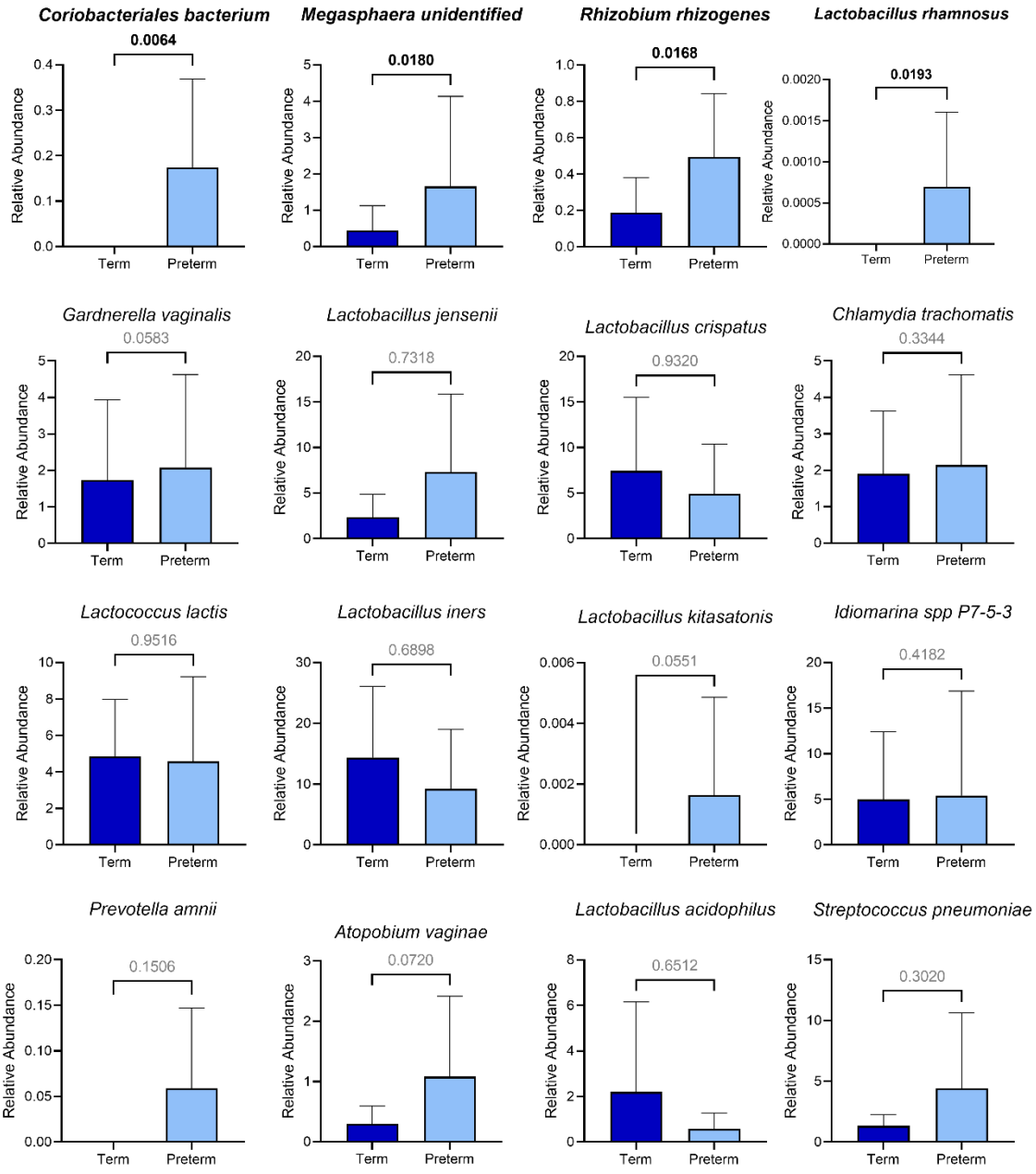


Figure 2.22. Relative Abundance of key vaginal species Term = birth >36+6 w (n=26) Preterm ≤36+6 w (n=17) at GTP 1, 2 & 3. Bars represent mean, error bars represent 95% confidence interval. Analysis performed with GraphPad Prism.

2.4.5.6 Correlation of Relative Abundance

The relative abundances of key organisms were correlated to identify relationships between vaginal organisms. Values closer to 1 indicate organisms positively correlated, in other words, commonly found together. Values closer to 0 indicate organisms negatively correlated, however, no organisms were found to be negatively correlated. Eighteen species were found to be significantly correlated in the term group while twenty-three species were found to be significantly correlated in the preterm group. Seventeen species were found to be significantly positively correlated at GTP1 (p>0.05) and seven species were found to be significantly correlated at GTP2.

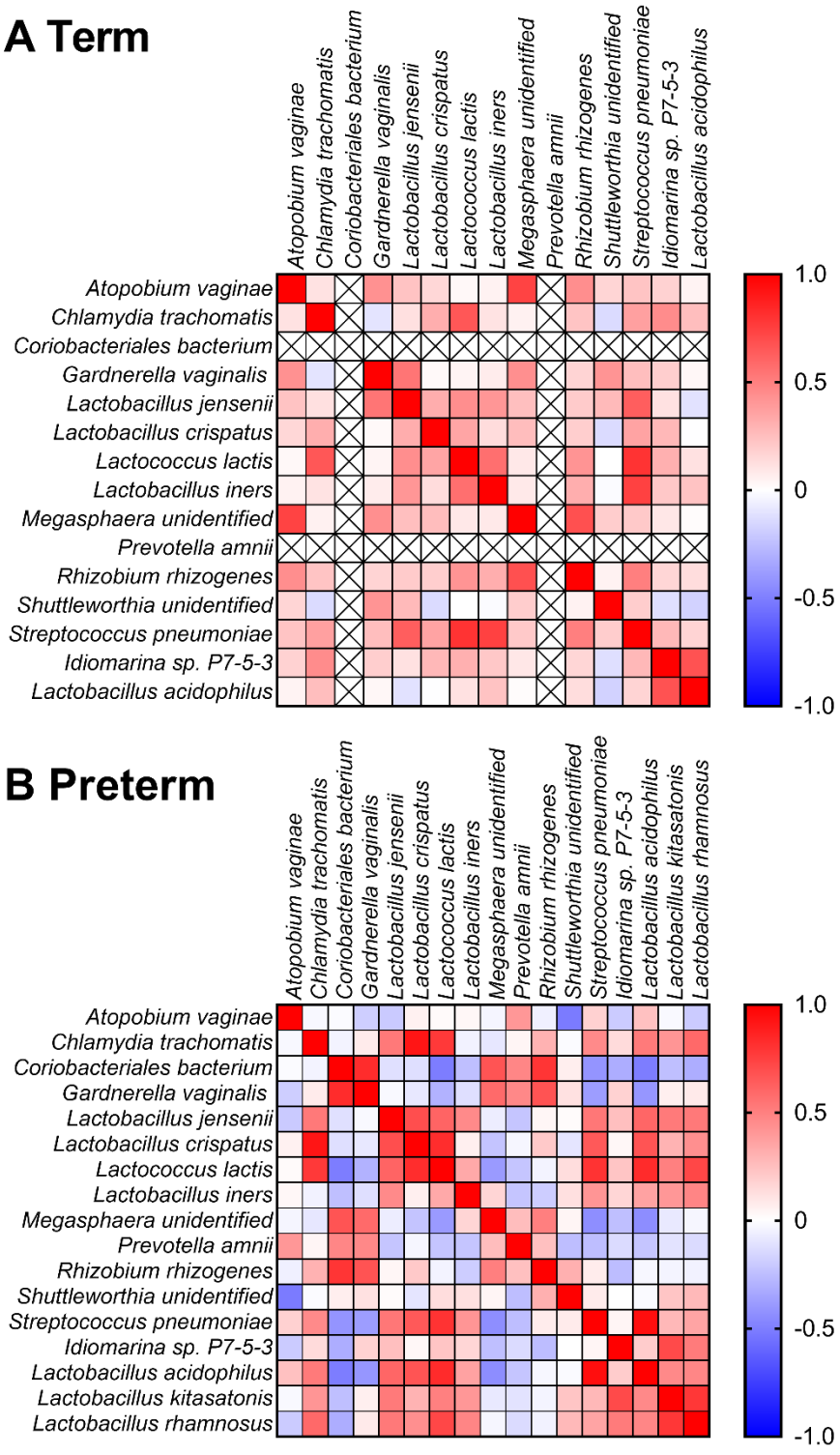


Figure 2.23. Pearson r correlation of bacterial relative abundance present in vaginal samples of pregnant women**. A)** Term = birth >36+6 w (n=26) **B)** Preterm ⩽36+6 w (n=17) at GTP 1, 2 & 3. 1 represents 100% positive correlation, 0 indicates no correlation, -1 indicates 100% negative correlation. Analysis performed with GraphPad Prism.

Table 2.12. Significant correlations of vaginal bacteria at Term >36+6 w (n=26) Preterm ≤36+6 w (n=17), GTP1 (20-22w), GTP2 (26-28w) and All GTP (GTP1, GTP2, GTP3 30-32w) (n=43). 1 represents 100% positive correlation, 0 indicates no correlation, -1 indicates 100% negative correlation. Pearson r correlation performed with GraphPad Prism.

Term	Bacterial Association		Pearson *r* Correlation	*p=*
	Gardnerella vaginalis	*Atopobium vaginae*	0.428	0.029
	Lactococcus lactis	*Atopobium vaginae*	0.735	0.000
	Rhizobium rhizogenes	*Atopobium vaginae*	0.439	0.025
	Lactococcus lactis	*Chlamydia trachomatis*	0.659	0.000
	Idiomarina sp. P7-5-3	*Chlamydia trachomatis*	0.451	0.021
	Lactobacillus jensenii	*Gardnerella vaginalis*	0.539	0.005
	Megasphaera unidentified	*Gardnerella vaginalis*	0.438	0.025
	Shuttleworthia unidentified	*Gardnerella vaginalis*	0.416	0.035
	Lactococcus lactis	*Lactobacillus jensenii*	0.440	0.025
	Lactobacillus iners	*Lactobacillus jensenii*	0.409	0.038
	Streptococcus pneumoniae	*Lactobacillus jensenii*	0.629	0.001
	Lactobacillus iners	*Lactococcus lactis*	0.564	0.003
	Rhizobium rhizogenes	*Lactococcus lactis*	0.417	0.034
	Streptococcus pneumoniae	*Lactococcus lactis*	0.798	>0.001
	Streptococcus pneumoniae	*Lactobacillus iners*	0.744	>0.001
	Rhizobium rhizogenes	*Megasphaera unidentified*	0.684	>0.001
	Streptococcus pneumoniae	*Rhizobium rhizogenes*	0.500	0.009
	Lactobacillus acidophilus	*Idiomarina sp. P7-5-3*	0.675	>0.001
Preterm	Bacterial Association		Pearson r Correlation	*p=*
	Lactobacillus crispatus	*Chlamydia trachomatis*	0.916	>0.001
	Lactococcus lactis	*Chlamydia trachomatis*	0.775	0.002
	Lactobacillus rhamnosus	*Chlamydia trachomatis*	0.585	0.023
	Gardnerella vaginalis	*Coriobacteriales bacterium*	0.825	>0.001
	Megasphaera unidentified	*Coriobacteriales bacterium*	0.670	0.008
	Rhizobium rhizogenes	*Coriobacteriales bacterium*	0.788	0.001
	Megasphaera unidentified	*Gardnerella vaginalis*	0.584	0.032
	Rhizobium rhizogenes	*Gardnerella vaginalis*	0.674	0.011
	Lactobacillus crispatus	*Lactobacillus jensenii*	0.692	0.009
	Lactococcus lactis	*Lactobacillus jensenii*	0.605	0.024
	Lactobacillus acidophilus	*Lactobacillus jensenii*	0.600	0.025
	Lactobacillus kitasatonis	*Lactobacillus jensenii*	0.519	0.049
	Lactobacillus rhamnosus	*Lactobacillus jensenii*	0.525	0.036
	Lactococcus lactis	*Lactobacillus crispatus*	0.821	0.001
	Streptococcus pneumoniae	*Lactobacillus crispatus*	0.647	0.016
	Lactobacillus acidophilus	*Lactobacillus crispatus*	0.669	0.012
	Streptococcus pneumoniae	*Lactococcus lactis*	0.798	0.001
	Lactobacillus acidophilus	*Lactococcus lactis*	0.827	0.001
	Lactobacillus rhamnosus	*Lactococcus lactis*	0.721	0.005
	Lactobacillus rhamnosus	*Lactobacillus iners*	0.478	0.050

	Lactobacillus acidophilus	*Streptococcus pneumoniae*	0.943	0.000
	Lactobacillus kitasatonis	*Idiomarina sp. P7-5-3*	0.708	0.027
	Lactobacillus rhamnosus	*Lactobacillus kitasatonis*	0.783	0.016
GTP1	Bacterial Association		Pearson r Correlation	p=
	Gardnerella vaginalis	*Atopobium vaginae*	0.444	0.044
	Prevotella amnii	*Atopobium vaginae*	0.458	0.037
	Streptococcus pneumoniae	*Atopobium vaginae*	0.808	<0.001
	Lactobacillus crispatus	*Chlamydia trachomatis*	0.943	<0.001
	Lactococcus lactis	*Chlamydia trachomatis*	0.934	<0.001
	Lactobacillus kitasatonis	*Chlamydia trachomatis*	0.468	0.032
	Lactobacillus rhamnosus	*Chlamydia trachomatis*	0.519	0.016
	Gardnerella vaginalis	*Coriobacteriales bacterium*	0.918	<0.001
	Prevotella amnii	*Coriobacteriales bacterium*	0.867	<0.001
	Prevotella amnii	*Gardnerella vaginalis*	0.991	<0.001
	Lactococcus lactis	*Lactobacillus crispatus*	0.944	<0.001
	Lactobacillus kitasatonis	*Lactobacillus crispatus*	0.476	0.029
	Lactobacillus rhamnosus	*Lactobacillus crispatus*	0.515	0.017
	Lactobacillus rhamnosus	*Lactococcus lactis*	0.512	0.018
	Rhizobium rhizogenes	*Megasphaera unidentified*	0.914	<0.001
	Idiomarina sp. P7-5-3	*Megasphaera unidentified*	0.761	<0.001
	Idiomarina sp. P7-5-3	*Rhizobium rhizogenes*	0.656	0.001
GTP2	Bacterial Association		Pearson r Correlation	p=
	Lactococcus lactis	*Chlamydia trachomatis*	0.766	<0.001
	Rhizobium rhizogenes	*Coriobacteriales bacterium*	0.732	<0.001
	Lactobacillus kitasatonis	*Lactobacillus jensenii*	0.774	<0.001
	Lactobacillus rhamnosus	*Lactobacillus jensenii*	0.774	<0.001
	Streptococcus pneumoniae	*Lactococcus lactis*	0.574	0.010
	Streptococcus pneumoniae	*Lactobacillus iners*	0.471	0.042
	Lactobacillus rhamnosus	*Lactobacillus kitasatonis*	1.000	<0.001

2.4.5.7 Relative Abundance of G. vaginalis, L. crispatus & L. iners

Studies have highlighted the clinical significance of *Gardnerella vaginalis*, *Lactobacillus crispatus* and *Lactobacillus iners* (Callahan et al., 2017). Based on this, we investigated the ratios of these key organisms (Figure 2.24). For example, Figure 2.24 A represents the ratio of *G. vaginalis : L. crispatus*, 1 signifies only *G. vaginalis* present, 0 signifies only *L. crispatus* present and 0.5 signifies an equal abundance of the two species.

We found a significantly higher relative abundance of *G. vaginalis* compared to *L. crispatus* and *L. iners* in the preterm group at both GTP1 and when GTP were grouped (Figure 2.24).

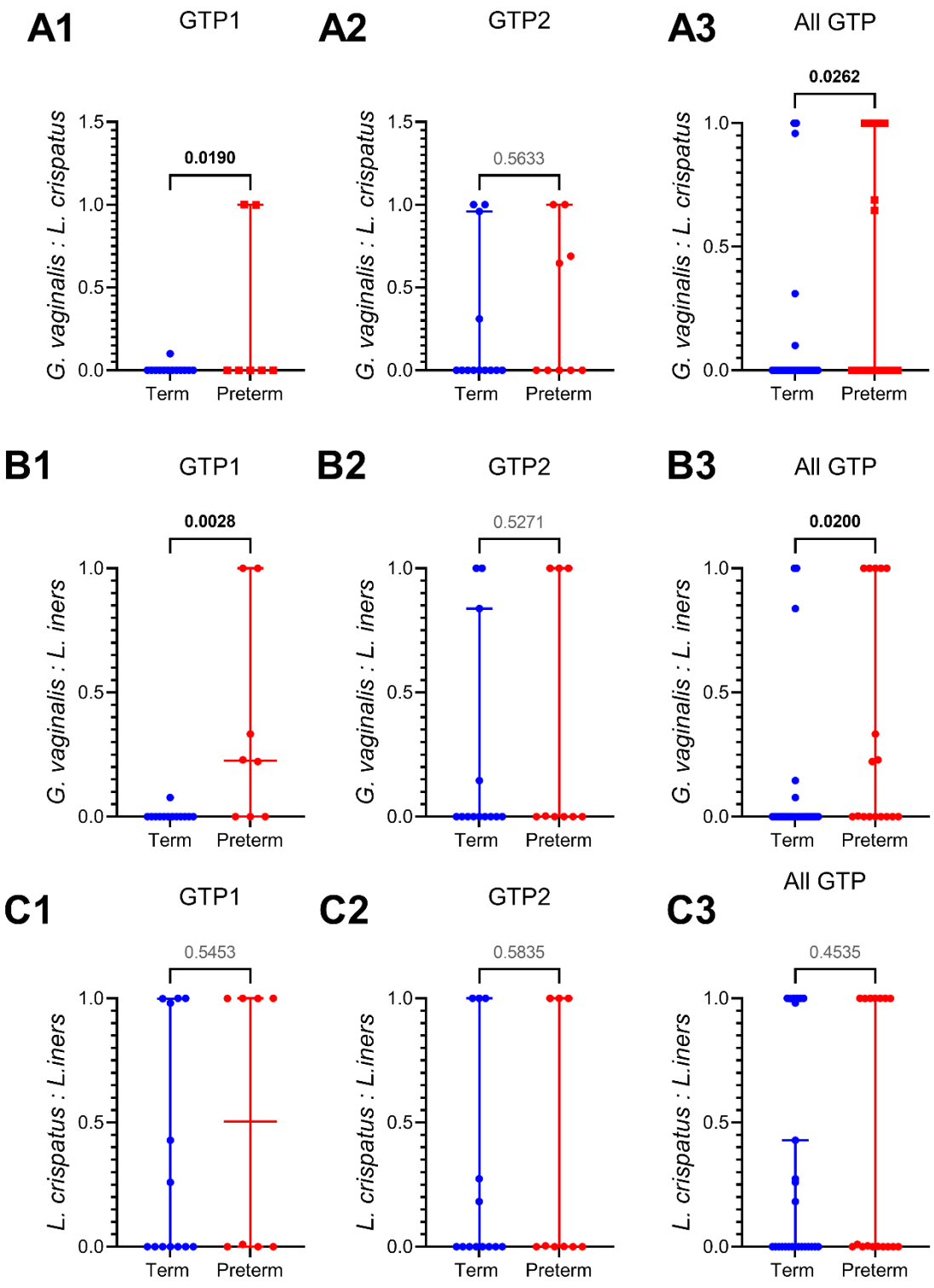


Figure 2.24. Relative abundance of clinically relevant vaginal bacteria A) Gardnerella vaginalis : Lactobacillus crispatus. **B)** Gardnerella vaginalis : Lactobacillus iners. **C)** Lactobacillus crispatus : Lactobacillus iners. All GTP = Term n=26, Preterm n=17, GTP1= Term =13, GTP1 Preterm=8, GTP2 Term =13, GTP2 Preterm =6. Middle line represents median, error bars represent 95% Confidence Interval. Analysis performed with Graphpad Prism

2.4.5.8 Community State type

CVF samples were categorised into the widely accepted CST groups from (Ravel et al., 2011) which reflects the dominant bacterial species present (Table 2.13). The most common CST was CSTIV at 25.6%, this group contains anaerobes with samples dominated by either *Gardnerella vaginalis, Gardnerella uncultured bacterium* or *Shuttleworthia uncultured bacterium* (see Figure 2.25). The next most common group was CSTIII at 18.6% dominated by *L. iners.* Notably, no samples were dominated by *Lactobacillus gasseri* (CSTII). However, 18/43 (41.9%) of samples did not fit into one of these categories and were dominated by either *Idiomarina P7-5-3, Lactobacillus acidophilus, Lactobacillus uncultured bacterium* or *Lactococcus lactis.*

Table 2.13 Proportion of samples per dominant bacteria identified from CVF samples (n=43). Dominant bacteria grouped into CSTIV anaerobes includes communities dominated by *Shuttleworthia uncultured, Streptococcus pneumoniae, Gardnerella vaginalis* and *Gardnerella uncultured,* (see Figure 2.25 for sample breakdown).

Dominant Bacteria		Number of Samples	Proportion (%)
Lactobacillus crispatus	(CSTI)	3	7.0
Lactobacillus gasseri	(CSTII)	0	0.0
Lactobacillus iners	(CSTIII)	8	18.6
Anaerobes	(CSTIV)	11	25.6
Lactobacillus jensenii	(CSTV)	3	7.0
Idiomarina P7-5-3		4	9.3
Lactobacillus acidophilus		1	2.3
Lactobacillus uncultured		7	16.3
Lactococcus lactis		6	14.0

Focussing on those samples where dominant species was successfully identified at more than one GTP, 3/16 patients had a consistent CST. Two patients transitioned from *L. lactis* to *Idiomarina* P7-5-3 and two patients transitioned from *L. iners* (CSTIII) to *L. crispatus* (CSTI) (Figure 2.25).

Sample ID	GTP1	GTP2	GTP3
AHR_22		33% L. uncultured	
AHR_23	CSTIII - 100% L. iners		
AHR_24	25% L. uncultured	CSTV - 30% L. jensenii	
AHR_25	CSTIII - 13% L. iners	CSTI - 100% L. crispatus	
AHR_28	41% Idiomarina P7-5-3	CSTIV - 20% G. vaginalis	
AHR_29	CSTIV - 67% S. uncultured	15% L. uncultured	
AHR_30	50% L. acidophilus	CSTIV - 64% G. uncultured	
AHR_31	28% L. lactis	23% L. uncultured	
AHR_35	25% L. lactis	17% L. uncultured	
AHR_37	33% L. uncultured	CSTIII - 14% L. iners	
AHR_38	CSTI -10% L. crispatus	13% L. lactis	
AHR_39	CSTIII -74% L. Iners		
AHR_40	CSTIII - 19% L. Iners	CSTIII - 66% L. iners	
AHR_41	50% L. uncultured		
AHR_44		CSTIV - 53% G. uncultured	
AHR_46		CSTIV - 33% S.uncultured	
AHR_47	CSTIII - 75% L. iners	CSTI - 9% L. crispatus	
AHR_53		13% L. lactis	CSTIII - 71% L. iners
AHR_55	26% L. lactis	92% Idiomarina P7-5-3	
AHR_65	CSTIV - 76% G. uncultured	CSTIV - 40% G. uncultured	CSTIV - 58% S. uncultured
SU0039	28% L. lactis	86% Idiomarina P7-5-3	
SU0055	CSTIV - 51% S. pneumoniae		
SU0067	CSTIV - 46% G. uncultured		
SU0097	CSTIV - 60% S. uncultured		
SU0108	CSTV - 30% L. jensenii	CSTV - 39% L. jensenii	CSTV - 50% L. jensenii

CSTI - *Lactobacillus crispatus*
CSTIII - *Lactobacillus iners*
CSTIV - Anaerobic Bacteria
CSTV - *Lactobacillus jensenii*
Lactobacillus uncultured bacterium

Figure 2.25 Dominant species for each sample at GTP1, GTP2 and GTP3. "uncultured" indicates that the sequence could not be matched to a named species. *Lactobacillus crispatus* (CSTI), *Lactobacillus gasseri* (CSTII), *Lactobacillus iners* (CSTIII), *Anaerobes* (CSTIV) *(*including *Shuttleworthia* uncultured*, Gardnerella vaginalis, Gardnerella* uncultured*, Streptococcus pneumoniae), Lactobacillus jensenii* (CSTV), *Idiomarina* P7-5-3*, Lactobacillus acidophilus, Lactobacillus* uncultured*, Lactococcus lactis.*

Comparing term and preterm, at GTP1, there was a higher frequency of CSTV and CSTIV in the preterm group compared to term. The largest difference was CSTIII which was present at increased frequency in the term group. At GTP2, there was a higher frequency of CSTV, and *Idiomarina P7-5-3* compared to the term group (Figure 2.26).

Within the preterm group, there was an increase of *Idiomarina P7-5-3* from GTP1 (20-22w) to GTP2 (26-28w) and a decrease in CSTIV and *Lactococcus lactis*. In the term group, CSTI, CSTIV, CSTV and *Lactobacillus* uncultured increased from GTP1 to GTP2, while CSTIII, *Lactobacillus acidophilus,* and *Lactococcus lactis* decreased (Figure 2.26).

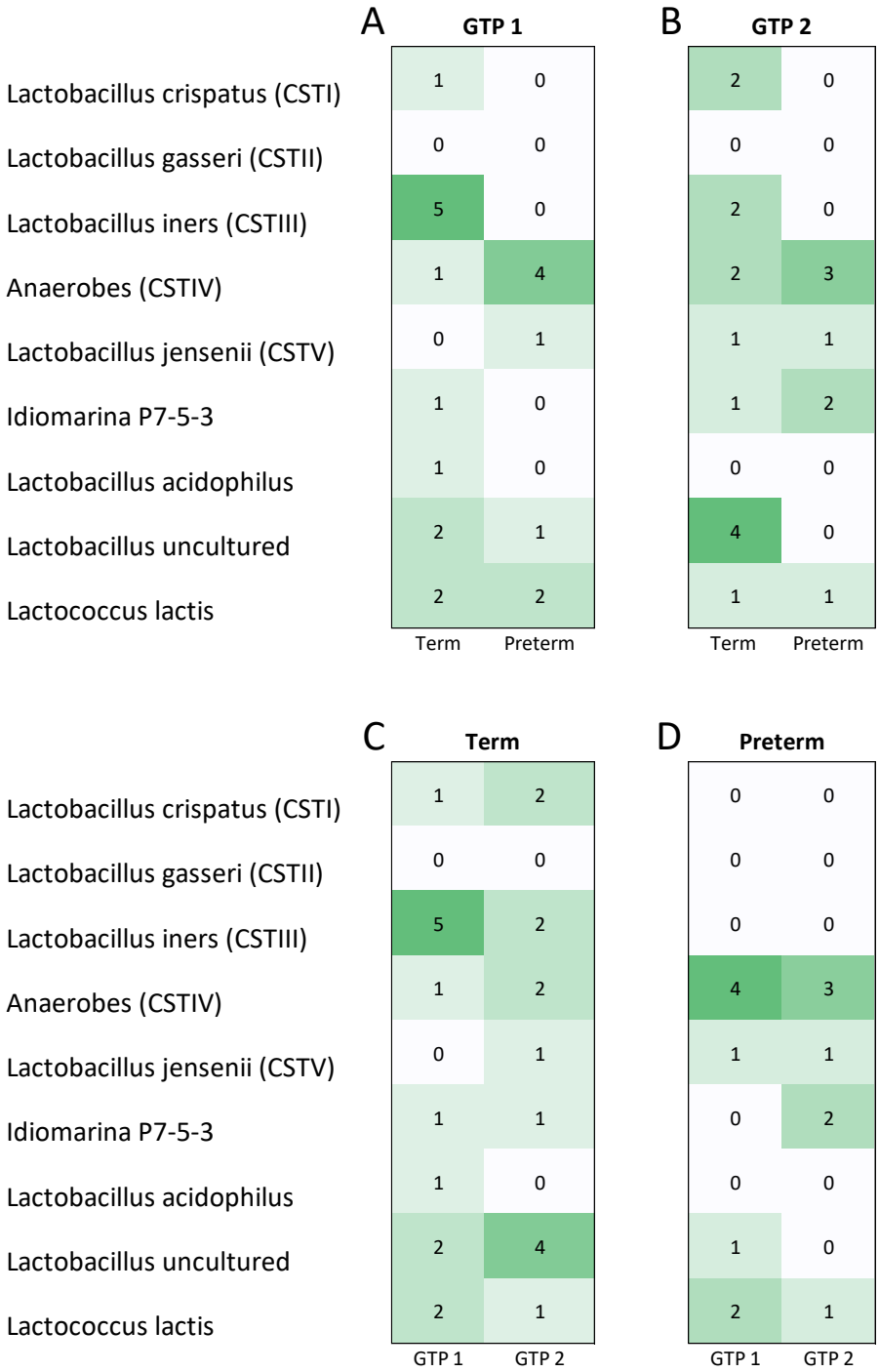


Figure 2.26 Community state type (CST) Heat abundance map. A) GTP1 = 20-22w, **B)** GTP2= 26-28w **C)** Term = birth >36+6 w gestation, **D)** Preterm ≤36+6 w gestation. CST indicates dominant bacterial species.

Based on the findings of previous studies, (Arena & Daccò, 2021; Brown et al., 2019; Callahan et al., 2017; DiGiulio et al., 2015; Usui et al., 2002) we analysed the percentage of vaginal community inhabited by *Lactobacillus* species (Figure 2.27).

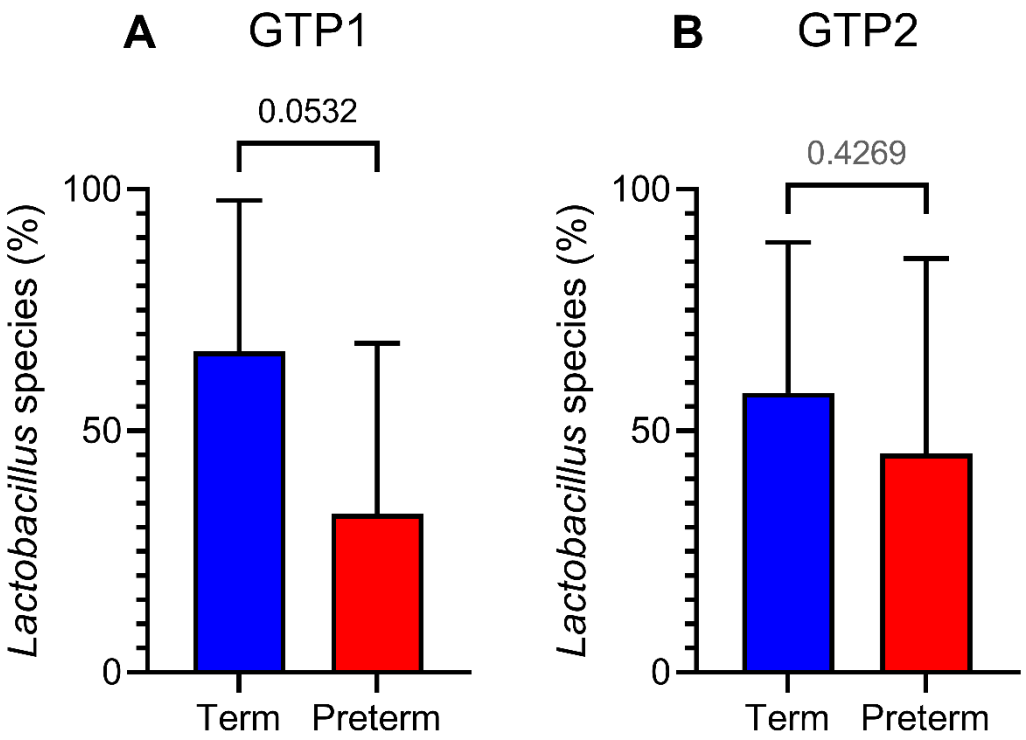


Figure 2.27 Percentage of vaginal community comprising of *Lactobacillus* species in **A)** Gestational time point (GTP) 1 22-24 weeks gestation, Term =13, GTP1 Preterm=8, **B)** GTP2 26-28 weeks gestation Term =13, GTP2 Preterm =6. Bars represent mean, error bars represent Standard deviation. Analysis performed with GraphPad Prism.

2.4.6 Bacterial Species Predictive of preterm Birth

ROC curves were used to analyse the predictive value of bacterial species and clinical factors. Area under the ROC curve is a representation of sensitivity and specificity. Values of 0.5 indicate no predictive value while 1 indicates 100% positive predictive ability. The relative abundance of *Atopobium vaginae* and *Gardnerella vaginalis* at GTP1 (p=0.0298, p=0.0426) and the relative abundance of *Rhizobium rhizogenes* at GTP 1, 2 & 3 (p=0.0327) was found to significantly predict PTB (Figure 2.28 & Table 2.14).

Table 2.14. Preterm birth prediction capability of vaginal bacteria using receiver operating characteristic (ROC) curve (Term n=26, Preterm n=17, GTP1 Term =13, GTP1 Preterm=8, GTP2 Term =13, GTP2 Preterm =6). Analysis performed on GraphPad Prism.

Organism	GTP	Area Under ROC Curve	95% Confidence Interval	P value
Atopobium vaginae	GTP1	0.79	0.57 to 1.0	***0.0298**
	GTP2	0.55	0.28 to 0.82	0.7257
	All	0.64	0.46 to 0.82	0.133
Gardnerella vaginalis	GTP1	0.77	0.54 to 1.0	***0.0426**
	GTP2	0.53	0.26 to 0.81	0.8264
	All	0.64	0.47 to 0.82	0.1176
Rhizobium rhizogenes	GTP1	0.69	0.45 to 0.93	0.1475
	GTP2	0.63	0.34 to 0.91	0.3805
	All	0.69	0.53 to 0.86	***0.0327**
Chlamydia trachomatis	GTP1	0.58	0.32 to 0.83	0.5623
	GTP2	0.62	0.33 to 0.90	0.4299
	All	0.58	0.40 to 0.75	0.4053
Coriobacteriales bacterium	GTP1	0.69	0.43 to 0.94	0.1579
	GTP2	0.58	0.29 to 0.88	0.5686
	All	0.65	0.47 to 0.83	0.1064
Idiomarina sp. P7-5-3	GTP1	0.65	0.42 to 0.89	0.2466
	GTP2	0.59	0.30 to 0.88	0.5393
	All	0.55	0.38 to 0.73	0.5511
Lactobacillus acidophilus	GTP1	0.53	0.27 to 0.79	0.828
	GTP2	0.5	0.22 to 0.78	>0.9999
	All	0.54	0.36 to 0.72	0.6728
Lactobacillus crispatus	GTP1	0.5	0.23 to 0.78	0.9711
	GTP2	0.53	0.24 to 0.81	0.8608
	All	0.51	0.33 to 0.69	0.9307
Lactobacillus iners	GTP1	0.6	0.35 to 0.84	0.4689
	GTP2	0.51	0.21 to 0.81	0.9301

	All	0.53	0.36 to 0.71	0.7094
Lactobacillus jensenii	GTP1	0.53	0.27 to 0.79	0.828
	GTP2	0.55	0.26 to 0.84	0.7257
	All	0.53	0.35 to 0.71	0.7468
Lactobacillus kitasatonis	GTP1	0.56	0.30 to 0.83	0.6378
	GTP2	0.58	0.29 to 0.88	0.5686
	All	0.59	0.41 to 0.77	0.3327
Lactobacillus rhamnosus	GTP1	0.63	0.36 to 0.89	0.3465
	GTP2	0.58	0.29 to 0.88	0.5686
	All	0.62	0.44 to 0.80	0.1965
Lactococcus lactis	GTP1	0.5	0.24 to 0.76	>0.9999
	GTP2	0.51	0.22 to 0.81	0.9301
	All	0.51	0.33 to 0.68	0.9505
Megasphaera unidentified	GTP1	0.63	0.38 to 0.89	0.3106
	GTP2	0.71	0.43 to 0.98	0.1605
	All	0.67	0.49 to 0.84	0.0679
Prevotella amnii	GTP1	0.56	0.30 to 0.83	0.6378
	GTP2	0.56	0.30 to 0.83	0.6378
	All	0.56	0.38 to 0.74	0.5184
Shuttleworthia unidentified	GTP1	0.57	0.31 to 0.82	0.6122
	GTP2	0.51	0.23 to 0.79	0.9301
	All	0.55	0.37 to 0.73	0.5847
Streptococcus pneumoniae	GTP1	0.55	0.29 to 0.81	0.6904
	GTP2	0.51	0.23 to 0.79	0.9301
	All	0.59	0.41 to 0.76	0.3327

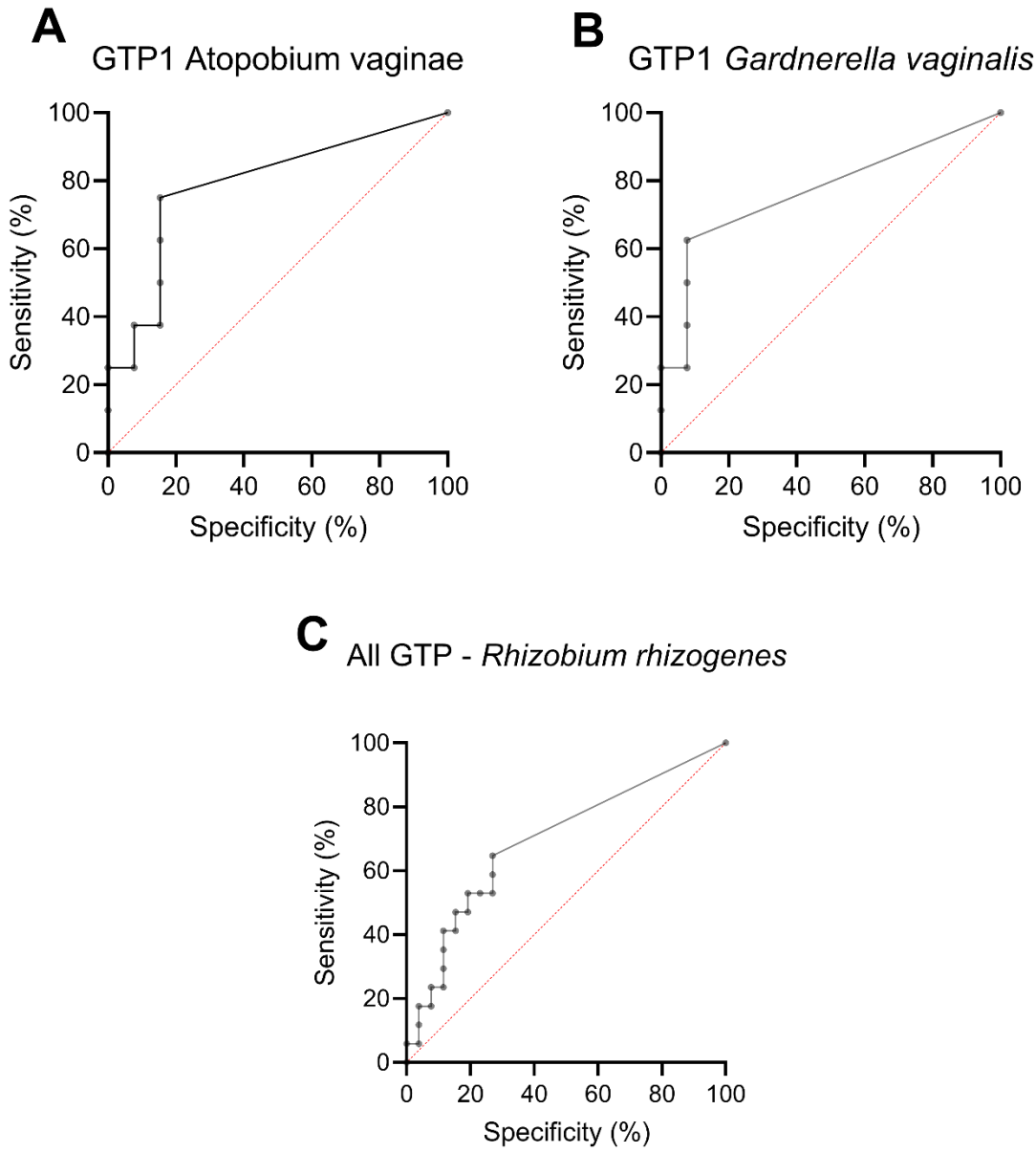


Figure 2.28. Predictive ROC curves A) relative abundance of *Atopobium vaginae* at GTP1 (term n=13, preterm=8) *p*=0.0298 **B)** relative abundance of *Gardnerella vaginalis* at GTP1 (term n=13, preterm=8) *p*=0.0426 **C)** relative abundance of *Rhizobium rhizogenes* at GTP 1, 2 & 3 (term =26, preterm=17) *p*=0.0327. Analysis performed with GraphPad Prism.

Additionally, species richness at GTP1 (p=0.0037) and the ratio of *Gardnerella vaginalis* and *Lactobacillus iners* at GTP 1, 2 & 3 (p=0.0248) was found to significantly predict PTB (Figure 2.29 & Table 2.15 & Table 2.16).

Table 2.15. Preterm birth prediction capability of vaginal community measures using receiver operating characteristic (ROC) curve (Term n=26, Preterm n=17, GTP1 Term =13, GTP1 Preterm=8, GTP2 Term =13, GTP2 Preterm =6). Analysis performed with GraphPad Prism.

Bacteria Ratio	GTP	Area Under ROC Curve	95% Confidence Interval	P value
G. vaginalis : L. crispatus	GTP1	0.75	0.50 to 1.0	0.0746
	GTP2	0.57	0.32 to 0.82	0.5932
	All	0.67	0.50 to 0.84	0.0625
G. vaginalis : L. iners	GTP1	0.8	0.57 to 1.0	***0.0248**
	GTP2	0.58	0.33 to 0.83	0.5478
	All	0.68	0.50 to 0.85	0.0527
L. crispatus : L.iners	GTP1	0.57	0.39 to 0.74	0.4713
	GTP2	0.58	0.31 to 0.85	0.5382
	All	0.57	0.32 to 0.82	0.5932
Factor	GTP	Area Under ROC Curve	95% Confidence Interval	P value
Shannon Diversity Index	GTP1	0.5	0.25 to 0.76	0.9711
	GTP2	0.62	0.33 to 0.90	0.4299
	All	0.52	0.34 to 0.70	0.8649
Species Richness	GTP1	0.75	0.52 to 0.98	0.0597
	GTP2	0.56	0.25 to 0.88	0.661
	All	0.78	0.63 to 0.93	***0.0037**

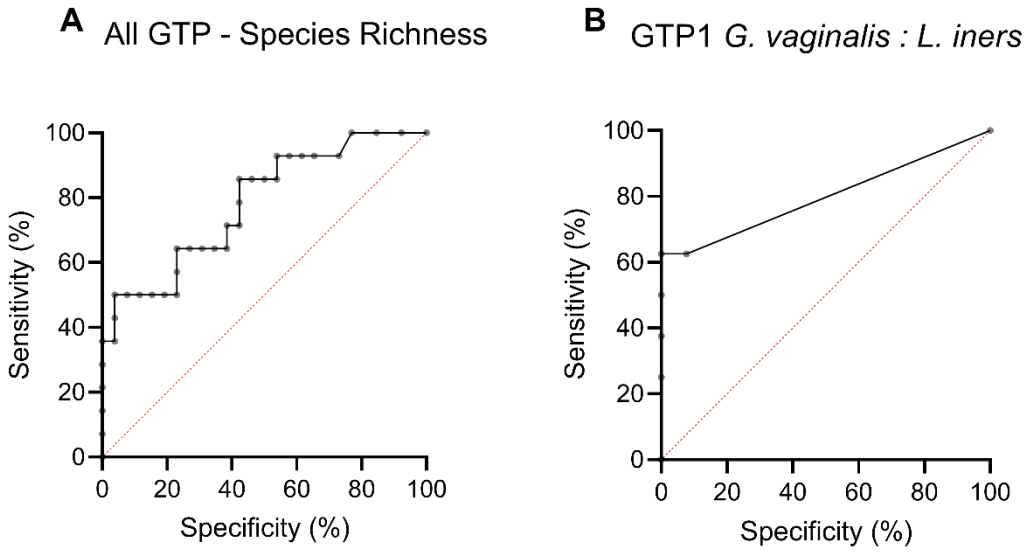


Figure 2.29. Predictive ROC curve A) Species richness at GTP 1+2+3 (term =26, preterm=17) *p*=0.0037 **B)** Ratio of *Gardnerella vaginalis* and *Lactobacillus iners* at GTP1 (term n=13, preterm=8) p=0.0248. Analysis performed with GraphPad Prism.

Table 2.16. Predictive cut-off values, sensitivity and specificity for PTB prediction using CVF swabs

Cut-off Value	Sensitivity %	95% CI	Specificity %	95% CI	Likelihood ratio
Relative Abundance of *Atopobium vaginae* at GTP1					
> 0.00014	75	41% to 96%	85	58% to 97%	4.9
Relative Abundance of *Gardnerella vaginalis* at GTP1					
> 0.00054	63	31% to 86%	92	67% to 100%	8.1
Relative Abundance of *Rhizobium rhizogenes* at GTP 1+2+3					
> 1.2	18	6.2% to 41%	96	81% to 100%	4.6
Abundance of *G. vaginalis* relative to *L. iners* at GTP1					
> 0.038	63	31% to 86%	92	67% to 100%	8.1
Species Richness at GTP 1+2+3					
> 133	50	27% to 73%	96	81% to 100%	13

2.4.7 Key Findings

Optimisation

- **Protocol A** - QIAmp DNA mini kit, adapted from (Stafford et al., 2017), was found to consistently isolate more DNA from cryopreserved CVF swabs.
- **Protocol C -** QIAamp DNA Microbiome Kit was found to isolate DNA from the whole cell standards but not from cryopreserved CVF swabs.
- A novel universal primer pair 319F/MCRevA was successfully optimised. This pair is better able to amplify key vaginal bacteria compared to 27F/1492R from Oxford Nanopore.

Microbiome Profile & PTB

- **Species richness** was significantly higher in the preterm group compared to term (Figure 2.14) and was able to predict PTB (Figure 2.29). However, number of reads was significantly higher in the preterm group which would likely account for the higher species richness.
- **Relative abundance** of *Atopobium vaginae* and *Gardnerella vaginalis* at GTP1 and *Rhizobium rhizogenes* at GTP 1 + 2 + 3 was found to be significantly higher in preterm and significantly predict PTB (Figure 2.28 & Table 2.14)
- Coriobacteriales bacterium relative abundance significantly higher in preterm at GTP1 (Figure 2.20)
- Abundance of *G. vaginalis* relative to either *L. crispatus* or *L. iners* abundance was significantly higher in the preterm group at GTP1 and when GTP were grouped (Figure 2.24)
- **Ratio of *G. vaginalis: L. iners*** was found to be predictive of PTB at GTP1 (Figure 2.29)
- **Lactobacillus dominance,** the percentage of *Lactobacillus* within a sample appears to be lower in those with PTB. This requires additional samples to be statistically significant (Figure 2.27)

2.5 Discussion

In this chapter, the overall aim was to produce a vaginal microbiome sequencing protocol suitable for Nanopore sequencing that could be used in a range of settings across the globe. Once completed, the second aim was to analyse the vaginal microbiome of pregnant women, comparing term and preterm samples in order to identify biomarkers for the prediction of PTB. The PRIME global health research group was collecting samples from: UK (Sheffield Teaching Hospitals), Bangladesh (icddr,b in Dhaka) and South Africa (Groote Schuur Hospital) to ensure that prediction methods are relevant to mothers around the world and not only those in high income countries. Unfortunately, the SARS-CoV-2 pandemic delayed sample collection from all research sites and so this work includes only samples from the UK. However, based on the methods optimised in this study, the PRIME research team continue to work with clinical samples from all partner countries.

2.5.1 Optimisation Bacterial DNA Extraction

This section looks at the optimisation of bacterial DNA extraction from CVF swabs stored at -80 °C in PBS. Both clinical samples and Vaginal Microbiome Whole Cell Mix standard was used to test 2 kits: the QIAmp DNA mini kit (QIAGEN, 51304) (Protocol A&B) and the QIAamp DNA Microbiome Kit (QIAGEN, 51704) (Protocol C).

Protocol A was adapted from a previous study from our group (Stafford et al., 2017). Our results show that this protocol successfully extracted total DNA from the swab and resulted in amplicons post-PCR. Protocol B is comparable to A with the addition of extended incubation at 56 °C an additional 95 °C incubation step. Based on testing, where we observed no amplicons for protocol B, we conclude that the additional heating steps may damage DNA and hinder subsequent amplification.

Protocol C has an additional host DNA degradation step compared to A and B which improves the ratio of host DNA to bacterial DNA after extraction. Testing showed that the host DNA degradation step was successful. Compared to protocol A, there was no visible amplicon produced from the swab sample before PCR indicating that the human DNA was degraded. This mirrors the bacterial standard which contains no human DNA. However, after amplification protocol C produced a visible amplicon for the bacterial cell standard but not for the swab which suggests that bacterial DNA present may have been degraded with the

host DNA. Comparatively, protocol A produced an amplicon for both the bacterial standard and the swab post PCR.

Host DNA depletion is desirable as it can hinder upstream steps such as quantification and sequencing. One study found 87.93% of DNA sequenced from vaginal samples of pregnant women was of host origin (Marquet et al., 2022). For our samples, which have been stored at -80 °C (some for several years) we suspect that bacteria in the sample have lysed and therefore microbial DNA is exposed prior to bacterial lysis and so is also degraded during the host depletion step. On the other hand, the whole cell standard contains intact bacteria, therefore, microbial DNA was not degraded as the host depletion step is performed before degradation of bacterial cell walls. This is supported by our testing of a fresh swab which underwent protocol C and produced an amplicon visible on a gel after PCR.

Therefore, we conclude that Protocol C is suitable for DNA extraction performed on fresh samples. However, as our samples and samples from partner countries were stored at -80 °C we chose to use protocol A for this study.

Many VMB sequencing studies store samples at -20 °C followed by long term storage at -80 °C (as was done in this study) followed by extraction using PowerSoil DNA isolation kit (MO BIO Laboratories) which does not contain a host depletion step but, includes inhibitor removal steps (Callahan et al., 2017; Stout et al., 2017; DiGiulio et al., 2015). However, a comparison of the PowerSoil kit and the QIAGEN DNeasy method showed that while the PowerSoil kit detected more diversity within the samples, the QIAGEN kit gave the highest DNA yield and quality (Mattei et al., 2019).

Other methods can be used to mitigate the effects of host DNA in sequencing experiments. Marquet et al, (2022) tested depletion of host DNA and enrichment of microbial DNA. They explain that “While a DNA molecule is sequenced in the nanopore, the data is already compared live with references to decide whether the DNA molecule should be sequenced further”. This is an exciting new method that builds on the newest sequencing platform. However, not all human sequences were excluded and 5.48% of *Gardnerella* reads were detected in the ‘rejected’ fraction. This technique requires prior knowledge of the species detected in the sample and may exclude species that are not commonly present but could potentially be clinically important. While very promising, novel methods that are still in

development such as this one, are prone to errors and so we chose not to use this technique in our study.

2.5.1.1 Optimisation Vaginal Microbiome Universal Primer Pair

As well as optimising DNA extraction method, universal primers specific for vaginal organisms were optimised. Oxford Nanopore sequencing provides the primer pair 27F/1492R in their kits for microbiome studies, however, this is not specific for vaginal organisms. Initially, we sought to assess the suitability of 27F/1492R for vaginal swab samples.

Firstly, we assessed if 27F/1492R was able to amplify the key vaginal species: *Gardnerella vaginalis, Lactobacillus gasseri, Mycoplasma hominis, Prevotella bivia, Streptococcus agalactiae, Lactobacillus jensenii*. From this small-scale *in-silico* analysis we determined that both the forward and reverse primer would not amplify *Gardnerella vaginalis* and only the forward primer would amplify the remaining species. This is supported by a previous study that assessed 27F/1492R for human vaginal samples, and re-designed primers which they report were better able to maintain the ratio of *Lactobacillus : Gardnerella* (Frank et al., 2008).

Based on this result, we aimed to find a primer pair that can amplify all key vaginal organisms. A literature search was performed, and 28 primers were identified for testing. Additionally, 2 novel reverse primers were created for this study. Small-scale in silico analysis including the species mentioned above and large-scale in silico analysis including bacterial sequences in the SILVA database was used to determine most suitable primers for amplification of key vaginal organisms such as *Gardnerella* species.

However, *in silico* analysis did not match results from laboratory experiments. In theory, primer pairs Fwd-p1/806R and Fwd-p1/785R are able to amplify all vaginal organisms present in the DNA standard but, when tested in the lab these pairs repeatedly did not produce an amplicon at a range of annealing temperatures. This phenomenon has been reported in other studies which observe that specificity and sensitivity vary significantly between *in silico* and *in vitro* experiments (Henriques et al., 2012; Morales & Holben, 2009). This discrepancy is likely due to the unpredictable nature of the PCR reaction which can be influenced by small experimental conditions such as mixing, reagent lot variation etc. which are not considered during *in silico* analysis.

From a combination of *in silico* analysis and lab work we compared 319F/MCRevA, 319F/MCRevB, and 27Fm/1492Rm with the standard pair for nanopore sequencing 27F/1492R (Figure 4.6). These pairs all reliably produced an amplicon of the expected size which was a suitable length and position on the 16S gene for species identification. However, specificity for vaginal organisms was mixed. Table 2.5 shows the % of each genus the primer pair is theoretically able to detect.

Sequencing results from the Nanopore Flongle showed that none of these primer pairs successfully amplified *Gardnerella vaginalis* (ATCC 14019) from the bacterial DNA standard but, were able to amplify the remaining species successfully. Insufficient detection of *Gardnerella* species is well documented in VMB studies (Srinivasan & Fredricks, 2008; Verhelst et al., 2004) and is due to mismatches in several popular regions for universal primers. One study tested samples with the primers 27f/189r and observed an absence of *Gardnerella vaginalis,* they then changed the forward primer to 338f and observed *G. vaginalis* was dominant species (Verhelst et al., 2004). This highlights the importance of primer choice when designing a microbiome study. Inevitably, no primer pair will equally amplify all species therefore primers should be assessed and biases considered when interpreting the data. In our case, *Gardnerella* will likely be underrepresented as only some species and or strains are amplified by our chosen primer pair.

Based on these results, we chose to use 319F/MCRevA for amplification of the clinical samples. This pair performed well in the lab and using *in-silico* analysis we predict can amplify most key vaginal organisms relevant to this study. 319F/MCRevA spans the variable regions V3-V6 which we believe will aid in the identification of vaginal bacteria. A recent study (Sirichoat et al., 2021) used vaginal samples to assess the performance of different variable regions in identifying vaginal bacteria. They concluded that population diversity was better assessed using the V3 region, followed by the V6–V7 and V4 regions. The V3 region was able to classify 69% of families and 63% of genera. Additionally, (Hugerth et al., 2020) found the V1-V3 region performed best *in silico*, but *in vitro* the V3-V4 region performed well and had good taxonomic coverage. The primer pair developed in this study spans the V3 region thought to be best for identification with additional regions that can further aid species identification. Now this has been established, this pair can be used consistently with the same methods to investigate the VMB of women around the globe.

2.5.2 Vaginal Microbiome sequencing

After optimisation, 60 clinical samples were sequenced using Oxford Nanopore technologies. Samples were split into 2 batches: one with a higher DNA concentration of 52.5 ng/sample and one with a lower concentration of 1.5 ng/sample. Splitting samples into the high and low batch enabled us to get as many reads as possible from every sample. If all samples were sequenced together, high concentration samples would have been normalised to match the lowest concentration resulting in fewer reads and decreased species identification. Alternatively, if the samples were not normalised samples with very low concentrations would have been overwhelmed by the volume of DNA from other samples resulting in very few reads. Sequencing in two batches allowed us to get the best out of each precious clinical sample. The high concentration batch produced more reads than the low concentration batch which was expected as there was more DNA available to sequence.

The majority, 68%, of the species identified from matching our sequences to the SILVA database were named “uncultured” or “unidentified”. Uncultured bacteria were observed in sequencing results during optimisation which used a community standard containing no uncultured species and so supports the idea that the high prevalence of uncultured organisms is due to methodologies rather than novel species. Errors during identification of base pairs during sequencing could lead to species being categorised as an “uncultured” sequence from the SILVA database despite being a known vaginal organism.

Only two studies to date have performed Nanopore sequencing on clinical vaginal swabs (Marquet et al., 2022; Kerry-Barnard et al., 2022). Marquet et al, (2022) found that the average concentration of DNA extracted from swabs was < 40 ng/µl per sample pre-PCR. We did not measure DNA concentration prior to PCR as the majority of DNA present is of host origin. Therefore, pre-PCR concentration does not corelate with concentration post PCR as only bacterial DNA is amplified during PCR. This study did not provide concentration data post PCR although did measure each sample for library preparation. Additionally, Marquet et al did not provide the number of bacterial reads per sample but, total reads were 2.73 million which is comparable to our high concentration batch (2.89 M) although not the low concentration batch (42.86 K).

Kerry-Barnard et al, (2022) was able to analyse sufficient reads from 106/148 frozen samples. No information on how long the samples were stored for or DNA concentration was provided. Additionally, no information regarding the Nanopore sequencing output e.g. number of reads. Based on the very limited available data we are unable to compare our findings to previous vaginal swab Nanopore sequencing results. Future studies should strive to include detailed results as supplementary material to allow the quality of samples to be appraised and compared.

2.5.3 Vaginal bacterial species

A total of 391 named bacterial species were identified from our clinical samples. This is in line with an exhaustive review of vaginal bacteria by (Diop et al., 2019) who identified 581 bacteria from all VMB research published at that time.

2.5.3.1 Lactobacillus spp.

As expected, the most abundant genus was *Lactobacillus* which is known to be the dominant genus of the VMB for most women (Ravel et al., 2011). *Lactobacillus* is known to dominate the VMB and unsurprisingly was the most abundant and prevalent genus from all samples.

Interestingly ***L. gasseri*** was not observed in any samples. This could be due to a mismatch within the primer, however, during optimisation, the selected primers successfully amplified *L. gasseri*. Therefore, this population likely has low to no abundance of *L. gasseri* compared to previous studies (Stafford et al., 2017) which observed 11% of patients (n=133) to have CSTII dominated by *L. gasseri.*

Relative abundance of ***L. acidophilus*** and ***L. crispatus*** was not significantly different between the term and preterm group. However, there was a trend of higher relative abundance in the term group. *L. crispatus* is considered to be the most health promoting bacteria within the VMB and has the best capacity for metabolising glycogen into lactic acid to maintain the low pH of the vagina (Argentini et al., 2022). *L. acidophilus* is a less common *Lactobacillus* species found in the vagina. One sample was dominated by *L. acidophilus* and this patient delivered at term. Similarly, three samples were dominated by *L. crispatus* all from term delivered women. However, additional patients are required to properly analyse these associations.

Larger studies have consistently observed significant association between *L. crispatus* dominated communities and term birth (Aslam et al., 2022; Payne et al., 2021; Stafford et al., 2017). Tabatabaei et al, (2019) found that communities dominated by *L. crispatus/L. acidophilus* at early gestations were associated with decreased risk of spontaneous preterm birth. Additionally, in our study we observed a significantly higher abundance of *G. vaginalis* relative to *L. crispatus* in the preterm group which indicates that when the abundance of *L. crispatus* is higher than *G. vaginalis*, there may be a decreased risk of PTB. Based on this, there is strong evidence to suggest *L. crispatus* is protective against PTB and there are a small number of studies that to suggest *L. acidophilus* is also protective against PTB.

L. jensenii has also been associated with term birth (Aslam et al., 2022; Payne et al., 2021). In our study we found that the relative abundance of *L. jensenii* was not statistically significant although was higher in the preterm group at all GTPs. Additionally, *L. jensenii* was significantly correlated with *Atopobium vaginae* which is associated with preterm birth (Odogwu, Chen, et al., 2021). In a previous study from our team 25% of the preterm group had communities dominated by *L. jensenii* compared to 10% of the term group (Stafford et al., 2017).

In the current study, three samples from one patient were identified as dominated by *L. jensenii*. From this, we can speculate that *L. jensenii* is stable within the vaginal environment. This is supported by DiGiulio et al, (2015) who observed the most stable communities to be *L. gasseri* followed by *L. jensenii*. Therefore, whether *L. jensenii* is truly associated with term or preterm birth, it is likely not due to a fluctuating community as seen with communities dominated by various species throughout pregnancy.

2.5.3.2 Lactobacillus iners

Lactobacillus iners AB-1 was identified in 44% of our samples. This bacterium was the most prevalent from the term group and was the dominant bacteria in 8 samples. Relative abundance was found to be higher in the term samples at GTP1. This finding is in line with previous research as *L. iners* is the most frequently detected bacterial species within the vagina (Macklaim et al., 2011).

Out of all the vaginal *Lactobacillus* species, *L. iners* is the most controversial. Some have associated it with PTB (Petricevic et al., 2014) and several studies have observed that *L. iners* dominated communities can transition into more diverse communities as seen in BV (Verstraelen et al., 2009).

However, *L. iners* AB-1 is known to have genes optimal for survival in the fluctuating vaginal environment (Kwak et al., 2020; Petrova et al., 2017). This includes the ability to tolerate normal vaginal pH but also pH >4.5 which is seen during menstruation and BV. This is supported by studies that have observed *L. iners* as the only remaining *Lactobacillus* species present after menstruation or BV (Lopes dos Santos Santiago et al., 2012).

This leads some to speculate that *L. iners* is a persistent well adapted bacteria not easily displaced by anaerobes but instead, can help restore the vaginal pH after menstruation or BV (Macklaim et al., 2011). The beneficial characteristics of *L. iners* are demonstrated in a study

where *L. iners* was able to reduce the viability of *G. vaginalis* and form a dense biofilm in its place (Saunders et al., 2007).

Our results show *L. iners* was found to positively correlate *with Lactobacillus jensenii, Lactococcus lactis* and *Streptococcus pneumoniae* in the term group. While in the preterm group significantly correlated to only *Lactobacillus rhamnosus.* This finding does not support the previous findings that *L. iners* often co-exists with anaerobes that are known to be associated with PTB such as *Gardnerella* (Lopes dos Santos Santiago et al., 2012). Or that *L. iners* contributes to a transitioning to a diverse community (Verstraelen et al., 2009).

In our study, no preterm patients were dominated by *L. iners*. Additionally, the relative abundance of *G. vaginalis* : *L. iners* was found to be significantly higher in the preterm group indicating that a higher abundance of *G. vaginalis* relative to *L. iners* leads to a higher risk of preterm birth. Further analysis showed that *G. vaginalis* : *L. iners* was able to predict PTB at GTP1. These findings implicate *G. vaginalis* in infection associated PTB and suggest that *L. iners* is more protective against PTB.

2.5.3.3 Gardnerella vaginalis

Gardnerella vaginalis was observed in 53% of the preterm samples and had a significantly higher relative abundance in the preterm group at GTP1. Abundance of *G. vaginalis* relative to either *L. crispatus* or *L. iners* abundance was significantly higher in the preterm group at GTP1 and was able to predict PTB (Figure 2.29). This supports previous studies that also found *Gardnerella vaginalis* to be predictive of PTB (Mcelrath et al., 2018; Bretelle et al., 2015).While *G. vaginalis* is thought to coexist with *L. iners*, it has been observed that *L. crispatus,* which is strongly associated with term birth, has an inverse correlation to *G. vaginalis* and does not coexist (Callahan et al., 2017). Our study does not support either of those observations, no bacteria were negatively correlated and *L. iners* was not positively correlated with *G. vaginalis* in any group (p= term 0.73, preterm 0.66, GTP1 0.58, GTP2 0.69).

High levels of cervical *G. vaginalis* have been observed in women with PPROM along with microbial presence in the amniotic cavity (Kacerovsky et al., 2021). However, these observations do not always correlate with inflammation so could go undetected throughout pregnancy. Identifying *Gardnerella* species at early gestations such as GTP1 in this study at 26-28 weeks could be a useful factor in a larger predictive model of PTB. Those with a high

relative abundance of *G. vaginalis* could be identified as at higher risk of PTB and given the appropriate clinical triage.

2.5.3.4 Streptococcus pneumoniae

The most prevalent bacterium from all samples was *Streptococcus pneumoniae* at 45.5%. The relative abundance of this species was found to be higher in the preterm group at GTP1 although not statistically significant.

S. pneumoniae is a common respiratory tract pathogen. Airway infections have been observed to hematogenously spread to the placenta resulting in inflammation and increased rates of PTB (Sandu et al., 2013). Endocervical and amniotic fluid infections of *S. pneumoniae* have also been reported in PPROM patients (Ouseph et al., 2019; Lajos et al., 2008). Based on this we can speculate that *Streptococcus pneumoniae* may have the capability to increase the risk of PTB and PPROM.

However, this research was conducted during an unusual time for respiratory viruses. The SARS-CoV-2 pandemic National government restrictions resulted in a 68% decrease in reported incidences of *S. pneumoniae* infections after 4 weeks and, an 82% decrease after 8 weeks across Europe and Asia including the UK (Brueggemann et al., 2021). Fewer studies have reported the incidence of *S. pneumoniae* after the removal of social distancing. However, trends reported in Australia, Israel and in paediatric patients in the UK indicate a rising number of respiratory infections including *S. pneumoniae* in 2021 (Amar et al., 2022; Lumley et al., 2022; Foley et al., 2021).

This decrease and subsequent increase of *S. pneumoniae* infection incidence may explain the significant difference in abundance between term and preterm samples. Seven samples included in the analysis were collected in December 2020 or later during the period of increasing respiratory infections. These swabs were taken from 3 patients who all delivered preterm (AHR65, SU0097, SU0108). In contrast, all samples included in this analysis from term delivered women were collected between November 2018- May 2019 before SARS-CoV-2 restrictions were in place. However, looking at the dominant bacteria present in each sample (Figure 2.25), *S. pneumoniae* was the dominant species only in SU0055 which was collected in September 2019. From this data, it is not possible to determine whether *S. pneumoniae* is a causative agent of PTB despite the significantly higher abundance in the preterm group.

Additional samples from term patients collected after December 2020 are needed as a comparison.

2.5.3.5 *Rhizobium rhizogenes*

Rhizobium rhizogenes (genus formerly known as Agrobacterium) was the most prevalent organism observed in 65% of the preterm samples and was found to be significantly higher in preterm patients when GTP were grouped. *R. rhizogenes* was able to predict PTB when GTPs were grouped but not at GTP1/ GTP2 alone. This is likely due to the small group numbers and additional patients may further support this finding. *R. rhizogenes* is a gram-negative soil bacterium that is usually pathogenic in plants (Velázquez et al., 2005). This genus has been reported in vagina but is a rare finding. More commonly, infections and sepsis are seen in neonates (Tiwari & Beriha, 2015; Khan et al., 2014).

A close relative of *R. rhizogenes* is *Rhizobium radiobacter* which has been described as the most common opportunistic human pathogen (Tiwari & Beriha, 2015; Lai et al., 2004) particularly in immunocompromised patients. The infections were strongly related to the presence of foreign plastic materials e.g., an intravascular catheter (Lai et al., 2004). This bacterium has been observed in the vagina, ovaries, urethra, sputum and endometrium among others (Riley & Weaver, 1977). Additionally, *Rhizobium pusense* is also known to be a human pathogen and has been isolated from blood, sputum and urine among others (Aujoulat et al., 2015).

Based on the observations of other members of the *Rhizobium* genus, we can speculate that this organism may be an opportunistic pathogen present in the hospital setting where these samples were taken. The higher prevalence and relative abundance of this bacterium in the preterm group could be due to the time spent in the hospital and an increased rate of clinical foreign plastic materials used for treatment. No current studies have reported an association between *Rhizobium* and PTB, however, *Rhizobium* genus is a good candidate to investigate in future studies.

2.5.3.6 *Other CSTIV Bacteria*

In addition to *G. vaginalis, S. pneumoniae and R. rhizogenes* other species classified as “diverse anaerobes” have been associated with PTB. One study found that 40% of samples from preterm delivered women did not contain any *Lactobacillus* species and was instead

dominated by diverse anaerobes (Aslam et al., 2022). In comparison, samples from term delivered women all contained either 1 or 2 *Lactobacillus* species (Aslam et al., 2022). Diversity of the VMB is known to increase during the 1st trimester (Kaur et al., 2020). Genera including *Atopobium, Sneathia, Gardnerella,* and *Megasphaera* are not commonly observed prior to pregnancy but were found to be common in the 1st trimester by Kaur et al, (2020). This observation correlates with our findings, as these genera were prevalent in our samples.

The relative abundance of ***Atopobium vaginae*** was found to be significantly higher in preterm patients and was found to predict PTB at GTP1. *A. vaginae* was also significantly associated with *Prevotella amnii* known to be associated with PTB (Elovitz et al., 2019; Nelson et al., 2016). Previous studies support our findings, for example a study of Nigerian women found *A. vaginae* predicted PTB (Odogwu, Chen, et al., 2021). Additionally, a targeted qPCR study identified that a high load (⩾108 copies/mL) of *A. vaginae* was able to predict PTB (Bretelle et al., 2015). *A. vaginae* appears to be a promising candidate for PTB prediction using the VMB.

Coriobacteriales bacterium **DNF00809** was identified in five samples. This bacterium has not previously been reported in association with preterm birth but has been reported in studies investigating BV (Plummer et al., 2021) and genital tract inflammation (Alisoltani et al., 2020) in non-pregnant women. Looking at the taxonomy identified from the SILVA database, we can see that this bacterium belongs to the order *Coriobacteriales* (of which *Atopobium* is also a member) within the family *Eggerthellaceae.* While this bacterium is not well characterised, *Eggerthellaceae* are known to be mostly anaerobic and are commonly isolated from human samples (Gupta, 2021). Other members of this family such as *Eggerthellaceae Eggerthella* have been identified from previous vaginal microbiome studies (Diop et al., 2019) and *Eggerthella* sp. MVA1 was found to significantly increase expression of IL-1α in cervical cells (McKenzie et al., 2021).

In this study, *Coriobacteriales bacterium* DNF00809 was significantly associated with *Gardnerella vaginalis*, *Megasphaera* unidentified and *Rhizobium rhizogenes* in the preterm group, all of which are associated with PTB. We also observed a significantly higher relative abundance in the preterm group at GTP1. *Coriobacteriales* is also likely to have a significantly higher relative abundance at GTP2 with additional participants as it was not present in term

samples. There is compelling evidence that *Coriobacteriales* and the *Eggerthellaceae* family are implicated in infection associated PTB and should be further studied in the future.

Megasphaera was found to have a significantly higher relative abundance in the preterm group when GTP were grouped. Additionally, *Megasphaera* was associated with *Gardnerella vaginalis, Rhizobium rhizogenes* and *Coriobacteriales bacterium*. *Megasphaera,* specifically *Megasphaera* phylotype 1 (MP1) has previously been associated with PTB (Elovitz et al., 2019; Hočevar et al., 2019; Fettweis et al., 2019; Nelson et al., 2014, 2016) and PPROM (Jayaprakash et al., 2016). We were unable to identify the species of *Megasphaera*, however, from the 14 named organisms known to be in the *Megasphaera* genus no species have been associated with positive pregnancy outcomes (Glascock et al., 2021). Targeted research is needed to characterise the individual associations of *Megasphaera* strains, similar to the various *Lactobacillus* species. *Megasphaera* species and strains may have differing relationships with the host and the vaginal environment.

Prevotella amnii was found to be associated with *A. vaginae*, *Coriobacteriales* and *G. vaginalis*. Interestingly, *P. amnii* was not present in any term sample. . However, a recent study observed a positive correlation between PTB and *Ureaplasma/ Prevotella* but interestingly, communities colonised by *Ureaplasma, Prevotella* **and *Lactobacillus*** were associated with term birth (Park et al., 2022). This study highlights the importance of investigating the whole community rather than targeting individual species.

The bacteria discussed in this section have all previously been associated with PTB and in this study are commonly found together, demonstrated by the significant positive associations. Dysbiotic organisms such as those discussed here are known to form biofilms within the vaginal environment (Castro et al., 2019), and on the choriodecidual surface of explanted human FM (Doster et al., 2017). *G. vaginalis* has been shown to initiate biofilm formation and is then often joined by other PTB associated organisms such as *A. vaginae* (Arroyo-Moreno et al., 2022). One study demonstrated that *A. vaginae* and *P. bivia* were able to incorporate into *G. vaginalis* biofilms *in vitro* (Castro et al., 2021). Biofilms are thought to be the cause of persistent BV infections (Muzny et al., 2020), partly due to being antibiotic resistant (Swidsinski et al., 2008). Antibiotic susceptible bacteria have been shown to produce antibiotic resistant biofilms (Rosca et al., 2022). Again, this is compelling evidence to

investigate the vaginal microbiome as a community rather than detecting the presence or absence of targeted organisms.

2.5.3.7 Lactococcus lactis cremoris

Finally, the second most prevalent bacteria from all samples was *Lactococcus lactis* subspecies *cremoris* which is a lactic acid producing bacteria commonly used in food production for milk products (Wegmann et al., 2007). This species is neither a common *Lactobacillus* species nor an anaerobic pathogen but was the dominant species in 6 samples. There was no significant difference in relative abundance in term vs preterm patients at any GTP. A few case-reports link *Lactococcus lactis cremoris* to PPROM, however, our study had insufficient cases of PPROM to draw conclusions.

One case of intrauterine-infection thought to be caused by *subspecies cremoris* was reported by (Slaoui et al., 2022). This case appears to be a descending infection as the patient presented with acute gastroenteritis after consumption of unpasteurized buttermilk. The day after admission, the patient presented with PPROM, malodorous amniotic fluid and fever. *Lactococcus lactis cremoris* was identified from a culture of placenta biopsy and the patient was successfully treated with amoxicillin. While *Lactococcus lactis cremoris* was identified in the placenta and is the likely cause of gastroenteritis, no vaginal or amniotic fluid samples were taken. Therefore, organism(s) present at the site of PPROM or in the amniotic fluid cannot be determined. Had molecular techniques been used in this case, a better insight into PPROM mechanisms could have been gained. Identification of organisms at these sites could determine whether PPROM was due to either local membrane infection by *Lactococcus lactis cremoris,* response to microbes in the amniotic fluid or, a general inflammatory response from gastroenteritis which induced fever.

An older case report claims “Chorioamnionitis due to *Lactococcus lactis cremoris*” (Azouzi et al., 2015). The patient presented with PPROM at 34w with no amniotic fluid malodour and without any gastrointestinal symptoms. Two weeks later the patient was admitted for labour induction but presented with fever and increased white blood cells. As in the previous case report, a biopsy of the placenta was taken and *Lactococcus lactis cremoris* was identified using culture techniques. Similarly, no vaginal or amniotic fluid samples were taken, and no molecular techniques were used to identify fastidious or non-culturable organisms.

These two case reports highlight the need for additional clinical samples and research. With this, we can better understand the mechanisms of PPROM and the organisms that may cause the highest risk of PPROM and ascending infection. Importantly, as molecular techniques become more accessible such as Oxford Nanopore sequencing used in our study, clinical settings should utilise these methods for clinical research to properly identify polymicrobial infections, fastidious organisms and unculturable bacteria.

Culture techniques bias our understanding of clinical infections. A review of the tree of life calculated that out of 92 bacterial phyla currently known we are unable to cultivate any species from 55 phyla (Hug et al., 2016). Another study estimated that ≤1 % of the microorganisms observed using a microscope could be isolated (Garza & Dutilh, 2015). Because of this culture bias we cannot confidently identify the infectious agent using culture methods alone, particularly in polymicrobial infections or infections of fastidious organisms.

Looking more specifically at vaginal colonisation of *Lactococcus lactis cremoris,* lactic acid is known to be beneficial to vaginal health producing antimicrobial and immune modulatory effects (Amabebe & Anumba, 2018; Aldunate et al., 2015). Therefore, if *Lactococcus* species can occupy this niche, preventing pathogens from colonising the vagina in the absence of lactobacilli, *Lactococcus* are likely to play a beneficial role in the human VMB. In support of this idea, other subspecies of *Lactococcus lactis* are currently being used to develop vaginal probiotics (Diaz-Dinamarca et al., 2020; Gao et al., 2011).

2.5.4 Alpha Diversity

Alpha diversity is a commonly used measure which represents the richness and evenness (also referred to as variety and abundance) of a community (Willis, 2019; B. D. Wagner et al., 2018). In this case, we're investigating the vaginal bacterial community of pregnant women.

Species richness (number of observed species) was significantly higher in the preterm group compared to term. Median number of species in the term group =23, in the preterm group =105.5 (p= 0.0134).

The most basic measure of diversity is species richness (observed number of species), which does not consider the relative abundance or evenness of a community. We found a significantly higher species richness in preterm-delivered patients compared to term-delivered (Median =105.5 vs 23 respectively, Figure 2.14, Figure 2.15). This is in contrast to (Nelson et al., 2016) who observed a higher median of 175.40 in term vs 117.90 species in preterm. However, the higher number of species may be due to the significantly higher number of sequencing reads in the preterm group as more reads results in more opportunity to identify different bacteria within a sample.

Samples were also assessed with Shannon Diversity Index (SDI) (Shannon, 1948) which is a more nuanced diversity measure that equally considers the number of taxonomic groups or richness and the distribution of abundances or evenness (Willis, 2019) (Figure 2.16, Figure 2.17).

In line with Romero & Hassan, et al, (2014), no significant differences in bacterial diversity were found. However, some trends were observed: in term-delivered patients diversity increased from GTP1 to GTP2 whereas in preterm-delivered patients diversity decreased from GTP1 to GTP2 (Figure 2.17a&b). Looking only at GTP1, diversity was higher in the preterm group compared to term (Figure 2.17c). This is consistent with Nelson et al, (2016) who observed the same non-significant trend at 9-24w.

Interestingly, a study from Haque et al, (2017) found that from 15 weeks gestation diversity gradually decreased in term patients but increased in preterm patients leading the study to suggest diversity in the first trimester can be used for PTB prediction. However, between 21-23 weeks gestation the preterm and term SDI intersected. Term diversity then remained

relatively stable between 23-37 weeks gestation but preterm diversity decreased leading to another intersect between 33-35 weeks gestation (Haque et al., 2017).

This study provides evidence to suggest that while diversity may significantly vary between term and preterm in early pregnancy, at later gestations diversity is more similar and may not be useful for PTB prediction. This may be the reason we see no significant differences within our samples at GTP1 (20-22w, 2nd trimester) and GTP2 (26-28w, 3rd trimester). To properly identify changes in diversity, earlier and more frequent time points should be taken.

Despite diverse anaerobes being repeatedly associated with PTB, when using SDI Hyman et al, (2014) found a significantly lower SDI in preterm samples compared to term. Additionally, when grouped by trimester Blostein et al, (2020) found the preterm group had a lower SDI than the control term group. These findings suggest that as the total diversity within the VMB does not correlate with PTB, it is only the presence of diverse anaerobes found in CSTIV that maybe useful for PTB prediction.

2.5.5 Community State Types

In order to assess the VMB at a community level, samples were categorised based on the dominant bacterium present into the widely accepted CST groups from (Ravel et al., 2011).

Despite numerous studies supporting Ravel's CST (Stafford et al., 2017; DiGiulio et al., 2015; Kacerovsky et al., 2015), 41.9% of our samples did not fit into one of these categories. These samples were dominated by either *Idiomarina P7-5-3, Lactobacillus acidophilus, Lactococcus lactis* or *Lactobacillus uncultured bacterium.* For this reason, we chose to report the % of dominant bacteria in addition to CST to better characterise each sample.

Similar to our findings, some studies also found that Ravel's groupings did not fit their results (Callahan et al., 2017; Romero, Hassan, et al., 2014; Gajer et al., 2012). Most different from Ravel's groupings was Blostein et al, (2020) who grouped their samples into three groups: diverse, *Lactobacillus ASV2* dominated or *Lactobacillus iners* dominated. Additionally, Callahan et al, (2017) observed that *L. iners* and *Gardnerella* often coexisted at near equal frequencies and therefore CST groupings did not well represent these communities. Based on this result, Callahan et al proposed analysis based on the quantitative frequencies of *G.*

vaginalis, *L. crispatus*, and *L. iners* which was also performed in our study. In our samples, *G. vaginalis* and *L. iners* were not found to coexist at equal frequencies. In all samples one species had a higher relative abundance, the majority of samples had a value close to 1 (only *G. vaginalis* present) or 0 (only *L. iners* present). This suggests that *L. iners* is not necessarily associated with PTB. The same is also true for *L. crispatus,* there were significantly more samples in the preterm group that had a higher abundance of *G. vaginalis* compared to *L. crispatus*. This finding can be attributed to *L. crispatus* ability to inhibit *G. vaginalis* growth (Amabebe et al., 2022).

Overall, the most common CST was CSTIV (dominated by anaerobes) these samples were dominated by: either *Gardnerella vaginalis, Gardnerella uncultured bacterium* or *Shuttleworthia uncultured bacterium*. We did not find a significant difference in the prevalence of CST between the term and preterm group but CSTIV was more prevalent in patients who delivered preterm.. This trend supports the evidence that diverse anaerobes are associated with PTB (Callahan et al., 2017; DiGiulio et al., 2015) with a greater sample number these associations may become more significant.

Notably, no samples were dominated by *Lactobacillus gasseri* (CSTII). As discussed in section 2.5.3, *L. gasseri* was not detected in any sample but as this bacterium was detected during optimisation, it is unlikely that this is due to a bias in methodology. Other studies have found a range of *L. gasseri* prevalence in vaginal samples from pregnant women: 1.8% (Dunlop et al., 2021), 8.6% (Stafford et al., 2017).

Studies report that some CST are more stable and beneficial to pregnancy outcome as they do not transition to another CST. Or inversely, some CST are thought to be “intermediate” and often transition to a different CST (DiGiulio et al., 2015; Gajer et al., 2012). Out of 16 patients with more than one data point, 3 had a consistent CST. Two patients transitioned from *L. iners* (CSTIII) to *L. crispatus* (CSTI) and both delivered at term. This supports *L. crispatus* association with term birth (Stafford et al., 2017), however, a larger sample size is needed to draw any conclusions. DiGiulio et al, (2015) found that CSTIV was able to transition to and from three other CSTs. This is reflected in our data as communities were found to transition from *Idiomarina P7-5-3* and *L. acidophilus* to CSTIV and from CSTIV to *L. uncultured*. As the sample size of patients with multiple time points is small, additional samples are needed to make any associations between transitioning vs stable communities and PTB.

2.5.5.1 *Lactobacillus deficient communities*

The most consistent finding from previous VMB research is a higher risk of PTB in patients with *Lactobacillus* deficient communities (Arena & Daccò, 2021; Brown et al., 2019; Callahan et al., 2017; DiGiulio et al., 2015; Usui et al., 2002). We analysed the percentage of each vaginal community containing *Lactobacillus* species. The % of *Lactobacillus* species did not significantly differ between the term and preterm groups. However, at GTP1 the preterm group had a lower average % of *Lactobacillus*. Additional clinical samples are needed to confirm this observation in our study. This would support other studies such as Arena & Daccò, (2021) who found a reduction or absence of lactobacilli in all women admitted with premature labour. Out of those admitted in premature labour, absence of lactobacilli was found in 18% of women who responded to the tocolysis and in 71.4% of women who did not respond. This suggests that in the absence of *Lactobacillus* species, colonising organisms produce a large inflammatory response which could be subclinical or difficult to treat resulting in a preterm birth.

2.5.6 Prediction of PTB

In order to establish which significant results were able to distinguish between term and preterm outcomes, we analysed the area under the receiver operating characteristic (ROC) curve to assess the predictive value of each factor. Unfortunately, due to the low sample size (n=3) we were unable to assess the predictive value for PPROM.

We found 5 factors were predictive of PTB. These were: the relative abundance of *Atopobium vaginae, Gardnerella vaginalis, Rhizobium rhizogenes, G. vaginalis: L. iners* and species richness (further predictive factors are discussed in chapter 5 after the addition of the metabolome data).

Assessing the relative abundance of *Atopobium vaginae, Gardnerella vaginalis, Rhizobium rhizogenes.* (Bretelle et al., 2015) demonstrated high loads of *A. vaginae* and *G. vaginalis* were predictive of PTB.

The proportion of ***G. vaginalis: L. iners*** was found to be predictive at GTP1. > 0.038 had a 63% sensitivity and 92% specificity. This indicates that if only *L. iners* is present there is a lower risk of PTB. When *G. vaginalis* is also present, even at low relative abundances, the risk of PTB is

greater. Our results are in contrast to Callahan et al, (2017) who observed that *L. iners* and *Gardnerella* often coexist at comparable frequencies, none of our samples had equal frequencies of *L. iners* and *Gardnerella* (Figure 2.24). Our findings indicate that *L. iners* may not be associated with PTB although often present due to its resilient nature, *G. vaginalis* on the other hand is again implicated in higher risk of PTB.

Species richness was found to be significantly higher in the preterm group and predictive of preterm birth (cut off, sensitivity, specificity). However, as discussed in section 2.5.4 this finding could be due to significantly more reads in the preterm group. More reads for these samples mean more opportunity for different bacterial species to be identified. Library preparation which includes quantifying the DNA concentration of each sample, normalising and re-quantifying should ensure an equal DNA input for each sample resulting in similar number of reads. This was not the case for our samples and could be due to poor quality DNA measured in the samples which did not result in a read able to identify a bacterial species.

One study investigated several library preparation methods on several different sequencing platforms and found that results from different library preparation methods on the same platform were more varied than results using the same library preparation method on different platforms (Jones et al., 2015). Library preparation was found to influence biases in error profiles, duplication rates, and loss of reads in organisms with a high G+C content (Jones et al., 2015). Despite the potential bias from number of reads, high diversity of the VMB has previously been associated with preterm birth (Shimaoka et al., 2019; Donders et al., 2009; Lee et al., 2009) and should be investigated in future studies.

Species of note that are frequently associated with PTB in other studies are *Ureaplasma* and Mycoplasma species (Haque et al., 2017; Parnell et al., 2017; Sweeney et al., 2017; Kwak et al., 2014; El-Khier et al., 2014). In a recent study, *Ureaplasma urealyticum* was detected in the cervix of 34.5%, *Mycoplasma hominis* in 17.2% and *Ureaplasma parvum* in 85.48% of patients who had a chronic cervical infection and delivered preterm (Barinov et al., 2022). In our study, *Ureaplasma urealyticum* serovar 7 str. (ATCC 27819), *Ureaplasma parvum* serovar 6 str. (ATCC 27818) and an uncultured *Ureaplasma* bacterium was detected in 1, 2 and 3 samples respectively. All were detected in patient AHR_53.3 who delivered preterm, however, due to the low prevalence and abundance of these bacteria no findings were significant.

2.6 Summary & Future work

We have optimised the isolation of bacterial DNA from CVF samples stored for extended periods in PBS at -80 ℃. For these samples it is important not to use a host DNA depletion step as this also degrades bacterial DNA which may have become exposed from lysed bacteria during storage. It is likely that better quality DNA could be obtained from fresh swabs using protocol C outlined in section 2.3.3. However, it is not always possible to isolate DNA from fresh swabs. This takes additional time as extractions cannot be performed on batches of swabs. Additionally, samples from partner countries had to be cryopreserved for transportation. The protocol optimised in this study is suitable for potentially lower quality samples stored in sub-optimal conditions. Current swabs stored for the PRIME study are not stored in PBS which allows for future analysis that may be incompatible with PBS. Reducing the storage time of CVF swabs to less than one year and storing the swabs dry may increase the yield of bacterial DNA and result in fewer samples excluded from analysis.

We also optimised a universal primer pair for amplification of vaginal bacterial sequences. This new pair successfully amplified *Gardnerella* species which are notoriously underrepresented in microbiome studies. Despite this success, it should be acknowledged that amplification using any primer pair will introduce bias as not all bacteria will be amplified equally. Future studies should assess the capabilities of primers prior to use and account for biases in analysis.

A major issue with current microbiome research is the lack of consistency in primer naming and sequences. For example, 27F/1492R is named in many papers, however, the sequence of the primers differs between studies. An accepted database of primer names and sequences would be beneficial for the wider field of microbiome research.

We hypothesised that women who give birth prematurely will have a significantly different VMB profile that can be used to identify those at a higher risk of PTB. We identified three bacteria that were able to predict PTB *(Atopobium vaginae* and *Gardnerella vaginalis* at GTP1 and *Rhizobium rhizogenes* at GTP 1 + 2 + 3) in addition to Ratio of *G. vaginalis* and *L. iners.* We had insufficient sample number to investigate the predictive value of VMB for PPROM. Future studies could aim to target PPROM patients in order to increase numbers.

Based on our analysis we can recommend further research into bacterial species significantly implicated in preterm birth. These are: *Coriobacteriales bacterium, Megasphaera* species, *R. rhizogenes, L. rhamnosus, G. vaginalis,* and *A. vaginae.* Future research should perform targeted analysis of these species while still investigating the vaginal community as a whole. Further *in vitro* research on these species of interest, such as the experiments detailed in chapters 2 and 3, would be beneficial as they would provide insight into the molecular and cellular mechanisms which increase the risk of PTB.

Additional clinical samples from the UK cohort are needed to increase sample size and draw more confident predictive models, particularly for the PPROM subgroup. Of note, we observed no significant findings at GTP2, this is likely due to the smaller sample sizes. Future studies should ensure sufficient samples for statistical significance after the exclusion of samples which did not meet the quality criteria.

The UK cohort can then be compared to the Bangladesh and South Africa cohorts to ensure that predictive methods are inclusive of women in a range of settings, not just those in high income countries. Throughout this project, CVF samples were collected using the same methods and GTP at partner sites in Bangladesh and South Africa. Future work is currently underway and includes the processing and analysis of clinical samples using the same methods, primer pair and analysis. This will allow us to confidently compare the vaginal microbiome of pregnant women around the globe without methodical factors impeding comparisons.

The comparison of VMB in pregnant women from 3 continents using the same methods will provide insight into the effect of lifestyle and setting on the VMB. Additionally, this study will provide strong evidence to determine if women from LMIC are commonly dominated by the *Lactobacillus* species that are considered health promoting in the US and Europe.

The overall aim of this chapter was to characterise and compare the vaginal microbiome of pregnant women delivering at term and preterm from UK (Sheffield Teaching Hospitals). To do this we created a standardised protocol in order to confidently compare vaginal flora throughout pregnancy and its relation to pregnancy outcomes specifically PTB. Future work will use the protocol developed in this work to analyse samples collected in Bangladesh (icddr,b in Dhaka) and South Africa (Groote Schuur Hospital). Future work will aim to collect samples from a range of settings to ensure that prediction methods are relevant to mothers around the world. VMB of women from the UK, Bangladesh and South Africa may significantly differ. Communities considered high risk for PTB from one setting may not have the same predictive power in a different population of women.

3 Chapter 3 Prediction of Preterm Birth using Microbial Metabolites in Cervico-vaginal Fluid

3.1 Introduction

The CVF metabolome provides a snapshot of the vaginal ecosystem which includes host epithelial cells, microbiota, and microbial transformation of host derived products. Bacterial diversity has been shown to strongly influence the metabolite composition of the CVF (McMillan et al., 2015). Up to 40% of all PTBs can be linked to infection (Romero & Dey., 2014; Goldenberg et al., 2008). Therefore, microbial associated metabolites may be able indicate the presence of dysbiosis and/or infection and consequently the risk of PTB.

Only a handful of studies have investigated both the microbiome and metabolome to assess the risk of PTB. A study by Thomas et al, (2015) found that the CVF metabolome was not predictive of PTB. However, other studies did observe significant associations. Metabolites including ethanol, ethylene glycol, glycolate, methanol, isopropanol and formate were observed at increased concentrations in PTB patients (Ansari et al., 2020). Additionally, acetate and the ratio of acetate/glutamate has been associated with preterm birth in several studies (Amabebe et al., 2019; Amabebe et al., 2016a; Amabebe et al., 2016b).

Table 3.1. Metabolomic studies using cervicovaginal fluid swabs from pregnant women. Participant characteristics include symptomatic/asymptomatic, risk level, gestation at sample collection (weeks), number of patients, sPTB = number of patients with spontaneous preterm birth, term = number of patients delivered at term.

Study	Metabolomic Methods	Location	Participant characteristics	Metabolomic findings
(Stafford et al., 2017)	^{1}H-NMR	UK	• Symptomatic at 24–36w (n=37) • Asymptomatic high risk: ○ 20-22w (n=80), sPTB=18, Term=62 ○ 26-28w (n=41), sPTB=12, Term=29	• Increased lactate and succinate were associated with CSTI and CSTII in term delivered patients. • Proposed that decreased lactate and/or succinate could increase the risk of PTB.
(Thomas et al., 2015)	GC-MS	New Zealand	• Low risk, 20w, sPTB=30, Term=30	• No significant metabolites. • No significant correlations with PTB.
(Amabebe, Reynolds, V. L. Stern, et al., 2016)	^{1}H-NMR, Spectrophotometry	UK	• Symptomatic 24–36w (n=82), sPTB=15, Term=67	• Increased acetate concentration is predictive of PTB within 2 weeks of sampling. • Predictive cut-off of >0.53 g/l acetate.
(Amabebe, Reynolds, V. Stern, et al., 2016)	^{1}H-NMR	UK	• Symptomatic 24–36w (n=65), sPTB=11, Term=54 • Asymptomatic low risk 20–22w (n=83), sPTB=0, Term=82 • Asymptomatic high risk ○ 20–22w (n=71), sPTB=26, Term=45 ○ 26-28w (n=58), sPTB = 17, Term=41	• In asymptomatic patients, lactate concentration was higher in low risk compared to high-risk patients. • High CVF acetate predictive of PTB

(Amabebe et al., 2019)	^{1}H-NMR, Spectrophotometry	UK	• Symptomatic, 19–36w, ○ sPTB=17 ○ Term=75	• Acetate was predictive of PTB. • Acetate/Glutamate ratio was associated with preterm delivery within 2 weeks of sampling.
(Ghartey et al., 2015)	UPLC-MS/MS, GC-MS	USA	• Asymptomatic, high-risk • 20–24w (n=20) • 24–28w (n=20) ○ sPTB=10 ○ Term=10	• In term patients, carbohydrate and lipid metabolism was downregulated. • Many dipeptides were significantly reduced in preterm patients. • significant increase in n-acetylneuraminate in preterm patients. • Significant metabolites did not correlate with a short cervical length.
(Ghartey et al., 2017)	UPLC-MS/MS, GC-MS	USA	• Symptomatic women 22–33w, • sPTB=20 • Term=30	• Lipid and carbohydrate metabolism were significantly up regulated in preterm patients • Many dipeptides were significantly reduced in preterm patients. • Significant metabolites did not correlate with a short cervical length.
(Ansari et al., 2020)	^{1}H-NMR	South Korea	• Asymptomatic & symptomatic, 15-35w (n=43) ○ PTB=22 ○ Term birth=21	• Acetone, ethylene glycol, formate, glycolate, isopropanol and methanol were significantly increased in preterm patients. • The same metabolites were predictive of PTB.

Vaginal bacteria associated with term birth such as *Lactobacillus* utilise carbohydrates as energy source exclusively and produce lactic acid which lowers the pH of the CVF (Amabebe & Anumba, 2018). Those organisms associated with PTB such as Gardnerella, Prevotella, Megsaphaera, Atopobium, Ureaplasma and Eggerthella are associated with increased acetate, butyrate, succinate and reduction in lactic acid and amino acids (Amabebe & Anumba, 2020). These anaerobic bacteria are able to metabolise the listed metabolites from carbohydrates and amino acids present in the CVF, which subsequently increases vaginal pH (Srinivasan et al., 2015).

To summarise, as the CVF represents a dynamic environment the metabolic profile can provide insight into the vaginal ecosystem which can be used to predict dysbiosis and PTB. There have been limited studies that identify microbial-metabolite markers. More studies are therefore required to generate robust data that can be used for prediction of sPTB and for the subsequent stratification of women at risk.

3.1.1 Mass Spectrometry

We have chosen to analyse the metabolomic signature of CVF swabs with mass spectrometry. Specifically, a method involving direct injection of liquid samples followed by electrospray ionisation and time of flight mass analysis was utilised similar to (Overy et al., 2005).

Electrospray ionisation (EIS) is a method which gives each particle a charge, either positive or negative depending on the mode. In the negative mode, analytes are charged through deprotonation resulting in a negative charge. While in the positive mode analytes are charged through protonation resulting in a positive charge. Depending on the analyte in question, one mode may be more suitable for detection(Ho et al., 2003). Because of this samples were first run on the positive mode followed by the negative mode and data was pooled.

This method is suitable for our samples as mass spectrometry detects a wide range of particle masses and particles are ionised directly from a volatile solvent. It also has a high ionisation efficiency and allows detection of small molecules (El-Aneed et al., 2009).

The liquid sample is held in a narrow metal capillary and a voltage is applied. The liquid is pulled into an elliptical shape, when a threshold voltage is reached the elliptical shape becomes a cone and a spray of particles is emitted from the point of the cone (Wilm, 2011) (Figure 3.1).

Once the particle droplets are in flight there are two current theories to how to the droplets become ionised particles, the ion evaporation model and the charge residue model. In both models, droplets reduce in size until the drops are the size of a single ion which continues through the machine to the time-of-flight mass analyser.

The time-of-flight mass analyser consists of charged plates which accelerate ions coming from the EIS using the same voltage. This acceleration will produce varying velocities based on the mass of the ion. Molecules with a smaller mass will travel faster and reach the detector quicker than molecules with a bigger mass (Mirsaleh-Kohan et al., 2008). The mass to charge ratio (M/Z) is then calculated for each ion.

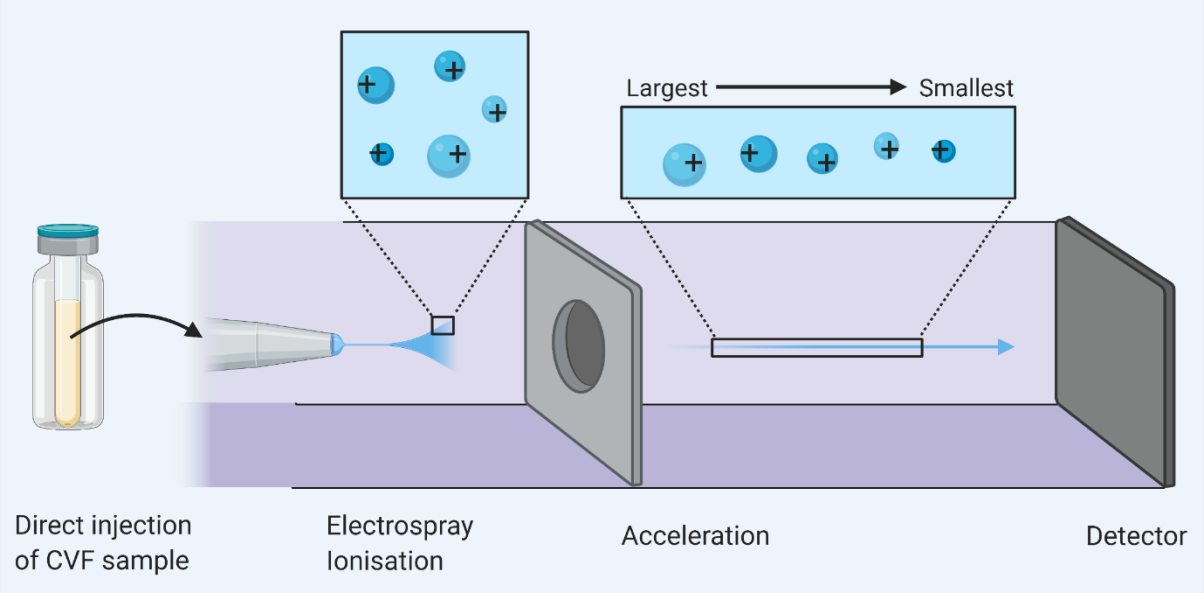


Figure 3.1 Mass spectrometry methods. Sample introduced via direct injection, ionised by electrospray ionisation, time-of-flight analysis.

3.1.2 Aims

The overall aim of this chapter was to characterise and compare the vaginal metabolome profile of pregnant women delivering at term and preterm from UK (Sheffield Teaching Hospitals).

Objectives:

1. To analyse the metabolome of clinical swabs from pregnant women using mass spectrometry
2. To compare results from term, preterm and PPROM delivered patients to identify potential biomarkers and vaginal organisms associated with PTB
3. To correlate the vaginal microbiome and metabolome data

We hypothesise that women who give birth prematurely will have a significantly different metabolite profile that can be used to identify a higher risk of PTB.

We hope to better understand which metabolite patterns suggest increased risk for PTB in order to develop PTB predictive models applicable to women around the world.

3.2 Methods

We aimed to characterise the cervicovaginal metabolome of pregnant women at 2 gestational time points to identify potential biomarkers for increased risk of PTB. Additionally, we aimed to correlate the vaginal microbiome data (presented in chapter 4) with the metabolome in order to gain a broader view of the host-microbiome interaction and to improve identification of microbial associated metabolite biomarkers.

3.2.1 Patient recruitment

Metabolomic experiments used the same swabs as the microbiome experiments therefore patient recruitment and data collection was identical. Please see section 2.3.1.

The ECCLIPPx II study was conducted with Human Research Authority approval from the Yorkshire & the Humber Research Ethics Committee (17/YH0179) in alignment with Sheffield Teaching Hospitals NHS Trust regulations, registration number STH19385. Between May 15th, 2018, and August 1st 2019

The PRIME study was conducted between 1st March 2018 and 25th March 2021 with approval by the Human Research Authority, the Health and Care Research Wales (HCRW) (IRAS project ID: 256135, REC reference: 18/LO/2044), and the Sheffield Teaching Hospitals NHS Trust research regulations (Protocol number: STH20635).

3.2.2 Sample preparation

To prepare clinical samples for storage and subsequent analysis swabs were processed as follows:

Samples from the PRIME study were stored dry at -20 °C for up to 3 days and were subsequently transferred to -80°C until processing and analysis. During processing, the tip of each swab, saturated with CVF, was cut off and placed into a 1.5 mL microfuge tube. Six hundred microliters of sterile PBS at pH 7.4 was added to the tube and was vortexed at 300 revolutions per minute (rpm) for 5 minutes. The swab tip was removed, and the elute was centrifuged for 3 min at 13,000 rpm. The supernatant was aspirated into a fresh tube and stored at -80 °C for a short time until mass spectrometry analysis. Storage of CVF samples at either -20 °C or -80 °C has been shown to have no effect on metabolomic analysis (Bai et al.,

2012). Control swabs containing no clinical sample were processed and stored with the samples until mass spectrometry analysis.

After thawing, samples were mixed and again centrifuged at 12,000 rpm for 2 minutes and diluted 1:10 with 50:50 methanol (Honeywell, LC-MS grade) and ultrapure water (ELGA water purification system) for mass spectrometry analysis.

3.2.3 Mass spectrometry

In order to identify the metabolite profile of the clinical samples, An ACQUITY ultra-precision liquid chromatography system (UPLC) (Waters, UK) coupled to a SYNAPT G2-Si Time of Flight mass spectrometer with electrospray sample introduction (Waters, UK) was used with the UPLC acting only as an automated injector (see Figure 3.1). Samples were analysed in both positive and negative ionisation modes to accurately measure both positive and negatively charged molecules. In the negative mode, analytes are charged through deprotonation resulting in a negative charge. While in the positive mode analytes are charged through protonation resulting in a positive charge.

Samples were analysed in triplicates, 10ul of each sample was directly injected into the mass spectrometer three times to give technical repeats. Data was collected over the mass range 50-800m/z with a scan time of 1sec per scan. Experimental conditions can be found in Table 3.2.

Table 3.2 Conditions for Sample Introduction into SYNAPT G2-Si Time of Flight mass spectrometer using electrospray sample introduction for positive and negative modes.

	Mode	
	Positive	**Negative**
Capillary Voltage	3kV	2kV
Sample Cone	100V	80V
Source Offset	50V	80V
Desolvation Gas Flow	600L/hr	600L/hr
Desolvation Temperature	280°C	280°C
Source Temperature	100°C	100°C

3.2.4 Data processing

The initial output from the mass spectrometer provided a mass spectrum for each technical repeat. The spectrum was converted into a list of m/z (mass to charge ratio) with intensity at each mass. To remove data set noise, an in-house macro was used to identify metabolites that were present in all 3 technical repeats as performed in (Overy et al., 2005). Metabolites not present in all technical repeats were discarded. Data was then grouped into 0.2 amu sized bins for ease of handling, as recommended by a previous study (Amabebe et al., 2021). Each metabolite was then normalised against the total intensity for that sample. This is to account for decreasing intensity as more samples are measured due to build-up of sample on the sensor.

3.2.5 Metabolite identification

Putative IDs were assigned by matching the accurate mass to the Humancyc database (https://humancyc.org/) using a tolerance of 20 ppm and were cross checked against the HMDB human metabolome database (https://hmdb.ca/)

3.2.6 Metabolomic Data Analysis

3.2.6.1 Multivariate Analysis

In order to visualise the variation within the samples Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) analysis was performed using SIMCA 15.0.2.

3.2.6.2 Bacterial correlation

In order to correlate the microbiome and metabolome data, Omu metabolomics analysis package in R (version 4.0.3) was used to produce heat plots. To identify differences in normalised % total ion count between preterm vs term and GTP1 vs GTP2, GraphPad Prism 9.4.1 was used to produce abundance and change in abundance graphs including a Kruskal Wallis test.

3.3 Results

We aimed to characterise the cervicovaginal metabolome of pregnant women at 2 gestational time points to identify potential biomarkers for increased risk of PTB. Additionally, we aimed to correlate the vaginal microbiome data (presented in chapter 2) with the metabolome in order to gain a broader view of the host-microbiome interaction and to improve identification of microbial associated metabolite biomarkers.

3.3.1 Demographic analysis

A total of 93 swabs from 51 patients were analysed. Most of the patients had samples collected at 2 time points (n=45, 31 term, 14 preterm). However, 6 patients only had one sample collected. Of those with one sample, 3 preterm patients delivered before GTP2 sample could be collected and 3 term patients had not reached GTP2 when analysis was performed (Table 3.3).

In our study population most participants were white (83.7%). PTB rate was 34.7% (17/49). In the preterm group, 23.5% of participants had PPROM. Maternal age, body mass index (BMI), and CVF fetal fibronectin concentration was not significantly different in the term vs preterm group (Table 3.3)

Table 3.3 Sample and patient numbers at each gestational time point (GTP)

		Term	Preterm	Total
Samples	GTP1	32	17 (4 PPROM)	49
	GTP2	31	14 (3 PPROM)	45
	Total Samples	63	30	93
Patients	Patients with 1 GTPs	3	3	6
	Patients with 2 GTPs	31	14	45
	Total Patients	34	17	51

PPROM (preterm pre-labour rupture of membranes).

Table 3.4 Patient demographic and clinical data

	GTP1		GTP2	
	Term (n=32)	Preterm (n=17)	Term (n=31)	Preterm (n=14)
Maternal factors				
Age (years)	31.28 ± 5.83	28.76 ± 6.09	31.65 ± 6.06	29.71 ± 5.54
BMI (Kg/m^2)	26.80 ± 5.84	26.18 ± 4.68	27.27 ± 6.28	26.94 ± 4.05
FFN (ng/mL)	4.0 ± 1.80	4.35 ± 0.93	3.65 ± 1.40	3.29 ± 1.64
GAP (days)	146.2 ± 7.86	140.3 ± 6.56	186.8 ± 6.69	182.9 ± 9.12
GAD (days)	273.2 ± 8.20	227.9 ± 28.28	273.2 ± 8.47	233.5 ± 21.49
Smoker n, (%)	8, (25)	1, (5.9)	7, (22.6)	1, (7.1)
PPROM	0	4	0	3
Ethnicity (n)				
White	27	14	26	12
Black	1	0	1	0
Asian	3	3	3	2
Hispanic	1	0	1	0
Treatment (n)				
Progesterone	1	1	3	2
Cerclage	0	0	0	0
Both	1	0	2	0

Age, BMI (body mass index), FFN (fetal fibronectin), GAP (gestation at presentation), GAD (gestation at delivery) are presented as Mean ±SD. PPROM (preterm prelabour rupture of membranes).

3.3.2 Multivariate analysis

A total of 1842 metabolites were identified using mass spectrometry. To visualise factors of variation within the dataset additional Orthogonal projections to latent structures Discriminant Analysis (OPLS-DA) were performed. The variation between GTP1 and GTP2 were investigated, a grouping of samples can be seen at both term and preterm in the negative mode. This can be seen to a lesser extent in the positive mode (Figure 3.2).

Similarly, in Figure 3.3 which shows variation between pregnancy outcome, some separation of term, preterm and PPROM samples can be seen at both GTP1 and GTP2 in the negative mode. The positive mode has less separation in pregnancy outcome and does not have distinct groupings (Figure 3.2).

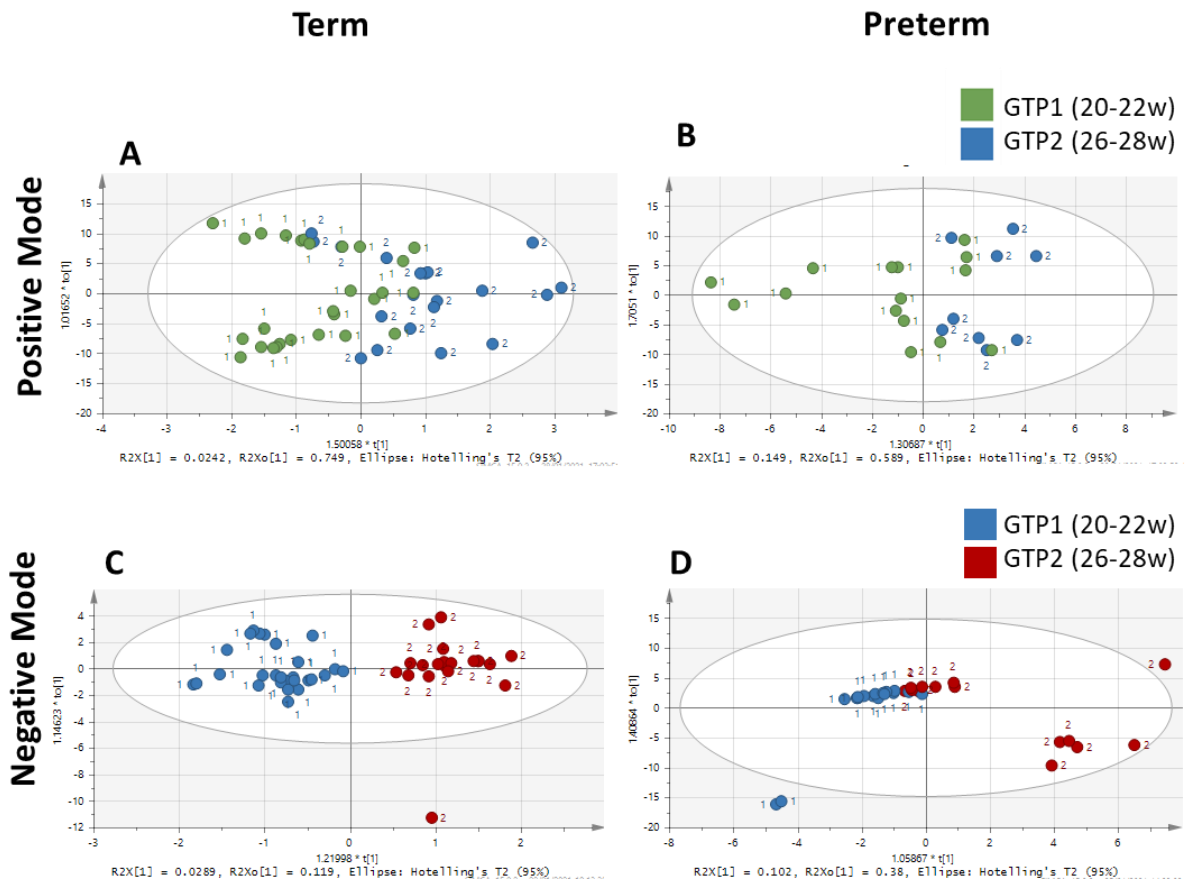


Figure 3.2 Orthogonal projections to latent structures Discriminant Analysis (OPLS-DA) of mass spectrometry from cervicovaginal swab samples, coloured by Gestational Time Point (GTP). (Term n=63, Preterm n=24, PPROM n=7). Graphs generated by Dr Heather Walker.

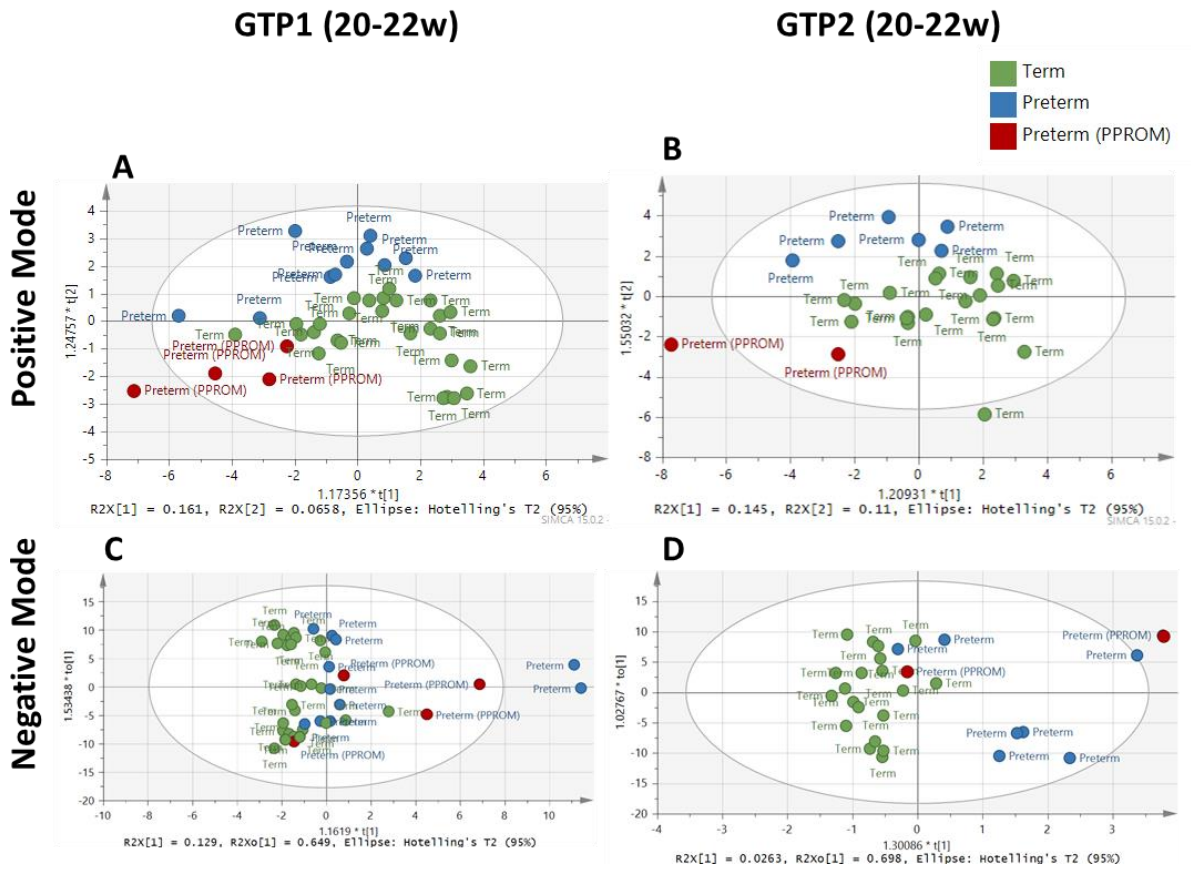


Figure 3.3 Orthogonal projections to latent structures Discriminant Analysis (OPLS-DA) of mass spectrometry from cervicovaginal swab samples, coloured by outcome. **A)** positive mode GTP1 **B)** positive mode GTP2 **C)** negative mode GTP1 **D)** negative mode GTP2. GTP1 = Term n=32, Preterm n=13, PPROM n=4. GTP2 = Term n=31, Preterm n=11, PPROM n=3. Graphs generated by Dr Heather Walker

3.3.3 Abundance

To investigate metabolite changes from GTP1 to GTP2 in term vs preterm we performed an ANOVA on the 4 groups of interest (GTP1 Term vs GTP1 preterm vs GTP2 term vs GTP2 preterm) (Figure 3.4).

Focussing on the preterm group, giganin and urate were found to significantly increase from GTP1 to GTP2. While cycloartenol, lanosterol and phytoene were found to significantly decrease from GTP1 to GTP2

In contrast, in the term group adenosine and deoxyguanosine significantly decreased from GTP1 to GTP2

3.3.3.1 Change in Abundance

To further investigate the metabolites that could be used for prediction of PTB, change in metabolite abundance from GTP1 to GTP2 in term vs preterm was plotted (Figure 3.5). This was calculated by subtracting the abundance at GTP2 from abundance at GTP1. Therefore, a positive value indicates increasing abundance and a negative value indicates a decreasing abundance from GTP1 to GTP2.

We observed that in the preterm group Adenosine, Pantothenate dehydrosafynol, deoxyguanosine, Urate, Giganin and Nonacosane abundance **increased** significantly compared to term samples. While Phytoene abundance **decreased** significantly.

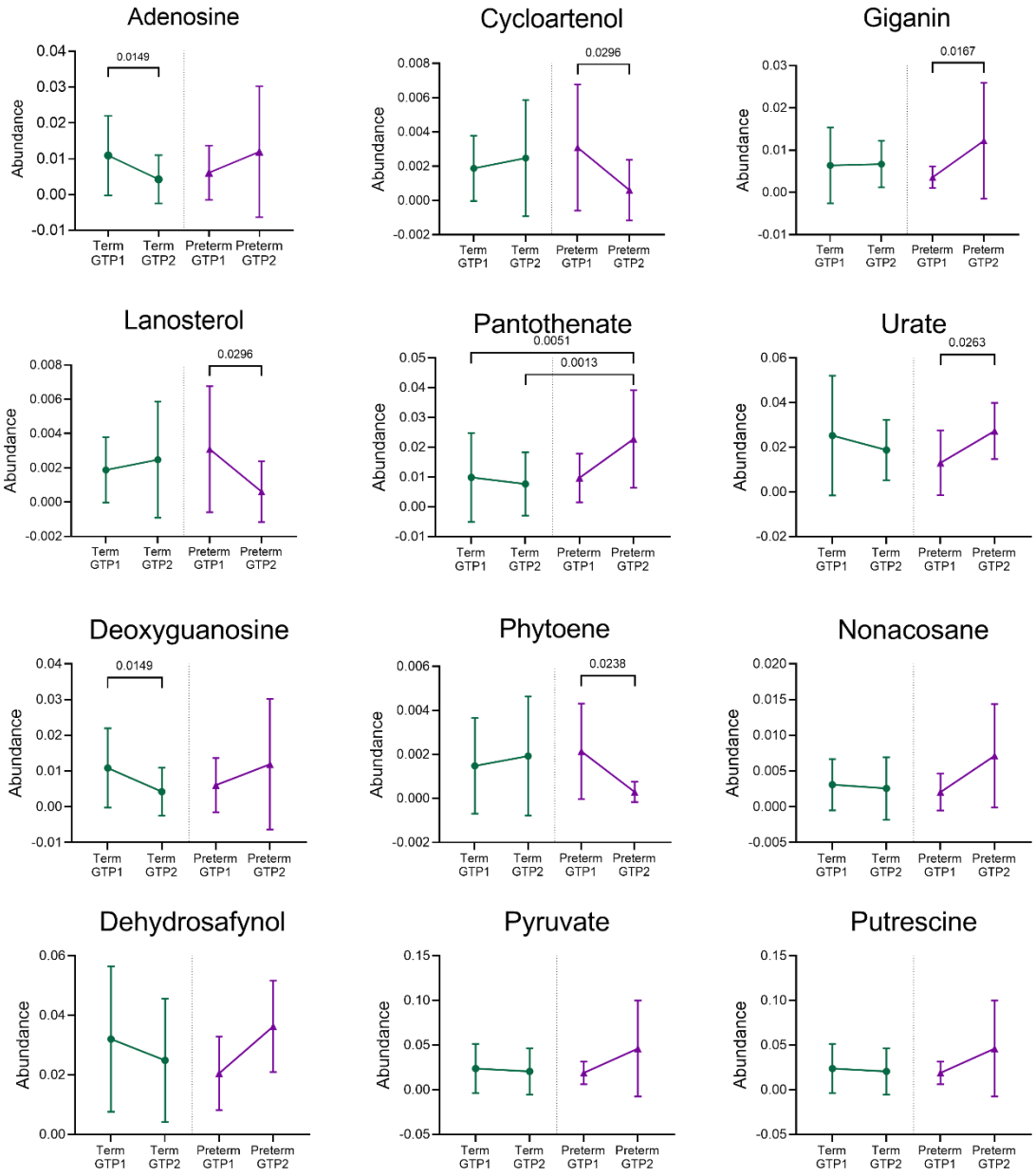


Figure 3.4 Metabolite abundance in term (>37 w gestation) and preterm (<36+6 w gestation) samples. Gestational time point (GTP) 1 = 20-22 weeks, 2 = 26-28 weeks. Term n=31, preterm n=14. Abundance = normalised % total ion count. Points represent mean, error bars represent standard deviation. Analysis performed on GraphPad Prism.

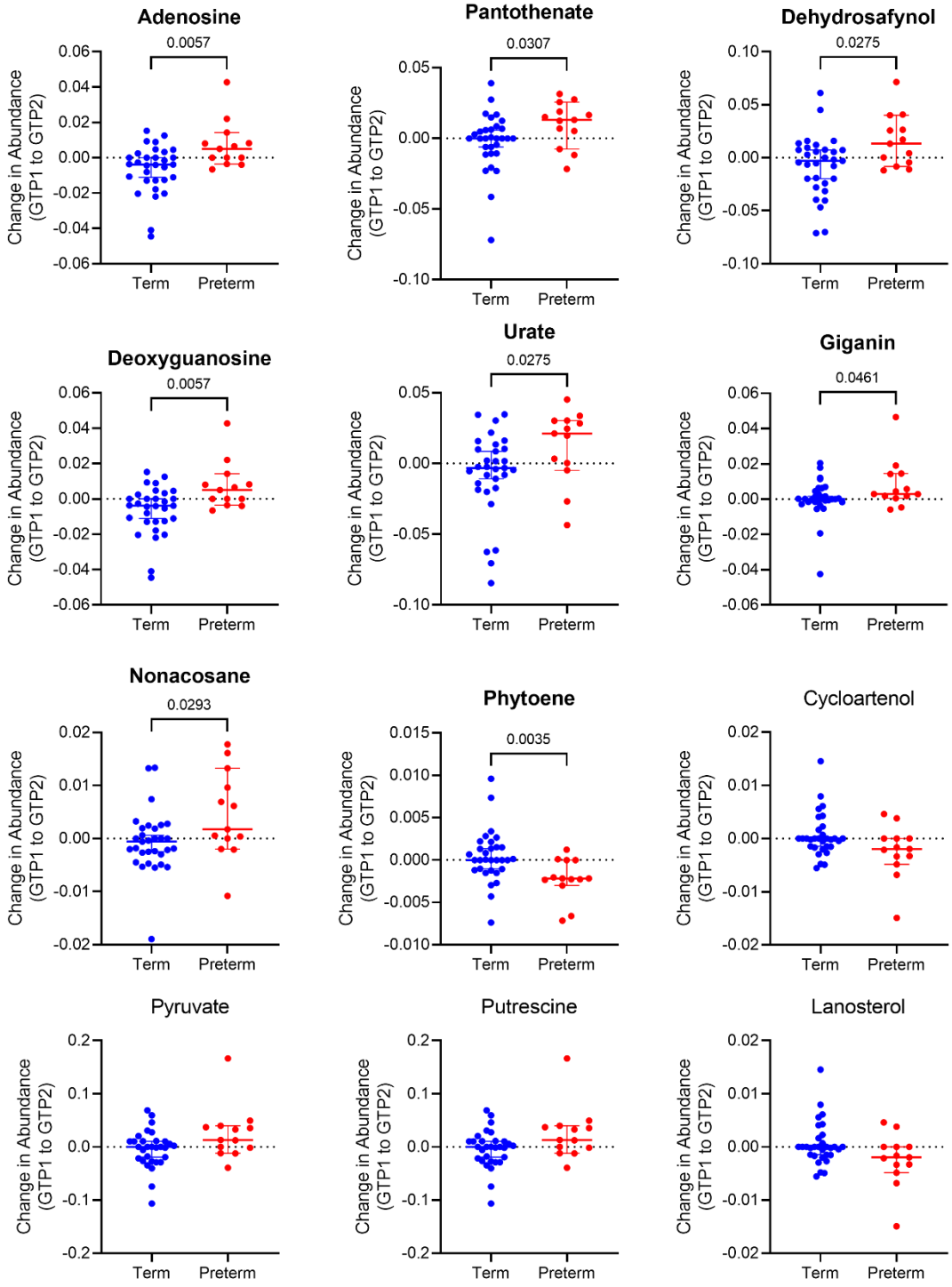


Figure 3.5. Change in abundance from GTP1 to GTP2 (GTP2 abundance minus GTP1 abundance) in term (>37 w gestation, n=31) vs preterm (<36+6 w gestation, n=14). Gestational time point (GTP) 1 = 20-22 weeks, 2 = 26-28 weeks. Abundance = normalised % total ion count. Line represents median, error bars represent 95% confidence interval. Analysis performed using GraphPad Prism.

3.3.4 Metabolome & Microbiome correlation

Metabolomic analysis was performed on a total of 94 samples, microbiome analysis was performed on a total of 60 samples, 45 samples had both microbiome and metabolome data. However, 17 samples did not pass the microbiome quality control. Therefore, 28 samples were included in metabolome/microbiome correlation (Figure 3.6).

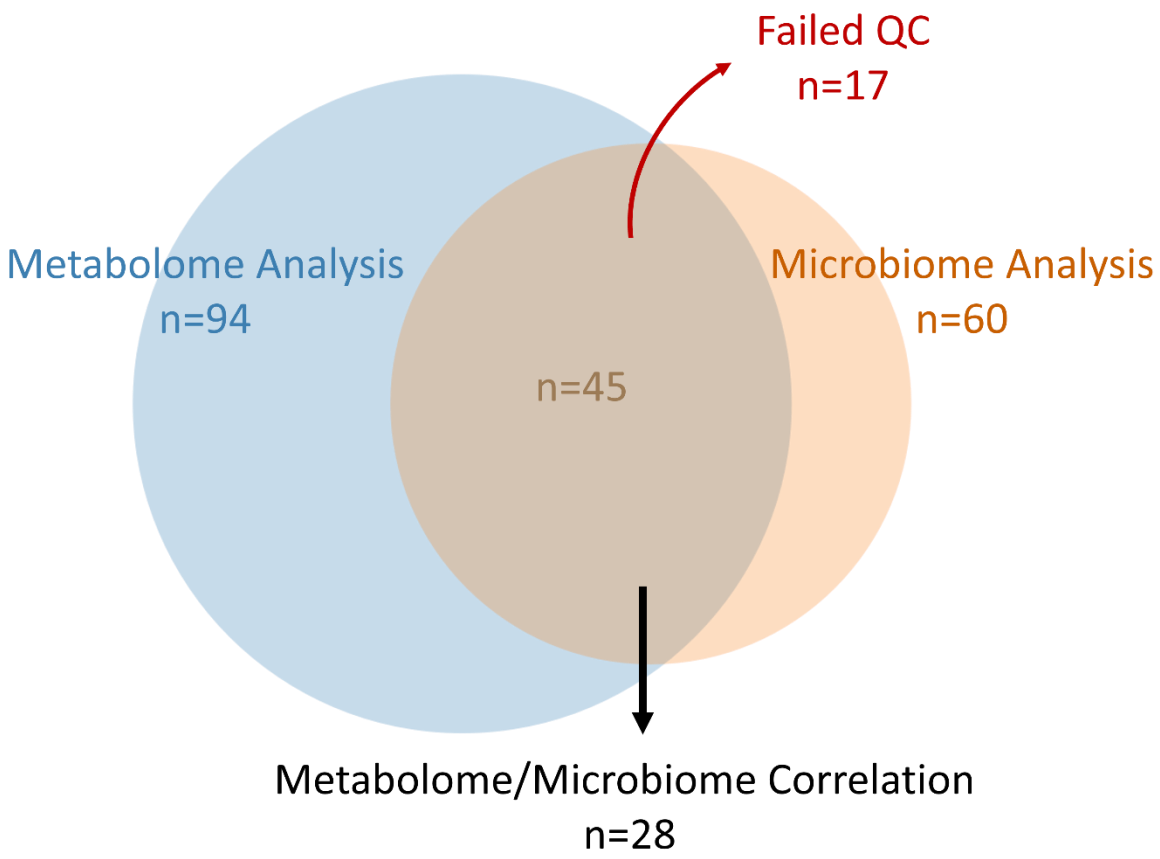


Figure 3.6 Sample numbers of metabolite and microbiome data. QC= Quality Control. GTP1 n=13, GTP2 n=15, Term n=26, Preterm n=2

3.3.4.1 Gestational Time Point 1 (20-22 weeks)

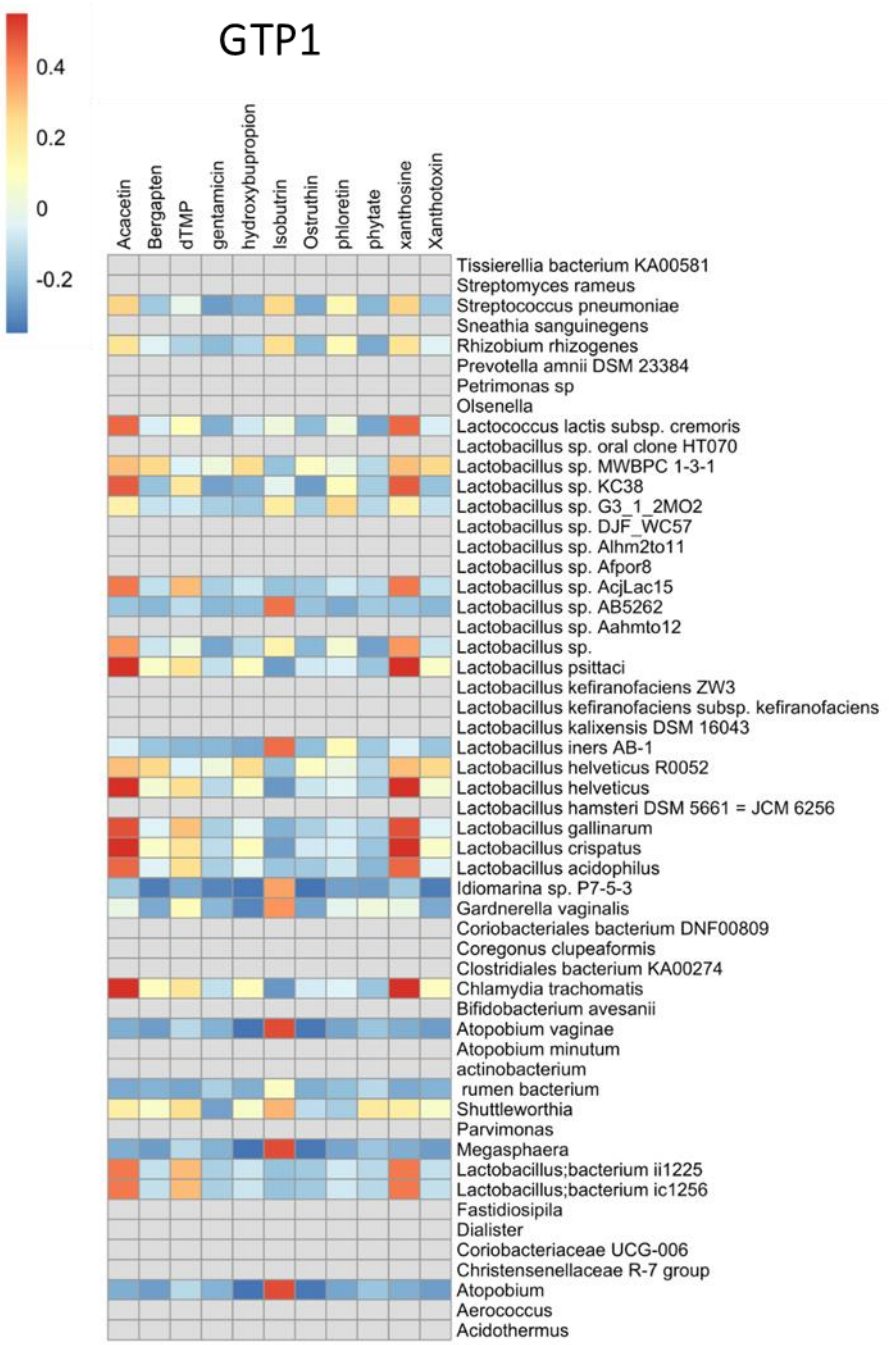


Figure 3.7 Correlation between bacterial species and metabolites identified from cervicovaginal fluid swabs at Gestational time point (GTP) 1 (20-22 w) (n=13). Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red). Only correlations with P values < 0.05 are represented. Graph generated by Dr Neha Kulkarni.

3.3.4.2 Gestational Time Point 2 (26-28 weeks)

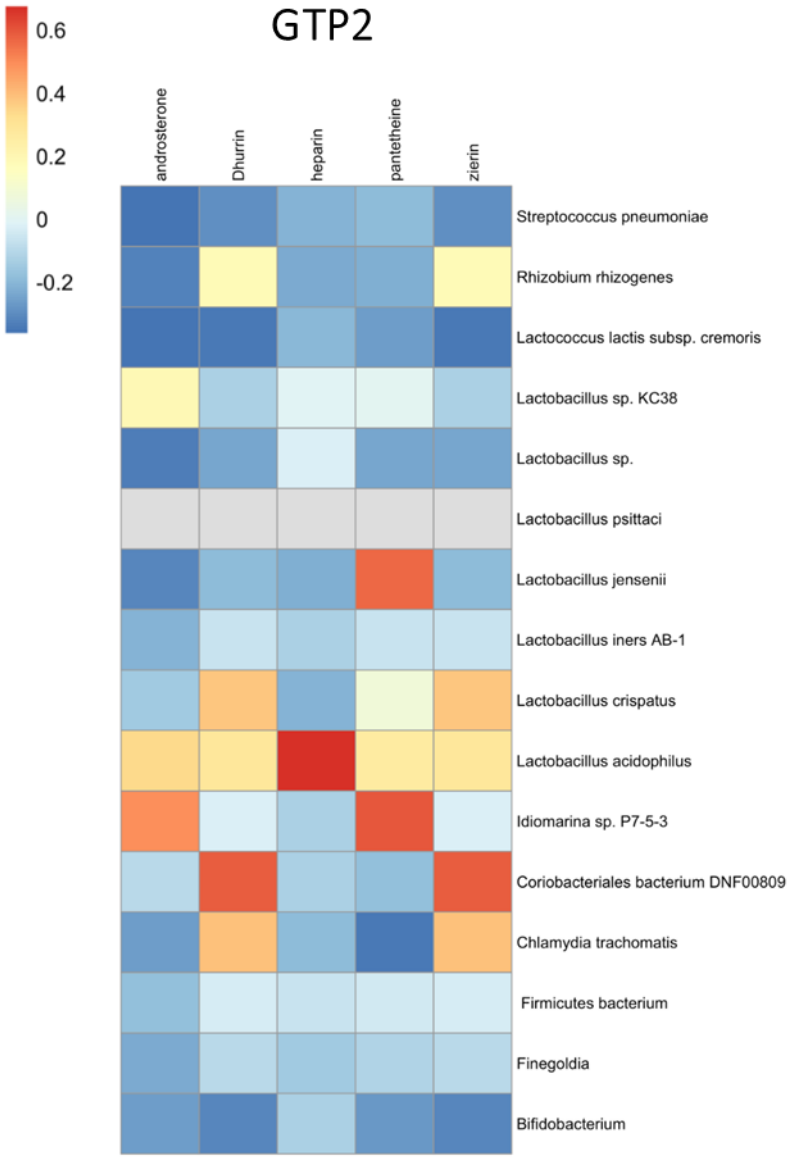


Figure 3.8 Correlation between bacterial species and metabolites identified from cervicovaginal fluid swabs at Gestational time point (GTP) 2 (26-28 w) (n=15). Pearson's correlation coefficients (r, colour) range from -1 (dark blue) to +1 (dark red). Only correlations with P values < 0.05 are represented. Graph generated by Dr Neha Kulkarni.

3.3.5 Predictive analysis

In order to identify metabolites that are able to predict PTB, we created ROC curves for each metabolite found to differ between term and preterm (Figure 3.4). Area under the ROC curve is a representation of sensitivity and specificity, 0.5 indicates no predictive value, 1 represents 100% positive predictive value, 0 represents 100% negative predictive value.

We found that Pantothenate and Phytoene abundance were able to predict PTB at GTP2. Additionally, the increase or decrease in abundance from GTP1 to GTP2 was found to be significant for pantothenate, phytoene, adenosine, dehydrosafynol, giganin, nonacosane and rate (Table 3.5).

Table 3.5 Predictive values of metabolites identified from clinical cervico-vaginal swabs. Analysis performed on GraphPad Prism.

Metabolite	GTP	Area Under ROC Curve	95% Confidence Interval	P value
Pantothenate	GTP1	0.58	0.41 to 0.74	0.3891
	GTP2	0.83	0.71 to 0.95	***0.0009**
	Change from GTP1 to GTP2	0.71	0.52 to 0.89	***0.0317**
Phytoene	GTP1	0.62	0.45 to 0.79	0.1722
	GTP2	0.71	0.56 to 0.86	***0.0324**
	Change from GTP1 to GTP2	0.77	0.63 to 0.92	***0.0045**
Adenosine	GTP1	0.65	0.48 to 0.81	0.097
	GTP2	0.61	0.41 to 0.80	0.2728
	Change from GTP1 to GTP2	0.76	0.62 to 0.91	***0.0067**
Cycloartenol	GTP1	0.59	0.42 to 0.76	0.3034
	GTP2	0.69	0.52 to 0.86	0.058
	Change from GTP1 to GTP2	0.67	0.49 to 0.86	0.0738
Dehydrosafynol	GTP1	0.61	0.44 to 0.78	0.2114
	GTP2	0.69	0.52 to 0.85	0.058
	Change from GTP1 to GTP2	0.71	0.55 to 0.88	***0.0279**
Deoxyguanosine	GTP1	0.65	0.48 to 0.81	0.097
	GTP2	0.61	0.41 to 0.80	0.2728
	Change from GTP1 to GTP2	0.76	0.62 to 0.91	***0.0067**

Giganin	GTP1	0.65	0.49 to 0.80	0.097
	GTP2	0.6	0.41 to 0.80	0.291
	Change from GTP1 to GTP2	0.69	0.51 to 0.87	***0.0462**
Lanosterol	GTP1	0.59	0.42 to 0.76	0.3034
	GTP2	0.69	0.52 to 0.86	0.058
	Change from GTP1 to GTP2	0.67	0.49 to 0.86	0.0738
Nonacosane	GTP1	0.63	0.47 to 0.79	0.1473
	GTP2	0.69	0.49 to 0.88	0.0617
	Change from GTP1 to GTP2	0.71	0.53 to 0.89	***0.0297**
Putrescine	GTP1	0.53	0.36 to 0.69	0.7687
	GTP2	0.66	0.48 to 0.85	0.0986
	Change from GTP1 to GTP2	0.68	0.50 to 0.86	0.057
Pyruvate	GTP1	0.53	0.36 to 0.69	0.7687
	GTP2	0.66	0.48 to 0.85	0.0986
	Change from GTP1 to GTP2	0.68	0.50 to 0.86	0.057
Urate	GTP1	0.66	0.49 to 0.83	0.0616
	GTP2	0.69	0.51 to 0.87	0.0546
	Change from GTP1 to GTP2	0.71	0.53 to 0.89	***0.0279**

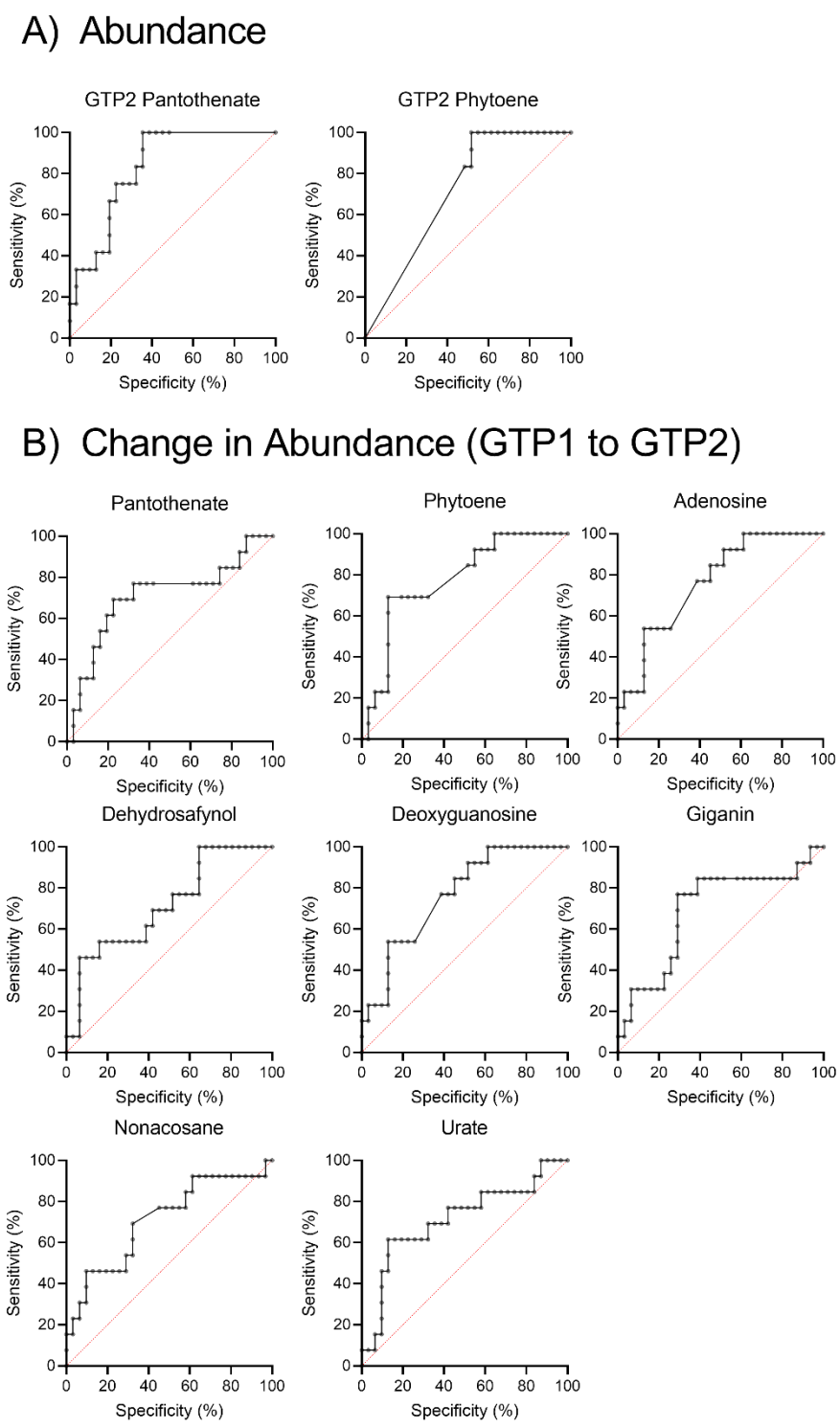


Figure 3.9. Predictive ROC curves for **A)** Abundance of pantothenate and phytoene at Gestational time point (GTP) 2, Term = 31, Preterm = 14 **B)** Change in abundance from GTP1 to GTP2 Term = 31, Preterm = 13. Analysis performed on GraphPad Prism.

3.4 Key Findings

- Putrescine, pyruvate, phytofluene and pantothenate were significantly higher and increased from GTP1 to GTP2 in the preterm group compared with the term group.
- Phytoene and isoliquiritigen were also higher in the preterm group at GTP1 but were found to decrease at GTP2 compared with the term group.
- Lanosterol and cycloartenol were higher in the preterm group at GTP1 but decreased in both groups at GTP2.
- Adenosine, dehydrostafynol, deoxyguanosine, jervine, nonacosane, thiamine and urate were found to be significantly lower in the preterm group at GTP1 but became increased at GTP2 compared with the term group.

PTB Prediction

- Pantothenate and Phytoene abundance was able to predict PTB at GTP2.
- The increase or decrease in abundance from GTP1 to GTP2 was found to be significant for Pantothenate, Phytoene, Adenosine, Dehydrosafynol, Giganin, Nonacosane and Urate.

Microbiome-Metabolome correlation

- At GTP1, Isobutrin Phloretin Phytate Xanthosine and Xanthotoxin were found to correlate with the microbiome data
- At GTP2, Androsterone Dhurrin Zierin Pantetheine Heparin were found to correlate with the microbiome data

3.5 Discussion

The CVF metabolome provides a snapshot of the vaginal ecosystem which includes host epithelial cells, microbiota, and their metabolic interactions. By investigating both the microbiome and metabolome we can gain more insight into the vaginal ecosystem and identify which bacterial species and their metabolic interactions can increase the risk of PTB. Identifying metabolites associated with PTB would enable prediction and earlier intervention. For example, preterm outcomes are generally associated with decreased lactic acid-producing bacteria and therefore lower lactate concentrations (Aldunate et al., 2015). While term pregnancies are generally considered to be associated with a lactate-rich CVF (Wiberg-Itzel et al., 2005).

Additionally, PTB is associated with increased mixed anaerobic bacteria and increased concentrations of SCFAs including acetate, propionate, butyrate, and succinate (Amabebe, Reynolds, V. Stern, et al., 2016; Aldunate et al., 2015)

In this study I attempted to identify metabolites predictive of PTB. CVF samples were collected from 51 patients. Maternal age, body mass index (BMI), and CV fetal fibronectin concentration were not significantly different in the term vs preterm group which allows us to confidently compare the two groups.

3.5.1 OPLS-DA

A supervised OPLS-DA was conducted to ascertain if our metabolomic methods were able to classify patients according to outcome. Our results in Figure 3.3 demonstrate that our metabolomics data was able to distinguish between term, preterm and PPROM at both GTP. With supervised methods there is a risk of overfitting (Lamichhane et al., 2018), this is a modelling error which results in prediction values only being applicable to the initial dataset. Our finding would benefit from validation using another cohort, however previous studies have identified metabolites ability to distinguish between term and preterm using this method with amniotic fluid (Romero et al., 2010). Additionally, studies using mass spectrometry have successfully classified term and preterm patients in several cohorts (Ghartey et al., 2015, 2017). Therefore, we are confident that this result is accurate.

In addition to predicting outcome, the OPLS-DA was able to distinguish samples taken at GTP1 and GTP2 (Figure 3.2). As each GTP represents a distinct timepoint during gestation, and has

been shown to have a differing microbiome and metabolome (Kaur et al., 2020), GTP was assessed separately for their capacity to predict PTB. Additionally, the change in metabolites from GTP1 to GTP2 was assessed as this provides more insight into the dynamic changes and demonstrates if the concentration of a metabolite associated with a high risk of PTB is stable, increasing or decreasing.

Abundance

The most significant metabolites were Phytoene and Pantothenate.

3.5.1.1 Phytoene

Phytoene, a part of the carotenoid biosynthesis pathway which includes vitamin A, was found to increase from GTP1 to GTP2 significantly more in the term than the preterm group and was predictive of PTB with AUC of 0.77. Carotenoids are found in green leafy vegetables and yellow/orange fruits (Thorne-Lyman & Fawzi, 2012). Lower abundance of Phytoene in preterm delivered women may be linked to poor maternal nutrition. One study found a 33% reduction in the prevalence of preterm birth and a 66% reduction in early preterm birth in women who took Vitamin A (Coutsoudis et al., 1999). However, this study is alone in its finding. A meta-analysis did not support this recommendation for vitamin A or carotenoid supplements (Thorne-Lyman & Fawzi, 2012). Coutsoudis' study investigated HIV positive pregnant women which may contribute to their finding. Additional research on Phytoene is required before a link to PTB can be established.

3.5.1.2 Pantothenate

Pantothenate or pantothenic acid or vitamin B5 plays a critical role in energy-yielding metabolic reactions including carbohydrate, fat and, to a lesser extent, protein metabolism (Pearlman & Almandoz, 2020). Pantetheinase is an enzyme that catalyses the breakdown of pantetheine to pantothenate and cysteamine (Boersma et al., 2014). As pantothenate is found in many food types, deficiency is rare in humans (Pearlman & Almandoz, 2020).

We found that pantothenate decreased in term but increased in preterm patients from GTP1 to GTP2. Abundance at GTP2 was found to be predictive of PTB with an AUC of 0.83 and change in abundance from GTP1 to GTP2 was also predictive of PTB with an AUC of 0.71. My findings are in line with previous work on pantothenate.

A previous study of CVF also found pantothenate to be significantly higher in the preterm group (Ghartey et al., 2017). The pantothenate pathway is thought to promote oxidative tissue damage and inflammation (Nitto & Onodera, 2013) which may account for its association with PTB which is characterised by inflammatory processes (Hadley et al., 2018). However, the metabolome is a complex system that is also influenced by the host and the microbiome.

From a host perspective, an Indonesian study investigated the impact of taking calcium supplements, including calcium pantothenate, on PTB but found no correlation (Brilian & Virginia, 2017). This highlights an additional dietary source of pantothenate which may influence concentrations within the CVF. Data on dietary supplements should be collected in future studies as this information is not commonly found in medical records.

From a microbiome perspective, Yao et al, (2018) found that the concentration of pantothenate in growth media for *Lactobacillus helveticus* affected the expression of the main enzymes of glucose metabolism. This suggests that pantothenate is an essential part of some *Lactobacillus* species metabolism. Unfortunately, similar research has not been performed on common vaginal species such as *L. crispatus,* or *L. iners.* Additional research into the metabolic pathways of both host and VMB would further elucidate the metabolic interactions which may allow co-colonisation and increase the risk of PTB.

3.5.1.3 Adenosine

Adenosine is a molecule implicated in a wide range of functions such as nucleotide biosynthesis, cellular energy metabolism. It is considered a signalling molecule (Eltzschig, 2009). We found that adenosine significantly increased from GTP1 to GTP2 in preterm patients compared to term and was predictive of PTB with an AUC of 0.76.

Adenosine concentration has been shown to be significantly higher in the third trimester of normal pregnancies (Yoneyama et al., 2000) but is also associated with preeclampsia (Salsoso et al., 2017). Previous studies have not yet linked adenosine and PTB. ATP is considered a danger associated molecular pattern (DAMP) and is released by hypoxic placental tissues, as a protective mechanism. ATP (Adenosine 5′-triphosphate) can be hydrolysed into adenosine which is anti-inflammatory (Spaans et al., 2014). Therefore, high levels of adenosine in the first and second trimester may indicate cell stress in gestational tissues.

3.5.1.4 Giganin

Giganin was first discovered by Fang et al, (1993) and is reported to be a "annonaceous acetogenin" meaning it belongs to a class of polyketide products found in plants of the family Annonaceae.

Annonaceous acetogenins have been found to have many biological properties including cytotoxicity against cancer cells (Neske et al., 2020). Additionally, they have been found to promote biofilm formation. Itrabin was found to strongly stimulate biofilm formation of *Pseudomonas monteilii* (Alessandrello et al., 2017) and squamocin was found to stimulate *Bacillus atrophaeus* CN4 biofilm formation. While these studies are not investigating microbiome biofilms, they provide potential insight into the relevance of giganin in the vaginal metabolome.

However, as giganin has not been identified in human metabolome studies before, additional experiments should be performed to confirm the identity of this metabolite.

In the current study, giganin was found to significantly increase from GTP1 to GTP2 in preterm patients. The increase in abundance was found to be predictive of PTB with AUC of 0.69. From the limited literature on giganin and annonaceous acetogenins the link to PTB is unclear. One possibility is that giganin is microbial-derived and is linked with vaginal microbe biofilm formation. Another possibility is that as the plant family Annonaceae includes some tropical fruits, giganin could be derived from maternal diet. However, giganin has not been reported in fruits and was identified in tree bark (Fang et al., 1993). Significantly more research and additional experiments to confirm the source of this metabolite in our samples is required before a link between giganin and PTB can be established.

3.5.1.5 Lanosterol

Lanosterol is a cholesterol precursor and differs from cholesterol only by three additional methyl groups (Miao et al., 2002). Interestingly, lanosterol is a precursor in both mammalian and fungal sterol pathways (Miao et al., 2002) including vaginal *Candida* species (Song et al., 2004).

In the current study we found that lanosterol significantly decreased in preterm patients from GTP1 to GTP2. Lanosterol has not been named in previous metabolomics studies in relation to PTB. However, an SNP on lanosterol-14α-demethylase (CYP51A1), a regulatory enzyme

involved in later stage of cholesterol biosynthesis, has been shown to increase the risk of PTB (Lewiń-ska et al., 2013).

Additionally, lanosterol has been shown to modulate TLR4-mediated innate immune response. Specifically, lanosterol was found to reduce macrophages capacity to secrete inflammatory cytokines but was found to enhance their phagocytic activity (Araldi et al., 2017). Therefore, lower lanosterol could result in higher inflammation which could lead to increased risk of PTB.

The association between lanosterol and PTB is not yet clear. However, based on our results there is evidence to suggest it has some relevance to PTB and should be investigated further. Additionally, a closely related metabolite, lathosterol which is also a cholesterol precursor has been found at lower concentrations in the amniotic fluid of women who gave birth preterm (Menon et al., 2014). In future research, the entire cholesterol pathway should be investigated to identify all potential biomarkers and elucidate the functional role of the related pathways.

One factor to consider when investigating sterols is medication. One study found that altered sterol biosynthesis can occur, likely due to medications including antidepressants commonly taken during pregnancy that inhibit enzymes that convert lanosterol (Genaro-Mattos et al., 2021). For this reason, future studies should aim to collect data on all medications taken by participants and control for this during analysis.

3.5.1.6 Urate

Urate or uric acid is the end product of purine metabolism in humans and is an important antioxidant (Devuyst & Igarashi, 2018). High levels of serum urate or (hyperuricemia) has previously been associated with low birth weight (Daise, 2018) and preeclampsia (Usman et al., 2021).

We found a significant increase in urate levels from GTP1 to GTP2 in preterm patients. The increase in abundance was found to be predictive of PTB with an AUC of 0.71. Our finding supports previous studies that observed increased levels of urate in saliva from 29 to 32 weeks gestation in those who had spontaneous preterm delivery (Püschl et al., 2020). Uric acid was shown to have a direct proinflammatory effect on macrophages (Martínez-Reyes et al., 2020) which could contribute to the increased risk of PTB.

Within the vaginal ecosystem, the makeup of the VMB could influence the levels of urate. A range of lactic acid producing bacteria have been observed to lower uric acid levels (Hsieh et al., 2021) including *Lactobacillus gasseri* PA-3 found in yoghurt (Yamanaka et al., 2019). Based on this evidence, there is reason to believe that vaginal lactic acid bacteria may also metabolise urate. Therefore, vaginal communities higher in *Lactobacillus* species are likely to have lower concentrations of urate as observed in the term delivered women compared to preterm.

3.5.1.7 Deoxyguanosine

Deoxyguanosine is one of four deoxyribonucleosides present in DNA and is involved in a pathway is involved in genetic and metabolic regulation (Mathews, 2014).

In the current study, deoxyguanosine was found to significantly decrease in CVF of term delivered patients and increase in preterm delivered patients from GTP1 to GTP2. The change in concentration was found to significantly predict PTB with an AUC of 0.76.

Deoxyguanosine has been linked to inflammation as it is a TLR7 agonist and was found to induce cytokine release from human peripheral blood mononuclear cells (Davenne et al., 2020). Additionally, a previous study found higher serum concentrations of 8-hydroxy-2'–deoxyguanosine, which is an oxidised derivative of deoxyguanosine commonly used as a marker of DNA damage, were found in patients who delivered preterm (Verma et al., 2019). No previous metabolomic study had reported deoxyguanosine in relation to PTB. Additional research is required before an association can be established.

3.5.1.8 Dehydrosafynol

Dehydrosafynol is a long-chain fatty alcohol commonly present in oils and waxes (Sandhir, 2014) and can be derived from dietary sources such as bees wax (Hargrove et al., 2004). Long-chain fatty alcohols from olive oil were found to reduce TNF-α and prostaglandin E2 production in macrophages (Fernández-Arche et al., 2009).

In the current study, dehydrosafynol was found to significantly increase from GTP1 to GTP2 in preterm patients. This increase was found to be predictive of PTB with an AUC of 0.71.

In addition to dietary sources, an additional source of long-chain fatty alcohols could be microorganisms. Waxes are thought to make up to 13% of the total lipid in several genera of

bacteria including *Acinetobacter* spp, *Micrococcus cryophilus*, and *Clostridium* spp (Sandhir, 2014). Additional research is required to establish a link between dehydrosafynol or any long-chain fatty alcohol and PTB.

3.5.2 Microbiome/ Metabolome Correlation

We were able to collect both metabolite and microbiome data from twenty-eight swabs (Figure 3.6). Unfortunately, due to exclusion of poor-quality samples only two samples were from patients who delivered preterm. Therefore, we were unable to perform predictive analysis or compare term and preterm metabolome/microbiome data.

However, by combining data from the vaginal metabolome and microbiome from the samples available we may be able to gain further insight into the vaginal ecosystem. The anaerobic vaginal environment is generally dominated by fermentation products such as lactic, succinic and acetic acid (Gajer et al., 2012). Previous studies have been able to establish associations between bacteria and vaginal metabolites.

Metabolites such as putrescine and pyruvate are usually associated with dysbiosis and BV (Srinivasan et al 2015, Ceccarani et al 2019), while Streptococcus dominated communities contained higher levels of acetate (Gajer et al., 2012). Yeoman et al, (2013) found that putrescine and 2-methyl-2-hydroxybutanoic acid were linked to increase activity of *Mobiluncus* species which has been associated with PTB (Dude et al., 2020).

3.5.2.1 Gestational Time Point 1

At **GTP1** eleven metabolites were found to have a correlation with the microbiome data although were not found to be significantly different in the term and preterm groups. These metabolites have not previously been reported in CVF metabolome studies and except for phytate, do not have clear links with microbial metabolism.

Three metabolites (gentamicin, hydroxybupropion and xanthotoxin) appear to originate from medications potentially taken during pregnancy. Five metabolites are commonly found in plants (acacetin, phytate, phloretin, isobutrin and ostruthin) and have evidence of anti-inflammatory properties which could influence pregnancy outcome.

Despite these metabolites and bacterial species being significantly correlated, this does not imply that there is a causal relationship, or a relationship at all (Armstrong, 2019). However, one possibility is the significant metabolites/ bacteria are both correlated to a third unmeasured factor which accounts for the significant *r* value we have found (Armstrong, 2019).

Gentamicin is an antibiotic with bactericidal activity against Gram-negative bacteria and is commonly used in obstetrics (Ward & Theiler, 2008). While the study protocol aimed to exclude vaginal swabs from women undergoing antibiotic treatment, we only had access to pregnancy health records and patient reported medications. If a patient was prescribed antibiotics for example by a general practitioner for something non-pregnancy related, this could potentially be missed if not reported by the patient. Antibiotic treatment can influence the microbiome as bacteria have varying susceptibility, surviving bacteria will colonise the environment and result in large changes in community composition (Bitew et al., 2021; Ahrens et al., 2020).

Alternatively, gentamicin is naturally produced by the bacterial genus Micromonospora (Weinstein et al., 1963) and could be produced by vaginal bacteria in the Actinobacteria phylum.

Hydroxybupropion is the main active metabolite of the drug bupropion which is used as a smoking cessation drug for pregnant women (Coles & Kharasch, 2007). This metabolite has been confirmed to cross the placenta and has been detected in cord blood and amniotic fluid (Fokina et al., 2016). Additionally, bupropion has been found to be anti-inflammatory in mouse models (Hajhashemi & Khanjani, 2014) which may decrease the risk of sPTL.

Xanthotoxin is a found in the plant family Apiaceae which includes parsnips and is known to have biological properties such as memory consolidation (Skalicka-Wozniak et al., 2018). Additionally, xanthotoxin was found to suppress LPS-induced expression of TNF-α, and IL-6 via NF-κB signalling (Lee et al., 2017), all of which are associated with inflammation induced PTB (Padron et al., 2020; Behnia et al., 2016). Xanthotoxin is also known as Methoxsalen or Oxsoralen and is used to treat psoriasis or eczema (Balakirski et al., 2022). There is no evidence that this drug increases the risk of preterm birth or other adverse pregnancy outcomes, but is not recommended for use during pregnancy due to lack of safety data and a theoretical risk of mutagenicity (Balakirski et al., 2022).

Deoxythymidine monophosphate (**dTMP**) is an essential metabolite required for replication of DNA in all cellular organisms (Myllykallio et al., 2009) including bacteria (Carnrot, 2006). Significantly higher rates of polymorphism were found in preterm delivered women on

Thymidylate Synthase (TYMS) which is an enzyme that catalyses the reaction of deoxyuridine monophosphate (dUMP) to dTMP. This may affect the concentration of dTMP.

Xanthosine is a nucleoside made up of xanthine and ribose and has been shown to increase the proliferation rate of bovine mammary stem cells (Choudhary & Capuco, 2012). Previous studies have not reported a link between xanthosine and PTB. However, three genes involved in the metabolism of xanthosine were identified in *E. coli* (Seeger et al., 1995). Therefore, the VMB may impact the concentration of xanthosine in the CVF.

Acacetin is a 4 methylated flavone of the parent compound apigenin. Acacetin was found to inhibit macrophage inflammatory response and regulate the composition of gut microbiota in mice (Ren et al., 2021). While apigenin was found to have anti-inflammatory properties in human gestational tissues and inhibited the transcriptional activity of NF-κB (Lim et al., 2013)

Bergapten which is commonly found in essential oils and grapefruit juice is thought to have antibacterial, anti-inflammatory effects (Quetglas-Llabrés et al., 2022).

Isobutrin and **ostruthin** are thought to be anti-inflammatory. Isobutrin was found to reduce production of TNF-α, IL-6, and IL-8 in human mast cells by inhibiting the activation of NF-κB (Rasheed et al., 2010). Ostruthin was found to inhibit TNF-α-induced nuclear factor κB in Human lung adenocarcinoma cells (Vo et al., 2021). Both have been shown to have some antimicrobial properties (Tiwari et al., 2012; Schinkovitz et al., 2003).

Phloretin is commonly found in fruits such as apple and is known to have antiviral, anti-inflammatory, antibacterial properties (Talarek et al., 2017). Specifically, phloretin has been shown to suppress TLR2-induced inflammation (Kim et al., 2018)

Phytate is commonly found in seeds and is known to decrease zinc absorption (Wang & Guo, 2021). However, a recent Cochrane review demonstrated that zinc supplementation did not reduce the rate of PTB (Carducci et al., 2021). Lactic acid bacteria including many *Lactobacillus* species produce phytases (Sharma et al., 2020) and therefore likely influence the concentration of phytate in the CVF. Additionally, 64% of vagina *Candida albicans* isolates were found to have a high level of phytase activity (Z. Mahmoudabadi et al., 2015). Further investigations into the host microbiome phytate metabolism interaction are required to better understand if this metabolite could be used as a biomarker.

3.5.2.2 Gestational Time Point 2

At **GTP2** we found five metabolites correlated with the microbiome data.

Androsterone, which is a steroid hormone was positively correlated with a number of bacteria. Previous studies have found association between altered metabolism of steroids including androsterone and PTB (Menon et al., 2014). Potential steroid biosynthesis pathway was identified in 14 bacterial phyla including Acidobacteria (Hoshino & Gaucher, 2021). Therefore, it is possible that the vaginal microbiome may impact androsterone levels in CVF however, we currently have insufficient data.

Both **Dhurrin** and **Zierin** are cyanogenic glycosides that act as plant toxins produced by plants as a defence compound. Dhurrin is produced in sorghum plants which include cereals (Laursen et al., 2016). This metabolite breaks down into cyanide upon disruption of plant cell walls (Laursen et al., 2016). In the context of beer production, lactic acid bacteria have been found to express β-d-glucosidase which reduces the concentration of dhurrin (Tokpohozin et al., 2016). Zierin has been identified from elderberries and is thought to be an anti-oxidant (Knudsen & Kaack, 2015). No previous studies have reported zierin in relation to PTB or bacterial metabolism.

Heparin is a naturally occurring polysaccharide belonging to the family of glycosaminoglycans but is well known as an anticoagulant (Oduah et al., 2016). Some research suggests that the similar molecule heparan sulphate is able to decrease bacterial ability to adhere to host cells leading to less cellular internalisation (Ivan Fernandez Vega, 2014). Heparin is not associated with PTB but has been shown to reduce the rate of preeclampsia (Karadağ et al., 2020).

Pantetheine is an oxidized form of pantetheine which can be cleaved by Pantetheinase into pantothenic acid and cysteamine (discussed earlier). The pantothenic pathway appears to be a good candidate for future research.

Associations between metabolites correlated with the microbiome in this study and PTB have yet to be established. However, many have been linked to inflammation. Further research is required to establish a correlation within the vaginal environment. These microbiome/metabolome interactions would benefit from bioinformatic pathway analysis to determine potential relationships. The ability to predict PTB from the vaginal metabolome remains understudied.

3.6 Summary & Future Work

The results presented in this chapter provides additional evidence that the CVF microbiome and metabolome are dynamic throughout pregnancy and provide new opportunities for improved PTB prediction, allowing triaging of pregnant women prior to them developing preterm labour symptoms.

We hypothesised that women who give birth prematurely would have a significantly different metabolite profile that can be used to identify a higher risk of PTB. We identified ten metabolites that appear to have some predictive value. These metabolites should be further studied to improve our understanding of the relationship between the CVF metabolome and PTB.

In particular, the pantothenate and cholesterol pathway should be investigated further. We found that pantothenate significantly increased and lanosterol significantly decreased in preterm patients. These metabolite pathways both have links to preterm birth (Ghartey et al., 2017; Menon et al., 2014) and *Lactobacillus* species (Yao et al., 2018).

Many of the significant metabolites found in this study have a link to cellular inflammatory processes, a well described feature of the PTB process (Cappelletti et al., 2016). For this reason, additional cytokine analysis on the same patients would provide useful insight into the inflammatory state of the vaginal community at the time of sampling. Future work could include an ELISA cytokine array to identify concentrations of inflammatory molecules which may indicate maternal immune response to vaginal bacteria.

One limitation of this study was sample size. Future work should recruit increased numbers of patients and their samples in order to obtain sufficient microbiome and metabolome data. This will lead to a higher number of preterm births as a primary outcome. Subsequent comparative and predictive analysis can then be performed. Additionally, future studies should aim to collect more extensive data on all medications taken by participants including those prescribed for non-pregnancy related issues.

Another limitation was that the CVF samples were eluted in PBS. Use of buffer containing salts for electrospray ionisation mode mass spectrometry can cause ion suppression and adduct formation in the mass spectrometer thus reducing signals and creating noisy spectrums (Wahab et al., 2016; Trufelli et al., 2011). Samples collected after 2020 have been stored dry

and will be eluted using water or an appropriate reagent for future techniques. In the current study, PBS controls were analysed to account for the additional salts in the samples. However, this approach does not reduce noise in the spectrums which may disguise other metabolites.

Metabolism data from vaginal organisms is available from sources such as the MetaCyc database (Caspi et al., 2020). However, compared to organisms such as *Staphylococcus aureus* which have experimentally elucidated pathways, vaginal bacteria are not well characterised. Additional studies are required to thoroughly characterise the metabolism of key vaginal microbiota. Once armed with robust bacterial metabolism data, future studies can investigate the metabolic pathway of the CVF environment accounting for host cells, vaginal bacteria and their interactions. This would require big data sets and therefore machine learning methods would be most useful. For example, the pathway modelling presented in (Cuperlovic-Culf, 2018). Mapping the vaginal metabolome pathway would allow us to better understand the link between the vaginal microbiome and metabolome and subsequently identify biomarkers of PTB.

In addition to swabs from the UK cohort, future studies analysing samples from South Africa and Bangladesh using the same methods are currently underway. These samples will be processed and analysed with the same methods allowing us to compare the metabolite profile of women from a range of settings. This will allow us to collect data that is more representative of all women not just those in high income countries. Differences between the microbiome and metabolome communities may provide further avenues for research.

Similarly to chapter 4, the overall aim of this chapter was to characterise and compare the vaginal metabolome of pregnant women from UK (Sheffield Teaching Hospitals), Bangladesh (icddr,b in Dhaka) and South Africa (Groote Schuur Hospital) with a standardised protocol in order to confidently compare vaginal metabolome and associated bacterial community. We aim to collect samples from a range of settings to ensure that prediction methods are relevant to mothers around the world.

Unfortunately, the SARS-CoV-2 pandemic delayed sample collection from all research sites and so this report includes only samples from the UK. However, based on the methods optimised in this study, the PRIME research team continue to work with clinical samples from all partner countries (discussed in future work).

In addition to the completed objectives, future aims include analysis of the metabolome of clinical swabs from pregnant women in Bangladesh and South Africa to identify potential biomarkers of PTB that will be applicable for women around the world. Additionally, comparison of the vaginal metabolome profile and risk of PTB across the three countries to assess the role of setting. This was not completed and is detailed in future works.

We hypothesise that the vaginal metabolome of women from the UK, Bangladesh and South Africa will significantly differ. Communities considered high risk for PTB from one setting may not have the same predictive power in a different population of women.

4 Chapter 4. Novel mechanical testing of fetal membranes exposed to infection associated molecules & assessment of *lactobacillus* degradation abilities using fluorescent gelatin.

4.1 Introduction

The fetal membranes (FM) are complex tissues consisting of many layers of cells, basement membrane, extra cellular matrix (ECM) and collagen fibres (Figure 1.7). Mechanically, FM is unusual as the tissue is under constant high stress in normal physiological conditions and is loaded close to the failure threshold (Lannon et al., 2014).

Increased levels of bacteria, immune cells and MMPs have been consistently observed in patients with PPROM (Dutta et al., 2016; Choi et al., 2012; Romero et al., 2011; Waters & Mercer, 2011). Pathogenic organisms such as *Streptococcus agalactiae* AKA Group B Streptococcus (GBS) have been shown to induce an inflammatory response in the cervicovaginal environment (Mitchell & Marrazzo, 2014; Bastek et al., 2011). This inflammatory response can initiate a range of biochemical signals such as release of MMPs and structural changes in the fetal membrane including apoptosis (Uchide et al., 2012). Additionally, microbial products such as gelatinase enzymes, MMPs and metabolites have been shown to reduce viability of cells and integrity of membrane tissue (Lithgow et al., 2022; Soares et al., 2008). These changes can ultimately lead to increased risk of membrane rupture (Figure 4.1).

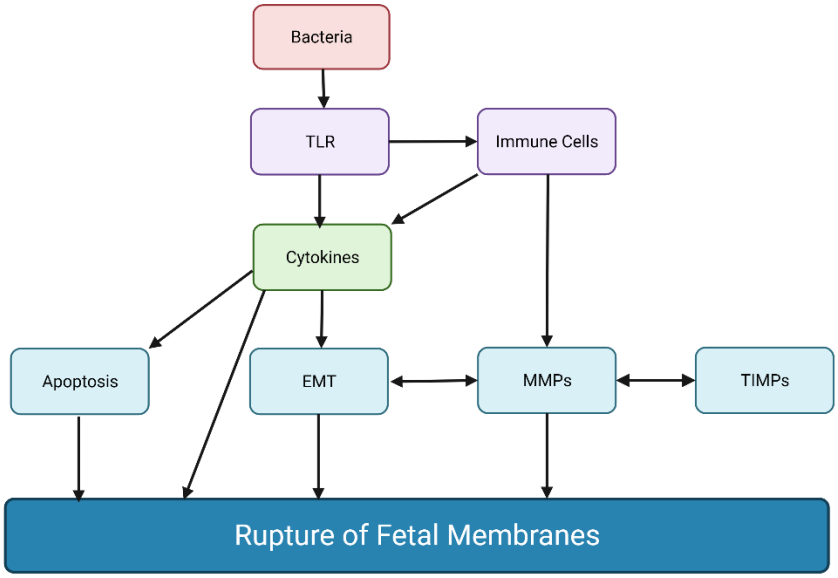


Figure 4.1 Mechanisms for bacterial weakening of fetal membrane. TLR= toll like receptor, EMT= epithelial to mesenchymal transition, MMPs= matrix metalloproteases, TIMPs = tissue inhibitor of MMPs. Created with BioRender.com

4.1.1 Bacterial Collagenase, collagenolytic proteases and Gelatinases

Bacteria have been shown to produce a range of enzymes that contribute to the breakdown of human tissues and contribute to disease (Duarte et al., 2016; Harrington, 1996). These enzymes include not only collagenase which is an uncommon enzyme produced by bacteria but also collagenolytic proteases (including MMPs) and gelatinase which are able to degrade denatured collagen or unfolded collagen present *in vivo* (Zhang et al., 2015; Leikina et al., 2002).

Gelatinases are able to degrade denatured collagen, specifically, gelatin, collagen (types IV, V, VIII, X, XI, XIV),elastin, proteoglycan core proteins, fibronectin, laminin, fibrilin-1, and TNF-α and IL-1b precursor (Laronha & Caldeira, 2020). While collagenases are able to cleave fibrillar collagen type I, II, III, IV and XI due to their ability to unwind triple helical collagen (Laronha & Caldeira, 2020).

However, in addition to bacterial products, MMPs can also originate from host cells. One study found MMP-9 was present in the fetal membranes of women with infection but was not present in those without infection (Weiss et al., 2007). During further investigation Weiss

et al observed that fetal membrane from non-labouring women produced MMP-9 when exposed to LPS.

4.1.2 Microbial Fetal Membrane Studies

Studies have used animal models (Bergeron et al., 2016; Vanderhoeven et al., 2014) and primary cell models (Boldenow et al., 2015; Reisenberger et al., 1997) to investigate the role of vaginal bacteria on membrane weakening. However, in this chapter we are focussing on the response and mechanical characteristics of **full thickness human fetal membrane tissue** as this is a more representative model of the tissue *in vivo*.

Studies measuring the responses of whole fetal membrane tissue have focussed on only a few bacteria. **1)** *Streptococcus agalactiae* (i.e. group B *Streptococcus* or GBS), which has been associated with poor maternal and fetal outcomes for decades (Larsen & Sever, 2008). **2)** A group of bacteria, genital mycoplasmas which includes *Ureaplasma parvum*, *Ureaplasma urealyticum* and *Mycoplasma hominis*, which have been associated with PPROM and sPTL (Murtha & Edwards, 2014; Passos et al., 2011; Novy et al., 2009; Kataoka et al., 2006). **3)** *Escherichia coli* which is associated with sPTL, PPROM, chorioamnionitis and other undesirable pregnancy outcomes (Cools, 2017). **4)** *Gardnerella vaginalis* also associated with PTB (Mcelrath et al., 2018; Bretelle et al., 2015).

These studies exposed fetal membranes to bacteria and measured a range of cytokines and MMPs: IL-1β was found to increase 30-fold after GBS exposure (Zaga et al., 2004). MMP-9 and TNF- α increased in response to GBS (Flores-Herrera et al., 2012). Potts et al, (2016) found increased IL-8 and MMP-2 after exposure to *Ureaplasma parvum*. *G. vaginalis* alone or in combination with genital mycoplasmas significantly increased IL-6 production (Noda-Nicolau et al., 2016). Finally, Menon et al, (2009) found *E. coli* increased concentrations of IL- 1β, IL-10, and TNF-α . *G vaginalis* and GBS stimulation resulted in a medium inflammatory response and *U. parvum* increased only IL-10 and TNF- α concentrations.

In summary, key bacteria that are widely associated with PTB and PPROM have been extensively shown to induce inflammation and production of MMPs in fetal membrane models. It is via these inflammatory molecules that bacteria are thought to contribute to fetal membrane weakening. However, there remains a need to elucidate novel mechanisms for bacterial-induced membrane weakening. For example, Feng et al, (2018) found that when

membrane tissue was exposed to *Ureaplasma parvum*, prothrombin production was increased. Thrombin not only plays a role in blood clotting but is also known to have proinflammatory properties and can contribute to membrane weakening (Feng et al., 2018).

A total of 581 bacterial species have been identified in the vagina using culture and/or molecular-based techniques (Diop et al., 2019) and yet there is very little research on how the normal vaginal microbiome could influence membrane weakening.

We aim to investigate the response of fetal membrane to a range of vaginal bacteria to identify non-pathogenic organisms that may increase the risk of PPROM. To measure the impact of bacteria on the mechanical properties of fetal membrane samples, we worked with the Natural Materials group at TUOS to test a novel mechanical method for assessing fetal membrane integrity, Dynamic Shear Analysis (DSA).

4.1.3 Mechanical Fetal Membrane Studies

To date, no studies have used dynamic shear analysis to test human fetal membranes. Previous mechanical studies have aimed to characterise the mechanical properties of FM using several types of methods.

Tensile testing, this can be either uniaxial or biaxial. Membrane samples are clamped and the distance between the clamps is increased at a steady rate while stress, strain and tensile strength are measured. (Bircher et al., 2019; Mauri et al., 2015; Buerzle & Mazza, 2013; Prevost et al., 2006; Pressman et al., 2002; Helmig et al., 1993; Lavery et al., 1982)

Burst testing, for this method membrane samples are clamped using a circular ring and increasing pressure is applied, usually using liquid. Either volume of liquid, pressure or height of the membrane is measured until rupture occurs. (Bircher et al., 2019; Perrini et al., 2013; Lavery & Miller, 1979; Miller et al., 1979)

Puncture testing, membrane samples are clamped, and probes of varying diameters are applied at a known force. Force and displacement are measured and some studies use a camera to visualise membrane integrity. (Buerzle & Mazza, 2013; Oyen et al., 2004; Schober, Kusy, & Savitz, 1994)

From these studies, we have discovered that several factors influence the risk of membrane rupture. It is well established that membranes from patients who undergo labour and vaginal

delivery require less stress to rupture than those who have a caesarean section (Lavery et al., 1982; Lavery & Miller, 1979). Additionally, less force is needed to puncture membranes at later gestations for both caesarean and vaginal deliveries (Benson-Martin et al., 2006; Oyen et al., 2004; Schober, Kusy, & Savitz, 1994). This is due to the biochemical and structural changes observed as gestation progresses but, more so shortly prior to and during labour (see section 1.1.1).

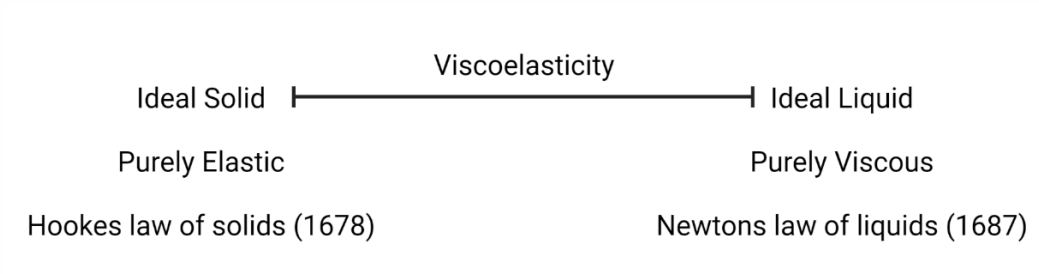
More recently, studies have shown a localised area of weakened membrane overlying the cervix (El Khwad et al., 2006; McLaren et al., 1999). Older studies did not control for sample location. For example, Al‐Zaid et al, (1980) who observed that membrane from PPROM patients required higher pressure before bursting compared to term but did not mention sampling location so was not able to investigate localised weakening at the rupture site. Similarly, Oyen et al, (2004) sampled from near the placental margin in singleton births so could not investigate localised weakening. However, in the same study, for multiple births, they sampled different locations on the membrane and found significant variation in pressure needed for rupture at the different sampling locations (Oyen et al., 2004).

4.1.4 Microbial & Mechanical Fetal membrane studies

A very limited number of studies have used any method of mechanical testing to measure fetal membrane response to bacteria. One of these few studies tested tensile strength (determined by breaking point loads) on fetal membrane that had been exposed to collagenase derived from Clostridium histolyticum and pseudomonas aeruginosa (McGregor et al., 1987). They observed a dose dependent decrease in membrane strength that was not observed when using collagenase deficient strains of bacteria. This indicates that in these experiments' membrane weakening is likely caused by bacterial products, rather than a membrane inflammatory response to the bacteria presence via TLR. Another study investigated GBS and neutrophil activation and found that exposure to GBS and activated neutrophils led to increased weakening of fetal membranes (Schoonmaker et al., 1989). To date, no studies have mechanically tested fetal membrane after exposure to commensal or pathogenic vaginal bacteria.

4.1.5 Rheology & Dynamic Shear Analysis

Rheology is the study of how materials deform and flow when stress (force) is applied. In response, all materials fall on a scale from an ideal solid to an ideal liquid (Barnes et al., 2001).



Ideal solid, defined as "a material that will not continuously change its shape when subjected to a given stress" (Barnes et al., 2001) and are considered purely elastic. Hookes law of solids (1678).

$$\text{Deformation } (\varepsilon) = \frac{\text{Tensile stress } (\sigma_E)}{\text{Elastic Modulus (E)}}$$
$$(aka \text{ Young's Modulus})$$

Ideal liquid, defined as "a material that will continuously change its shape (i.e. will flow) when subjected to a given stress, irrespective of how small that stress may be" (Barnes et al., 2001) and are considered purely viscous. Newtons law of liquids (1687)

$$\text{Shear strain } (\gamma) = \frac{\text{Shear Stress } (\sigma)}{\text{Viscosity } (\eta)}$$

Viscoelasticity is the behaviour of materials that fall between the extremes of solid and liquid and describes a material that has both viscous and elastic properties (Barnes et al., 2001).

A rotational shear rheometer applies and measures:

- **Torque (M)** = A measure of how much a force (F) acting on an object causes that object to rotate)
- **Angular Displacement (θ)=** The angle, in radians, through which an object moves on a circular path.
- **Angular velocity (Ω)** = The change in angular displacement (θ) per unit time of measurement

By measuring these, the rheometer can calculate:

- **Shear stress (σ)** = Torque (M) × Stress constant (K_σ)
- **Shear strain (γ)** = Angular Displacement (θ) × Strain constant (K_γ)

Shear strain is a measure of deformation (in this case fetal membrane). Stress and strain constant are measurements from the geometry on the Rheometer.

The Modulus: Measure of materials overall resistance to deformation.

$$G^* = \frac{\text{Shear stress } (\sigma)}{\text{Shear strain } (\gamma)}$$

Which can be further split into:

The Elastic (storage) Modulus: Measure of elasticity of material. The ability of the material to store energy.

$$G' = \frac{\text{Shear stress } (\sigma)}{\text{Shear strain } (\gamma)} cos\delta$$

The Viscous (loss) Modulus: The ability of the material to dissipate energy. Energy lost as heat.

$$G'' = \frac{\text{Shear stress } (\sigma)}{\text{Shear strain } (\gamma)} sin\delta$$

Briefly, samples are compressed to a known force between two parallel plates (Figure 4.2), the upper plate (geometry) rotates at a fixed strain using a digitally mapped bearing and the torque on the motor required to achieve this is converted to stress response of the sample at a range of oscillation frequencies. Within each sinusoidal deformation at a particular frequency the strain peak (γ0), stress peak (σ0) and difference in phase (δ) is measured. These values are then used to calculate the complex modulus G* as well as G'' (the viscous modulus) and particularly informative to Dynamic Shear Analysis (DSA), G' (the elastic modulus).

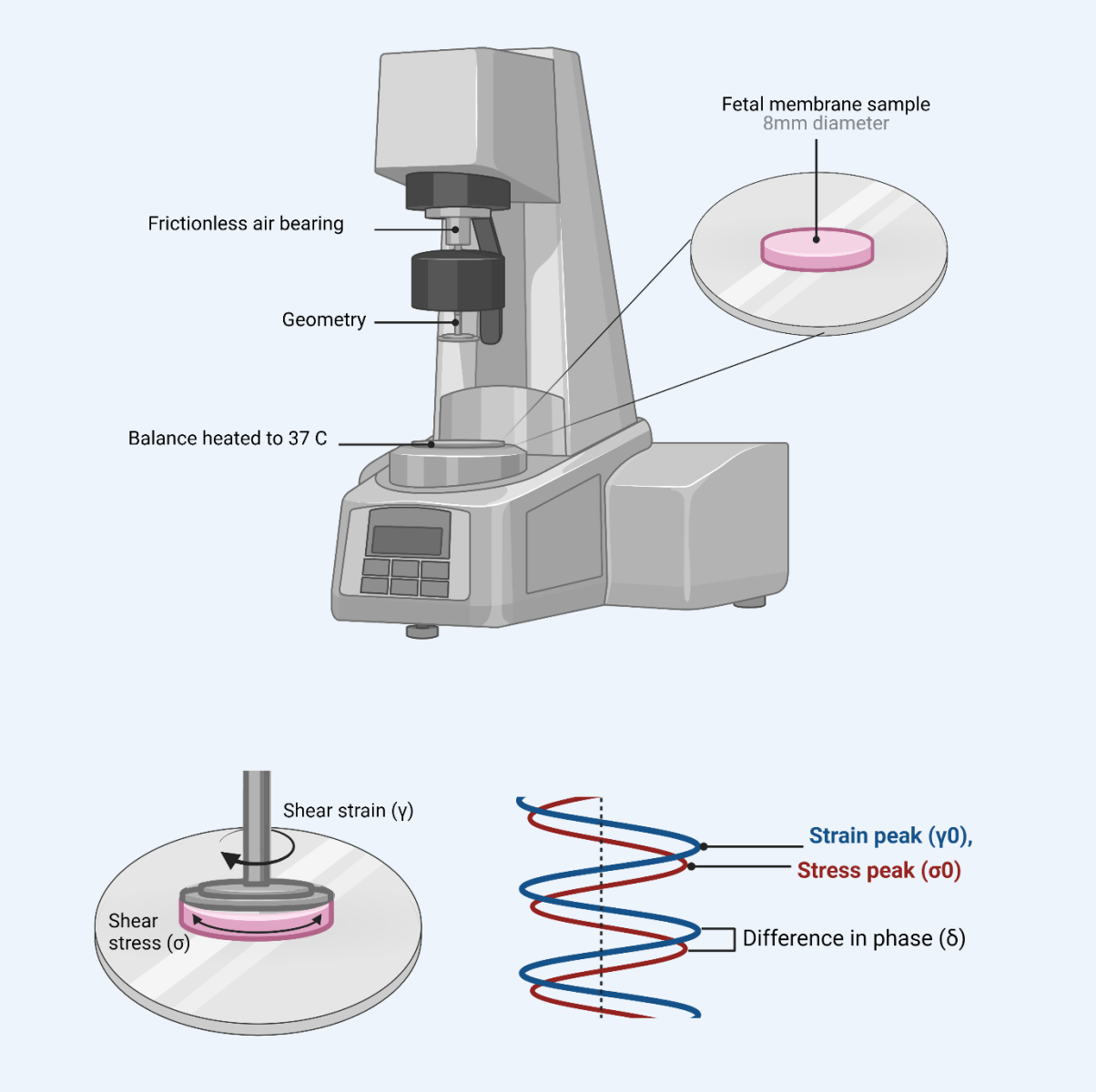


Figure 4.2 Dynamic Shear Analysis of fetal membrane samples using a rheometer. Samples are compressed to a known force between two parallel plates, the upper plate moves at a fixed strain and the digitally mapped bearing is used to record the absolute displacement, which is converted to stress response of the sample at a range of oscillation frequencies. strain peak (γ0), stress peak (σ0) and difference in phase (δ) measured for each frequency. These values are used to calculate G′ (the elastic modulus). Created with BioRender.com

This novel method for measuring FM has many benefits, a frequency sweep measures the tissues response to increasing rate of deformation and so gives information about both structure and dynamics. By measuring the viscoelasticity of fetal membrane, we can infer the relative risk of rupture. Samples with a higher G' are "stiffer" and samples with lower G' are more likely to rupture. Models that try to replicate natural loading often use clamps which can reduce or alter the structure and integrity of the tissue. Our method does not use clamping.

While this is a novel technique for fetal membrane samples, dynamic shear analysis has been applied to bovine and equine tibial cartilage to provide insight into the pathology of osteoarthritis in humans (Holland et al., 2014). This study was able to identify significant differences in tibial cartilage and it is speculated that this technique could precisely quantify the pathology of osteoarthritis. Additionally, this technique has been utilised to study torn shoulder rotator cuff tendons (Chaudhury et al., 2011). This study discovered that torn rotator cuff tendons have a significantly lower shear modulus compared to healthy rotator cuff tendons which is thought to translate into structural weakness. Dynamic shear analysis could therefore be used to determine appropriate treatment for these injuries.

Tissues such as fetal membrane are complex structures with many cell types and functions (Bryant-Greenwood, 1998) (see Figure 1.6 for a cross section of fetal membrane). Often investigations focus on cellular or molecular mechanisms and do not investigate the tissue as a whole. It is important to consider not only the molecular signalling and cell behaviour but also the physical characteristics and interaction between the two.

4.2 Hypothesis, Aims & Objectives

Our overall hypothesis is that vaginal bacteria can influence the structure and integrity of fetal membranes via collagen degradation and tissue immune response e.g., cytokines, MMPs. We hypothesise that vaginal bacteria may contribute to the weak area known to be present over the cervix at term and prematurely in PPROM patients.

To test this, we aim to investigate **1)** the mechanical properties of fetal membrane using dynamic shear analysis (DSA) in addition to **2)** the degradation abilities of bacteria using fluorescent gelatin model.

Objectives – Mechanical Studies:

1. To optimise dynamic shear analysis methods on fetal membrane.
2. To expose fetal membranes to inflammation associated molecules (TNF-α and LPS) and measure the mechanical properties using DSA.
3. To expose fetal membranes to bacterial metabolites (lactic acid, and acetate) and measure the mechanical properties using DSA.

Initially, we also aimed to expose fetal membranes to a range of commensal bacteria and measure the mechanical properties. However, this was not completed and is detailed in future works.

We hypothesise that samples exposed to infection- and inflammation-associated molecules, bacterial metabolites or bacteria will be significantly less stiff than the untreated controls.

However, we expect that bacteria and their metabolites associated with term pregnancies e.g., *Lactobacillus crispatus* and D-Lactate will decrease the stiffness of the tissue **less** than other bacteria, including PPROM associated bacteria and metabolites such as acetate, *Ureaplasma urealyticum* or *Gardnerella vaginalis*.

Objectives – Degradation Studies:

1. To optimise the time and dose response of bacteria and bacterial products on DQ™ gelatin assay.
2. To assess a range of commensal bacteria's degradation abilities using DQ™ gelatin assay.
3. To assess a range of commensal bacteria's enzyme inhibition abilities using DQ™ gelatin assay.

We hypothesise that bacteria associated with PPROM will have significantly greater degrading abilities compared to term associated bacteria and controls. Additionally, we hypothesise that bacteria associated with term birth can inhibit the collagenase abilities of other bacteria.

By testing a range of organisms commonly found in the vaginal microbiota we aim to identify potential communities which may increase the risk of membrane rupture despite not being pathogenic. Overall, we aim to elucidate the role of the vaginal microbiome in PPROM.

4.3 Methods – Dynamic Shear Analysis of Fetal Membrane

4.3.1 Recruitment and eligibility

Studies were undertaken at the Jessop Wing Maternity Unit of the Royal Hallamshire Hospital in Sheffield. Jessop Wing has an annual birth rate of approximately 7000 deliveries, and a preterm birth rate of 9-10% (800 births per year). Of those 800, around 150 births occur before 34 weeks' gestation following spontaneous labour. Therefore, the larger PRIME study that is work is part of, aimed to collect 50 term and 50 preterm placentas and fetal membranes from patients that had been in spontaneous labour. Exclusion criteria included patients who had an elective caesarean section without prior labour, multiple pregnancies, established clinical vaginal infection and patients with uncertain pregnancy duration. After March 2020, all patients were required to take a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) PCR test on admission to Jessop Wing. A negative PCR test result was required before processing fetal membrane samples.

Study participants were recruited either by prior antenatal consent, when admitted with preterm labour symptoms or after delivery. Patients attending Jessop Wing for an antenatal appointment who met the study criteria were made aware of the study via advert leaflets. Following an explanation of the study and time to read the patient information sheets, those who wished to take part in the study gave written consent and a research sheet was placed in their notes to indicate that the research team should be contacted to collect the placenta and fetal membranes.

A significant proportion of preterm births are transfers from adjacent hospitals therefore it was not always possible to recruit preterm participants prior to labour. In these cases, patients were approached after admission with preterm labour symptoms, provided they were not in established labour, thus allowing them sufficient time to decide whether to participate or not. Those patients who were admitted when in term or preterm labour were approached with information sheets shortly after birth. Consent was taken once all medical procedures had concluded; this is when placentas are routinely obtained for clinical histopathology in preterm patients.

The studies were approved by the Fulham NRES and the Human Research Authority approval from Health and Care Research Wales (HCRW) Approval, IRAS project ID: 256135, REC reference: 18/LO/2044 in alignment with Sheffield Teaching Hospitals NHS Trust regulations,

protocol number: STH20635. Samples were obtained between 1st March 2018 and 25th March 2021.

4.3.2 Sample collection

All fetal membrane samples were processed within 6 hours of delivery in order to maintain the viability of the tissue. However, the majority were processed within 2 hours after delivery. Due to the unpredictable turnaround times for SARS-CoV-2 PCR test results, many tissue samples were discarded when test results were not available within the 6-hour window for processing. This especially affected participants who delivered quickly after admission.

Placentas including the fetal membranes were transported to the laboratory in sealed bags and all work was done in biosafety cabinet. The fetal membranes were spread out from placenta with the amnion facing upwards as shown in Figure 4.3. The tissue was examined for signs of infection such as separation of the chorion and amnion, a green or yellow colour or malodour (Yetter, 1998). If infection was suspected, membrane samples were discarded as it is important that the membrane layers remain attached for rheological assessment. Additionally, to assess the impact of the infection model the tissue samples should not already be infected. An 8 mm biopsy punch (Kai medical, Japan) was used to cut 8 mm diameter discs of fetal membrane. Samples were then washed twice in room temperature phosphate buffered saline (PBS) to remove blood.

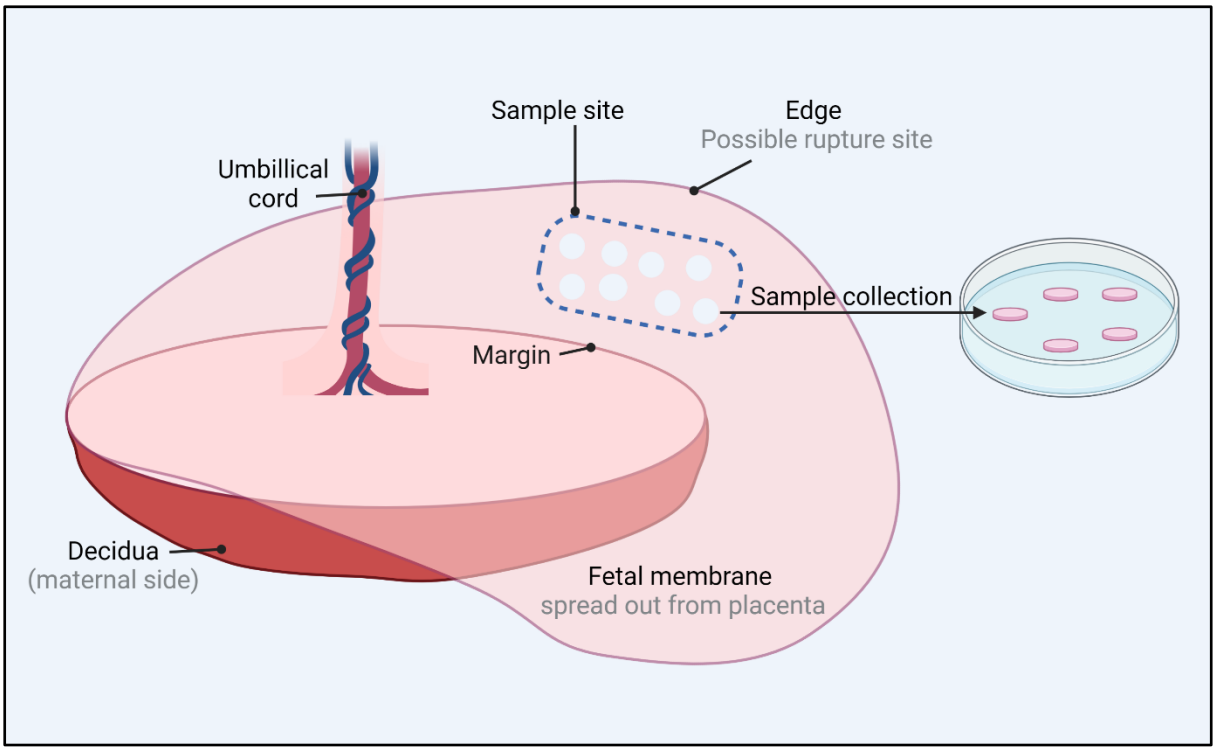


Figure 4.3 Fetal membrane sample collection. Created with BioRender.com

4.3.2.1 Cryopreservation of fetal membrane

Due to laboratory restrictions during the SARS-CoV-2 pandemic, some of the fetal membrane samples were collected and frozen at -80℃ for experimentation when the laboratories were re-opened. Fetal membrane discs were placed into cryomedia made up of sterile PBS containing 10% dimethylesulphoxide (DMSO) (Merck, Germany), 10% Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco, USA), 10% Fetal bovine serum (FBS) (Gibco, USA). Samples were allowed 8 min equilibrium time at room temperature and stored rapidly at −80 °C as previously detailed by (Tehrani et al., 2013). Prior to experimentation, tubes were thawed in a room temperature water bath. Samples were removed from the cryomedia as soon as possible and washed three times with room temperature PBS to remove DMSO.

4.3.3 Treatment of samples

Samples were placed into a 24 well plate containing treatment media and incubated at 37°C, 5% CO_2, for 1 - 24 hours. Treatment media was made up of DMEM F12 + 10% FBS + LPS or TNF- α or a metabolite. The untreated condition contained DMEM F12 + 10% FBS only. Treatment conditions contained concentrations of study compounds comparable to those

found *in vivo*: TNF-α 100 ng/mL (Luo et al., 2010), LPS 1000 ng/mL (Flores-Espinosa et al., 2014), D-Lactate 0.03 mg/mL, L-Lactate 0.03 mg/mL, Acetate 0.6 mg/mL. Lactate concentrations employed were similar to median concentrations measured in vaginal samples of women dominated with *L. gasseri* (Witkin et al., 2013). Furthermore, they matched with D-lactate concentrations measured in previous unpublished work from our group Narice (2019) *Novel and potentially non-invasive techniques to predict preterm birth*. Appropriate acetate levels were determined from a previous metabolomics study (Amabebe, Reynolds, V. Stern, et al., 2016) where acetate concentration was found to be significantly higher in the women who delivered preterm vs term (0.63 ± 0.04 vs 0.54 ± 0.01 mg/mL; p = 0.006) optimal cut-off value for prediction of PTB was >0.53 mg/mL acetate. Therefore, we chose to treat the membrane samples with 0.6 mg/mL.

4.3.4 Dynamic shear analysis

In order to assess the mechanical properties of fetal membrane samples after exposure to infection associated molecules Dynamic Shear Analysis was performed using an AR2000 rotational shear rheometer (TA Instruments, Delaware, USA). This technique is novel for fetal membrane samples but has previously been used to measure human rotator cuff tendons (Chaudhury et al., 2011). Fetal membrane samples were loaded and gently compressed to 0.2 N between two parallel plates, the upper being 8 mm in diameter and the lower plate being heated to 37°C. Samples then underwent a standard oscillatory test (0.623 rad/s to 6.283 rad/s, 0.02 strain) as done in (Chaudhury et al., 2011). To ensure consistent contact between upper and lower plates during testing, samples were compressed until a 0.2 N rise in Normal Force was observed. The upper plate moves sinusoidally (smooth periodic oscillation) at a fixed strain and records the stress response from the samples over a range of frequencies (ω) by measuring the torque on the drive motor. The digitally mapped bearing of this motor is used to record the absolute displacement, which is converted to strain. The resulting strain peak (γ0), stress peak (σ0) and difference in phase (δ) was measured for each frequency. These values were used to calculate the elastic (storage) modulus (G'). The mean storage modulus across all frequencies was used to indicate the mechanical integrity of the fetal membrane.

4.4 Methods – Bacterial Degradation of fluorescent gelatin

In order to study the degradation abilities of vaginal bacteria, an *in vitro* gelatin model was used which allowed visualisation of gelatin degradation when exposed to bacteria and their products.

4.4.1 Bacterial culture

Bacteria were cultured to an exponential growth phase in order to assess their degradation and inhibition abilities. De Man, Rogosa & Sharpe (MRS) bacterial broth (Oxoid, CM0359) was inoculated with *L. gasseri* (ATCC 33820), *L. jensenii* (ATCC 25258) and *L. crispatus* culture (ATCC 33323) from frozen stocks stored at -80°C. Bacteria cultures were grown for 24hrs in an anaerobic chamber (Don Whitley Scientific DG-250) containing 10% hydrogen, 10% CO_2 and 80% nitrogen at 37°C. Bacterial cultures were sub-cultured every 24hrs by adding 500µl of cell suspension into 20 mL fresh MRS media.

In order to ensure the same number of bacteria and therefore bacterial products were present in each condition, bacterial cell suspensions were counted using a 1/400 2 (depth 0.02 mm) Helber counting chamber (Hawksley, Z30000). Suspensions were diluted with fresh MRS to reach a concentration of 10^7 CFU/mL which is representative of CFU in vaginal fluid (Li et al., 2019). Once diluted, cell suspensions were centrifuged at 5000 x g for 5 minutes. The supernatant was removed and filtered (0.2µ filter) to produce conditioned cell free media for use in experiments. The bacterial cell pellet was resuspended in either MRS or PBS, counted and diluted to 10^7 CFU/mL.

4.4.2 Fluorescent gelatin assay

To measure the degradation and inhibition abilities of bacteria an EnzChek gelatinase assay kit (Molecular probes, D12054) was used to quantify collagenase/gelatinase activity from bacteria and bacterial products. 20 µl of 25 µg/mL DQ™ fluorescent gelatin from pig skin, 80 µl of reaction buffer and 100 µl of sample (see conditions listed below) was added to each well of a black 96 well plate with a clear bottom.

L. gasseri, *L. jensenii* and *L. crispatus* conditioned cell free media was used at 1:1, 1:10, 1:20 dilutions in order to provide insight into a dose response. PBS and MRS were used as negative controls, MRS was also diluted in PBS 1:10 and 1:20 and of collagenase A was used (0.005 and 0.001 U/mL). A Varioskan Flash plate reader was used to measure fluorescence intensity

(excitation 495 nm, emission 515 nm) every 15 minutes for 5hr (21 timepoints), all conditions were run in duplicate.

To investigate bacterial collagenase inhibition, *L. gasseri*, *L. jensenii* and *L. crispatus* conditioned cell free media (1:10 dilution) was mixed with 0.005 and 0.001 U/mL collagenase. In addition to the PBS, MRS and collagenase controls as described above, collagenase (0.005 and 0.001 U/mL) reconstituted 1:10 and 1:20 in MRS was used to control for the brown colour of this media which may obscure fluorescence. Fluorescent measurements were taken every 15 minutes for 5hr, all conditions were run in duplicate. Collagenase conditions were added to the plate at the last possible moment before loading into the plate reader in order to measure the initial rapid increase in fluorescence as the collagenase efficiently degraded the DQ^{TM} gelatin.

Raw fluorescence readings were exported from the plate reader into an excel sheet. Fluorescence values were either plotted as the raw arbitrary units or were normalised to timepoint one in order to visualise an increase/ decrease in fluorescence. Normalised values were not used for conditions containing collagenase as the degradation and fluorescence increase begins rapidly before the first reading and therefore normalisation does not give an accurate rate of degradation. Analysis was performed using GraphPad Prism 9. Statistical analysis was performed on all experiments with sufficient repeats, area under the curve was calculated for each repeat of each condition and was plotted on a bar plot ANOVA and Tukey's multiple comparisons test were used to assess statistical significance.

4.5 Results

4.5.1 Dynamic Shear Analysis of Fetal Membrane

4.5.1.1 Cryopreservation

We tested membrane samples from a total of 11 patients. Samples from 6 patients were cryopreserved within 6 hours of delivery, samples from 2 patients were processed within 6 hours of delivery and stored at 4°C in PBS for up to 48hr, while samples from 3 patients were processed and tested within 6 hours of delivery however, 1 patient had chorioamnionitis confirmed by histology and therefore was not included in analysis. G' measurements ranged from 594 - 12 Pa. There was considerable variation between technical repeats, demonstrated by the large standard deviation (SD) in Figure 4.4. Statistical analysis showed membrane samples tested within 6 hours of delivery (n=2) had a significantly higher G' than cryopreserved samples (n=6) (p= 0.0032) (Figure 4.5).

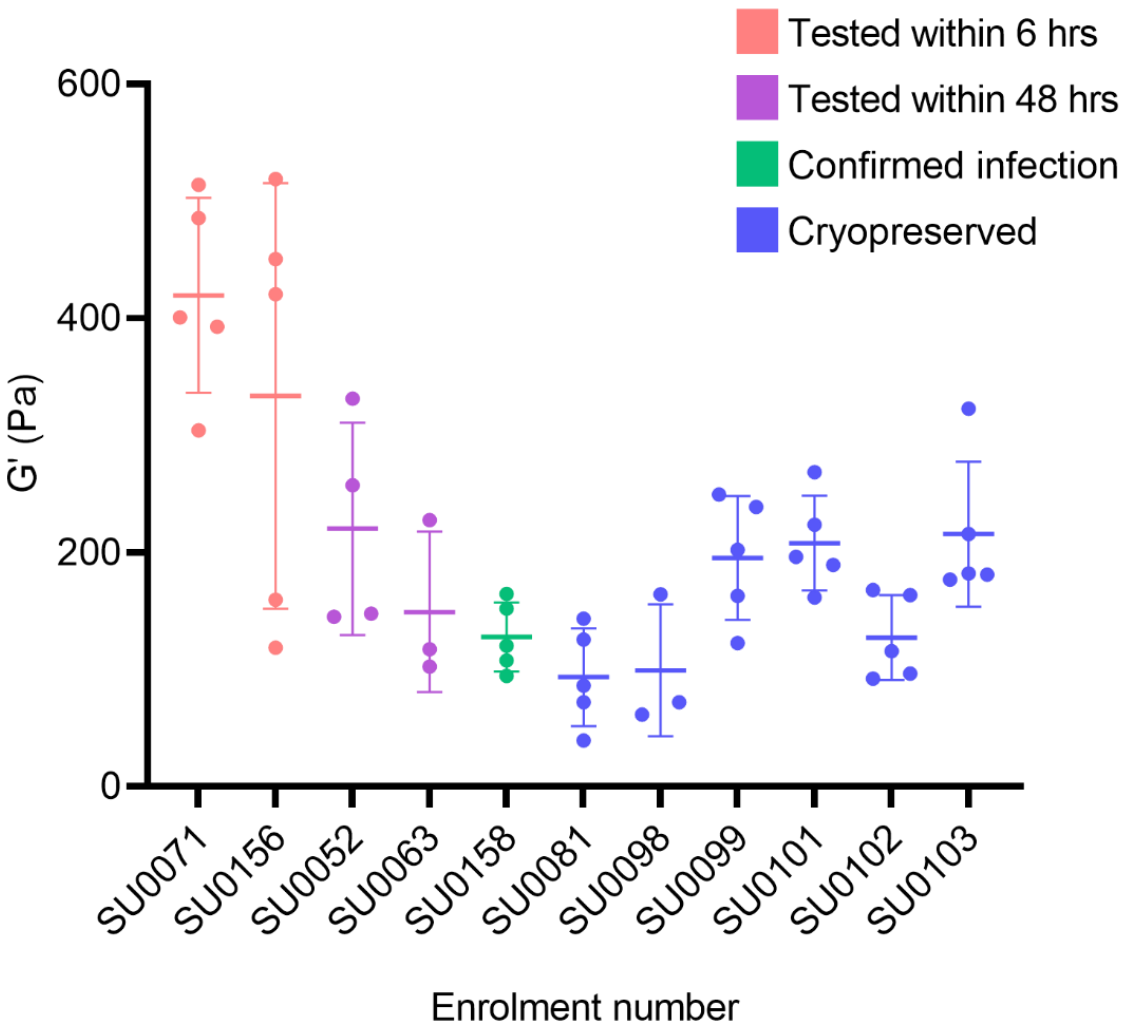


Figure 4.4 Measure of elasticity (G') of fetal membrane samples from 11 patients. Pink = samples processed and tested within 6 hours of delivery, purple = processed within 6 hours of delivery and stored at 4°C in PBS for up to 48hr, green = chorioamnionitis confirmed by histology, blue = cryopreserved within 6 hours of delivery. Measured at angular frequency 0.6283-6.283 rad/s. Mean storage modulus across frequencies for each technical replicate are plotted for each patient, error bars represent standard deviation. Analysis performed on GraphPad Prism.

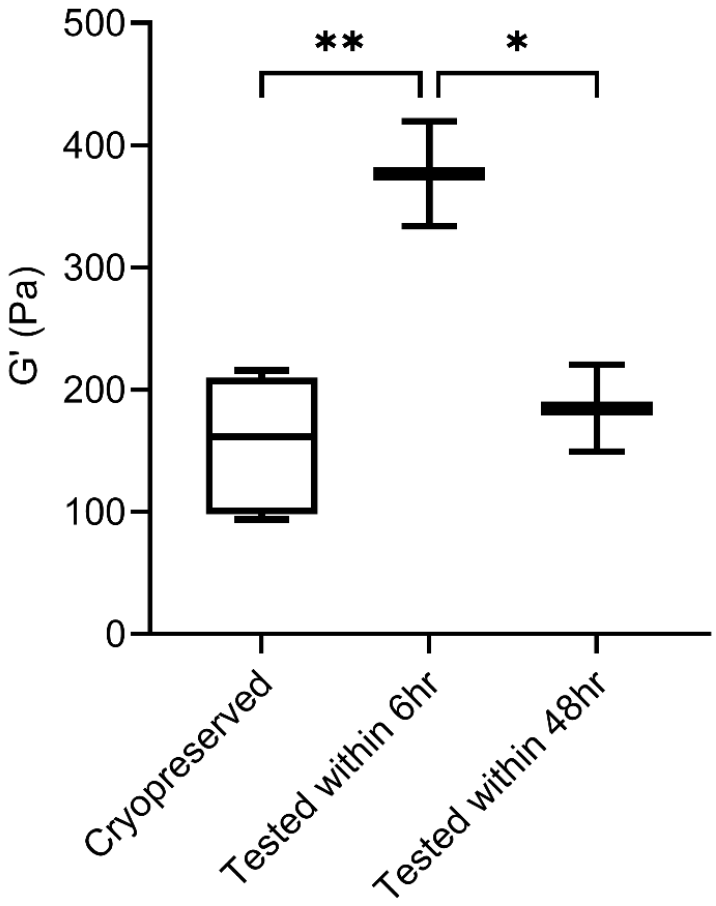


Figure 4.5 Mean G' (measure of elasticity) of cryopreserved and fresh fetal membrane. Angular frequency was measured at 0.6283-6.283 rad/s. Biological repeats from cryopreserved (n=6), fresh tested within 6hr (n=2) and fresh stored in PBS at 4°C for up to 48hr (n=2) fetal membrane samples, each biological repeat included 5 technical repeats per condition. T test, **p= 0.0032, *p=0.026 respectively, mean storage modulus across frequencies is plotted, bars represent range. Analysis performed on GraphPad Prism.

4.5.1.3 Artificial degradation

To assess the ability of the rheometer to detect differences within the fetal membrane samples, fresh samples were artificially degraded using a range of trypsin concentrations. As shown in Figure 4.6, this technique was able to detect differences in artificially degraded samples, however, we did not have enough repeats for statistical analysis.

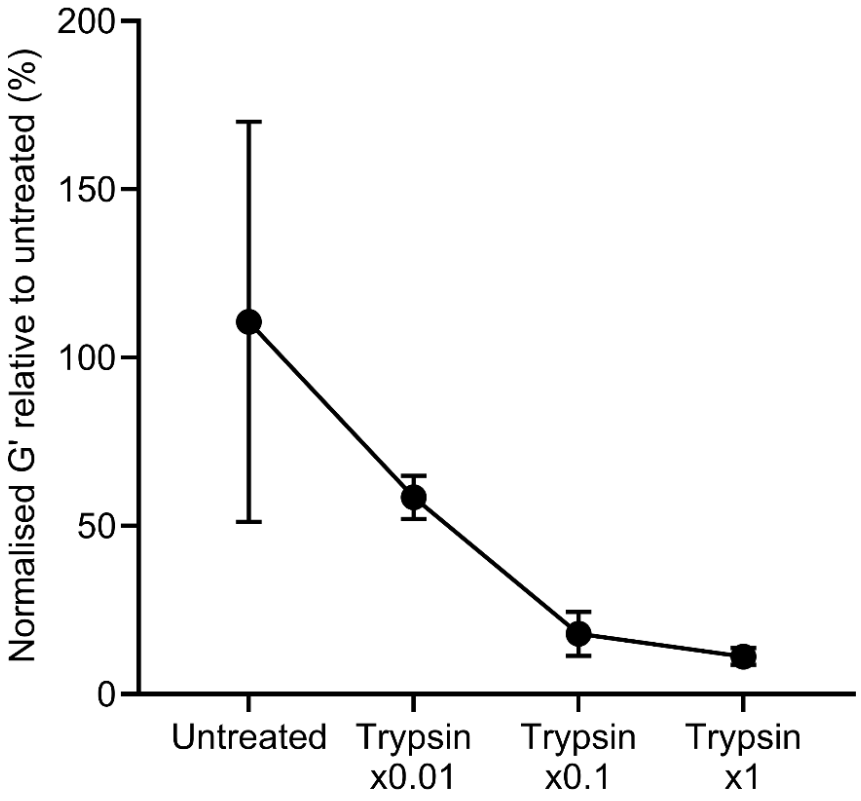


Figure 4.6 Artificial degradation of fetal membranes using trypsin, tested within 48 hours of delivery. Measured at angular frequency 0.6283-6.283 rad/s. Points represent mean, error bars represent SD (n=1, 5 technical repeats per condition). Analysis performed on GraphPad Prism.

4.5.1.4 *Infection Associated Molecule Testing*

In order to assess if infection associated signalling within the tissue could change the sample characteristics enough for us to measure a difference, we mechanically tested samples after stimulation with TNF-α which replicates an inflammation and LPS which replicates bacterial infection. We were able to observe a significant difference between treated and untreated conditions. Treated samples were normalised relative to the untreated controls to account for individual differences in membrane strength between patients. This resulted in the average normalised G' of the untreated samples to be 100%. In comparison, the average G' of samples treated with TNF- α was 53% (± 9.4, p=0.0088), the average G' of samples treated with LPS was 54% (± ± 20.8, p=0.0103) (n=3) (Figure 4.7). This indicates that the viscoelasticity and therefore the mechanical integrity of the treated membrane is roughly half of the untreated and would likely be more susceptible to rupture.

Due to SARS-CoV-2 restrictions in laboratories and clinical settings, some fetal membrane samples were cryopreserved for experimentation at a later date. Experiments with TNF-α and LPS were repeated on cryopreserved samples, however, we did not observe the same % decrease in these samples. Samples treated with TNF-α had a normalised average G' of 106% (± 37.03) compared to the untreated control and samples treated with LPS had a normalised average G' of 77% (± 33.99) (Figure 4.8a).

4.5.1.5 *Metabolite Testing*

In contrast to fresh tissue where the start of the experiment is dependent on clinical factors such as time of delivery, experiments on cryopreserved samples could be planned and therefore we had time to perform an additional metabolite experiment. Membrane samples were treated with bacteria-associated metabolites L-Lactate, D-Lactate and acetate. All metabolites were similar to the untreated control (100%), average G' relative to untreated were: L-Lactate 127% ± 25.5, D-Lactate 108% ± 85.5, acetate 118% ± 19.2 (Figure 4.8b).

For both experiments using cryopreserved samples, we did not have sufficient repeats to perform statistical analysis (n=2).

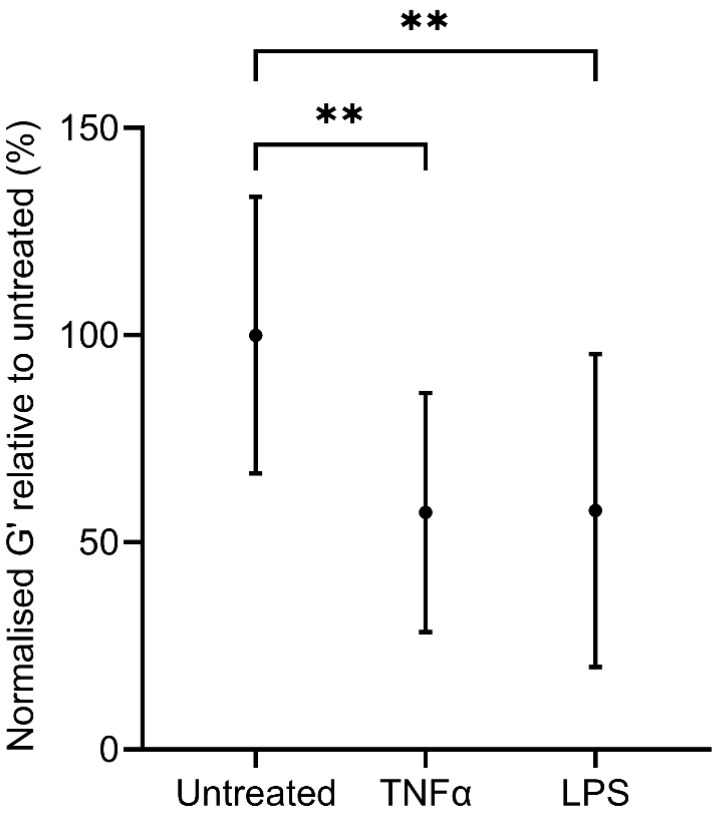


Figure 4.7 Elasticity (G' Pa) of fetal membrane samples tested within 6 hours of delivery after exposure to TNF-α (100 ng/mL) or LPS (1000 ng/mL). Measured at angular frequency 0.6283-6.283 rad/s. Treatment conditions normalised to untreated control (%), circle represents average, error bar represents standard deviation. Untreated vs LPS p=0.0036, untreated vs TNF-α p=0.0027. Data from 3 patients, 5 membrane samples per patient (n=15). Analysis performed on GraphPad Prism.

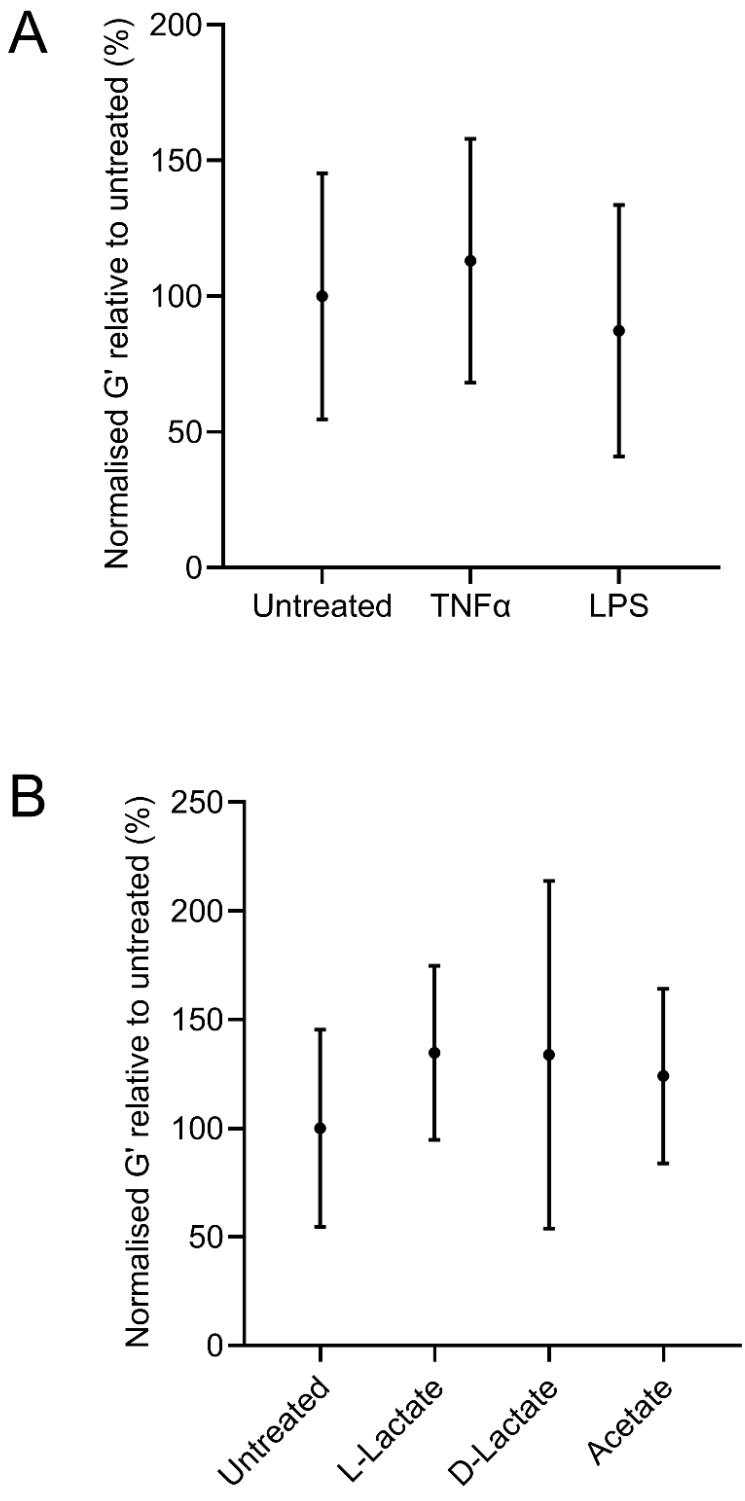


Figure 4.8 Elasticity (G' Pa) of cryopreserved fetal membrane samples (n=2) treated with A) TNF-α (100 ng/mL) or LPS (1000 ng/mL); B) D-Lactate (0.03 mg/mL), L-Lactate (0.03 mg/mL), Acetate (0.6 mg/mL). Measured at angular frequency 0.6283-6.283 rad/s., circle represents average, error bar represents standard deviation. Analysis performed on GraphPad Prism.

4.5.1.6 Tissue Viability

To test the viability of tissue samples after thawing and subsequent incubation with TNF-α or LPS, trypan blue was used to stain and identify non-viable cells. We demonstrated that the tissue was viable using trypan blue after the stimulation experiment compared to a control (Figure 4.9).

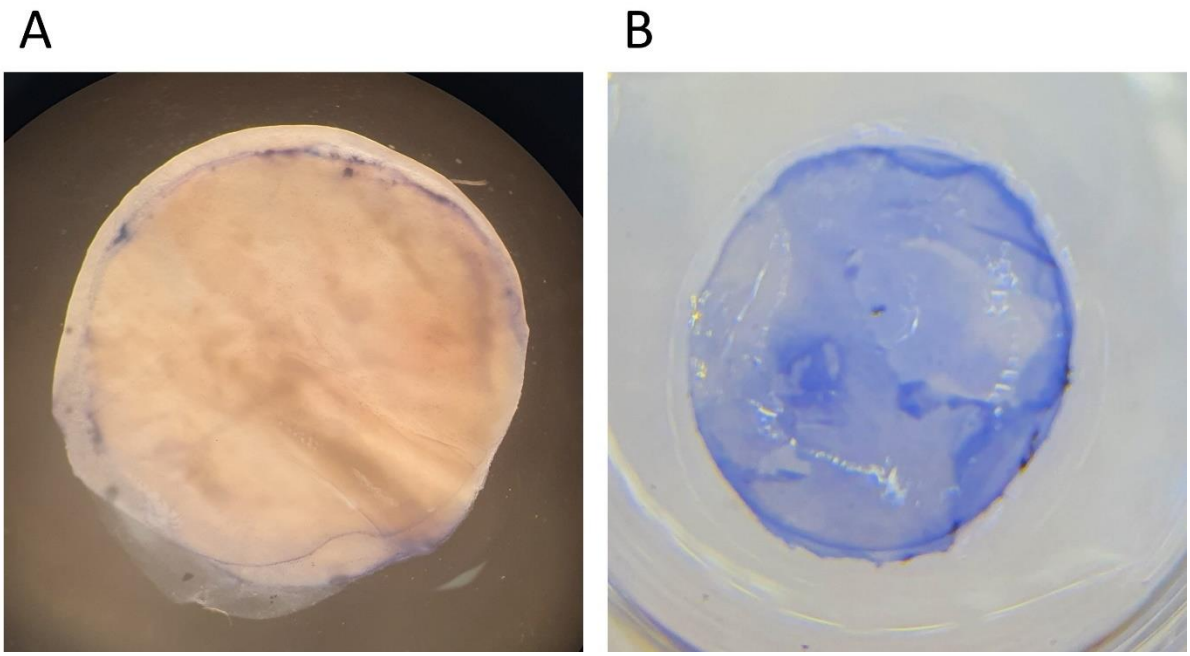


Figure 4.9 Fetal membrane 8 mm discs. Samples were placed in trypan blue for 10 seconds, removed and washed with PBS x3. **A)** after 24hr incubation with LPS; **B)** Control after 1 hr in 100% ethanol.

4.5.1.7 Tissue Relaxation

As the gap between the parallel plates decreases and makes contact with the sample Normal Force increases and indicates contact with the geometry (Janmey et al., 2007). All samples were compressed to 0.2 N Normal Force prior to the frequency sweep to ensure sufficient contact to avoid slippage of the tissue. Once the frequency sweep began the gap between the plates remained stationary and therefore as the tissue relaxes during dynamic shear analysis Normal Force decreases. We can therefore use Normal Force to visualise the rate of tissue relaxation (Figure 4.10). Looking at only one patient (including 5 technical repeats), a visible difference in the rate of relaxation can be seen between the conditions (Figure 4.10a). However, when considering samples from 3 patients this visible difference is no longer present and statistical analysis of area under the curve reveals no significant differences (p>0.05).

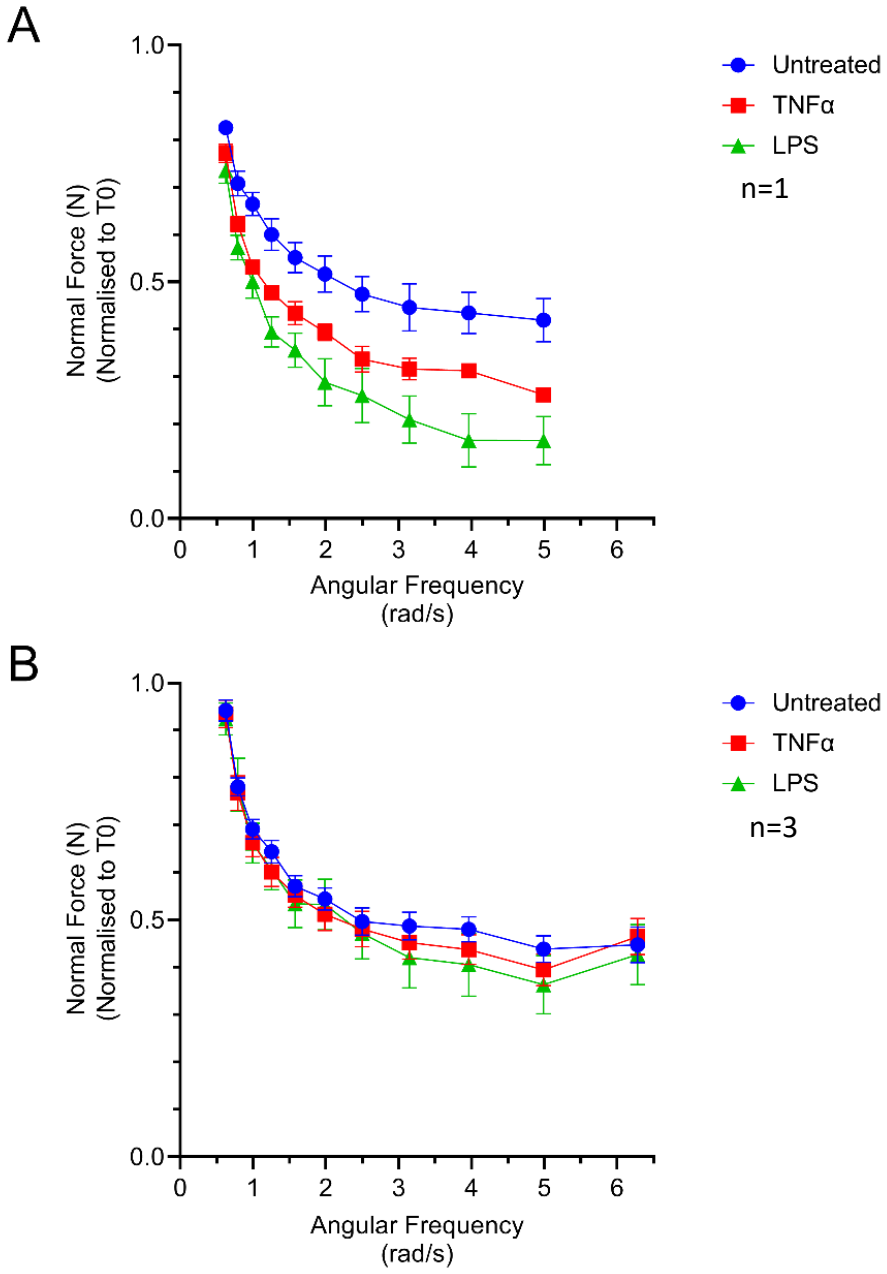


Figure 4.10 Relaxation of fetal membrane samples tested within 6 hours of delivery after exposure to TNF-α (100 ng/mL) or LPS (1000 ng/mL). Normal force (N) measured at angular frequency 0.6283-6.283 rad/s. Each measurement was normalised to the first reading at 0.6283 rad/s for each condition. A) Points are an average of 5 technical repeats (n=1), error bars represent standard error of the mean. B) Points are an average of 5 technical repeats per experiment (n=3), error bars represent standard error of the mean. No significant difference between conditions was observed, measured by area under the curve. Analysis performed on GraphPad Prism.

4.5.2 Collagenase & Gelatinase Assay

4.5.2.1 Collagenase & gelatinase activity

Based on a pilot experiment (detailed in Appendix A), the collagenase/gelatinase activity of conditioned cell-free media from *L. gasseri, L. jensenii* and *L. crispatus* was investigated, this contains bacterial products from optimal growth conditions. The conditioned cell-free media was diluted 1:10 or 1:20 in PBS to reduce the opacity and allow fluorescence to be measured every 15 minutes for 5 hours. All conditions were run in duplicate (n=3). Statistical analysis was performed by calculating the area under the curve (AUC) for each repeat (Figure 4.11) and plotting AUC in a bar plot (Figure 4.12).

The rate of fluorescence intensity increase and therefore gelatin degradation was significantly higher for the conditioned media compared to the controls for both 1:10 (p=0.0010) (Figure 4.11a) and 1:20 (p=0.0005) dilutions (Figure 4.11b). Significance was calculated in Figure 4.12.

The rate of fluorescence intensity increase in the 1:10 dilution appears to differ between *Lactobacillus* species (Figure 4.11a). However, using Tukey's multiple comparisons test we found that each bacterial media condition was significantly different from the negative control but were not significantly different from each other (p>0.05).

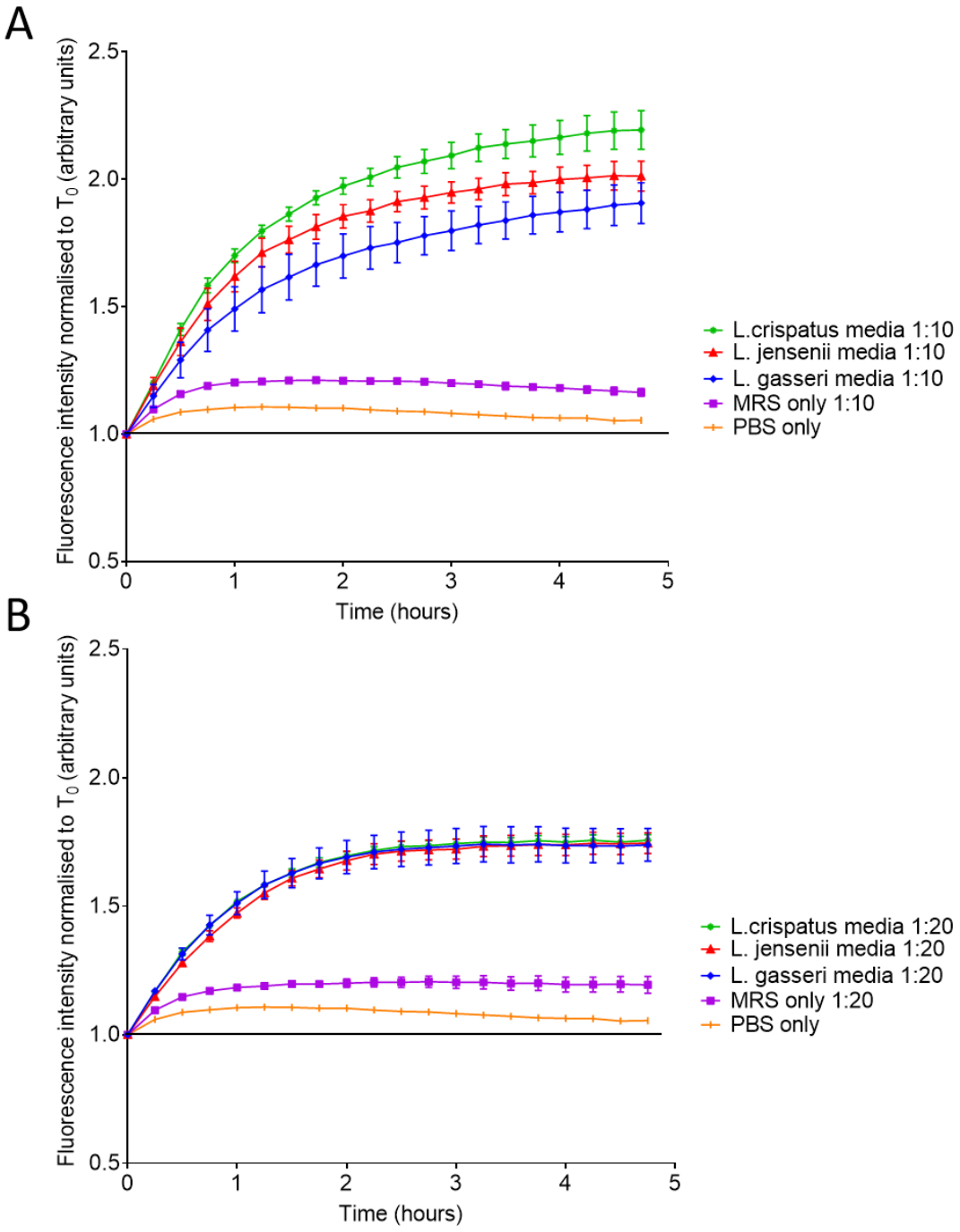


Figure 4.11 Collagenase/gelatinase activity by *L. gasseri*, *L. jensenii* and *L. crispatus* conditioned cell free media **A)** 1:10 dilution **B)** 1:20 dilution Fluorescence intensity (arbitrary units) normalised to the first time point for each condition, measured over 5 hours, all conditions were run in duplicate n=3. Conditioned media contains bacterial products from 10^7 CFU/mL diluted 1:10 or 1:20 PBS. Analysis performed on GraphPad Prism.

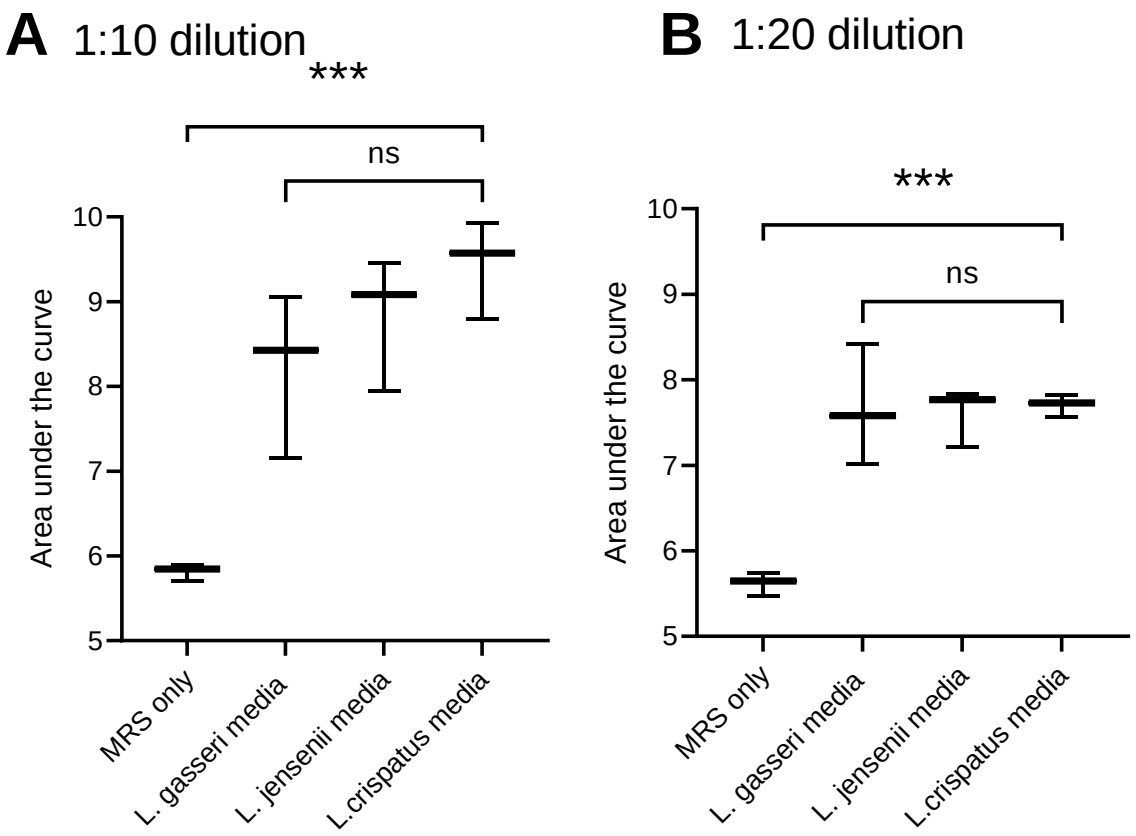


Figure 4.12 Bar plot of Area Under the curve from Figure 4.11.

Curve represents Collagenase/gelatinase of Conditioned cell free media from *L. gasseri, L. jensenii* and *L. crispatus* measured fluorescence **A)** 10^7 CFU/mL diluted 1:10 PBS. ****p*=0.0010 and ns, *p*= 0.2479 **B)** 10^7 CFU/mL diluted 1:10. ****p*=0.0005 and ns, *p*= 0.9658. ANOVA. Bars represent min/max values, line represents median, n=3. Analysis performed on GraphPad Prism.

4.5.2.2 Collagenase inhibition

In addition to collagen degradation, we also investigated the collagenase inhibiting effect of conditioned cell-free media from *L. gasseri*, *L. jensenii* and *L. crispatus* (1:10 dilution) by incubating with 0.005 and 0.001 U/mL collagenase. Inhibition was assessed by comparing fluorescence of collagenase + conditioned cell-free media vs collagenase + fresh bacterial media (MRS) vs collagenase alone. This was measured over 5 hours; all conditions were run in duplicate (n=3).

We were unable to use normalised values for conditions containing collagenase as the degradation and fluorescence increase begins rapidly before the first reading and therefore normalisation does not give an accurate rate of degradation. Collagenase was added to the plate at the last possible moment before loading into the plate reader and starting the first reading. However, the first measurement of the control containing only 0.005 U/mL collagenase ranges from 754 – 1009 arbitrary units, compared to all other conditions which range from 20 -90 arbitrary units.

To test how the opacity of MRS affects fluorescence readings from collagenase degradation, we compared collagenase alone vs MRS + collagenase (1:10) which represents the opacity of the other conditions. Although the rate of increase is visibly different (Figure 4.13a), the AUC was not significantly different. However, MRS + collagenase is a more representative control for conditioned media + collagenase and therefore was used for statistical analysis.

To investigate the collagenase inhibiting abilities of *Lactobacillus* species we compared cell-free conditioned media from *L. gasseri*, *L. jensenii* and *L. crispatus* (1:10 dilution) + collagenase vs fresh MRS media + collagenase (1:10). The rate of degradation was not significantly different between the control and the *Lactobacillus* conditions when AUC was compared using ANOVA and Tukey's multiple comparisons test (Figure 4.14). However, the trend in the data suggests that *Lactobacillus* may be able to inhibit degradation to some extent (Figure 4.13). Future research is required to establish this and should investigate a wider range of enzymes at a range of concentrations.

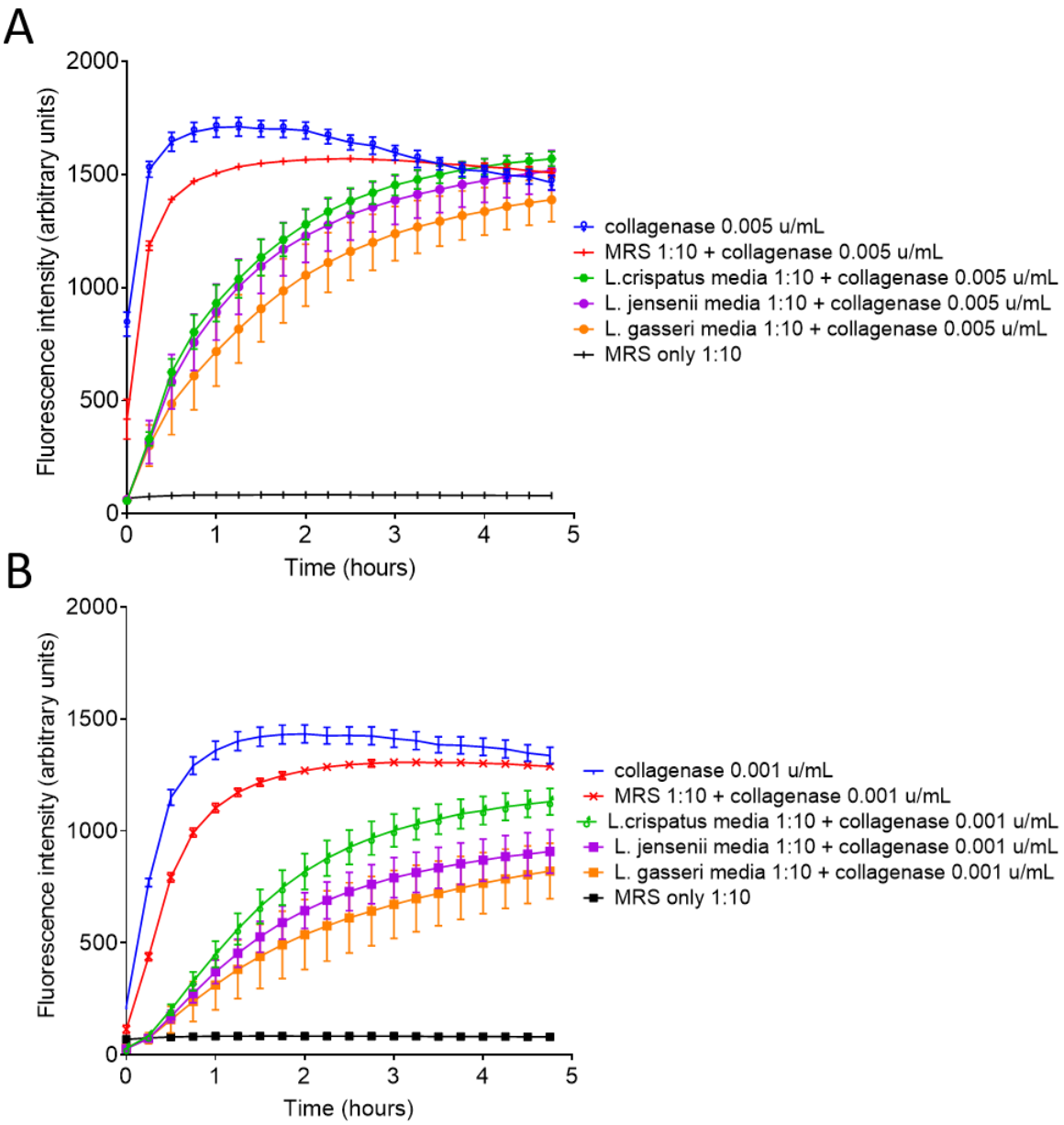


Figure 4.13 Collagenase inhibition by *L. gasseri*, *L. jensenii* and *L. crispatus* conditioned cell-free media 1:10 dilution mixed with **A)** 0.005 U/mL collagenase; and **B)** 0.001 U/mL collagenase. Fluorescence intensity (arbitrary units) measured over 5 hours; all conditions ran in duplicate (n=3). Analysis performed on GraphPad Prism.

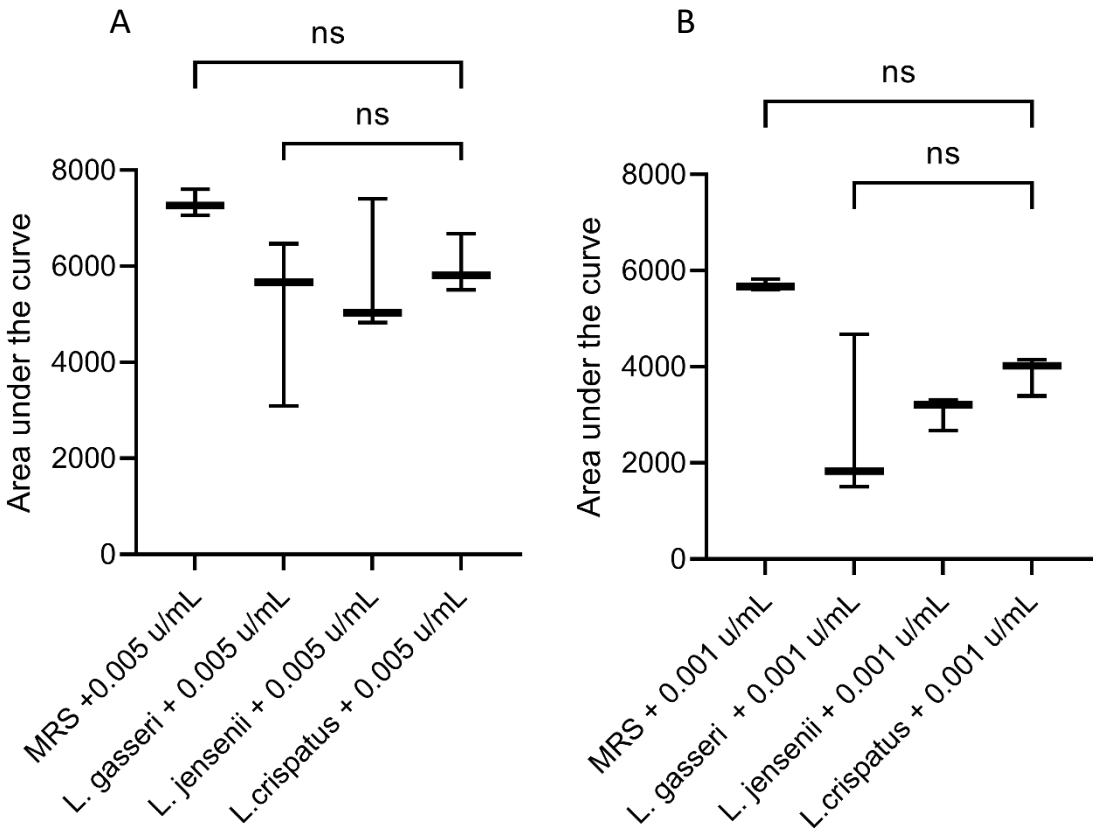


Figure 4.14 Collagenase/gelatinase inhibition significance of *L. gasseri, L. jensenii* and *L. crispatus* measured by area under the fluorescence curve from Figure 4.13. Conditioned cell free media contains bacterial products from 10^7 CFU/mL diluted 1:10 PBS. **A)** 0.005 U/mL collagenase; and **B)** 0.001 U/mL collagenase. Bars represent min/max values, line represents median, n=3. Analysis performed on GraphPad Prism.

4.5.3 Key Findings

Dynamic Shear Analysis of Fetal Membrane

- Membrane samples tested within 6 hours of delivery (n=2) had a significantly higher G' than cryopreserved samples (n=6) (p= 0.0032) (Figure 4.5)
- Dynamic shear analysis is able to detect differences in artificially degraded samples (Figure 4.6)
- Infection associated molecules significantly decreased the stiffness of fresh membrane samples compared to the control. Untreated vs LPS p=0.0103, untreated vs TNF-α p=0.0088 (n=3) (Figure 4.7)
- Infection associated molecules did not significantly decrease the stiffness of cryopreserved membranes (Figure 4.8a)
- Bacterial associated metabolites did not significantly decrease the stiffness of cryopreserved membranes (Figure 4.8b)

Collagenase & Gelatinase Assay

- The rate of fluorescence intensity increase and therefore gelatin degradation was significantly higher for the conditioned media compared to the controls for both 1:10 (p=0.0010) (**Figure 4.11**a) and 1:20 (p=0.0005) dilutions (n=3) (Figure **4.11**b & Figure 4.12)
- The rate of fluorescence intensity increase was not significantly different between bacterial species (Figure **4.11**a) (p>0.05)
- Inhibition of collagenase degradation by *Lactobacillus* conditioned media was not significant (Figure 4.13 & Figure 4.14) but is an area for future research

4.6 Discussion

We aimed to investigate the effect of exposing FM samples to bacteria. Our hypothesis was that some organisms would decrease the stiffness of the membranes via collagen degradation and/or inflammatory mechanisms. However, due to SARS-CoV-2 restrictions we were unable to complete live bacteria testing on fetal membrane. Instead, FM were exposed to infection associated molecules (section 4.6.1) and the degradation abilities of bacteria were tested using a gelatin assay (section 4.6.2). Based on other studies such as (Jayes et al., 2016) who investigated the effect of bacterial collagenase on uterine fibroids, we anticipate a decrease in stiffness of the FM in the presence of bacteria able to fully or partially degrade collagen or components of the ECM. This promising area of work will be explored further through future studies (see future work section).

4.6.1 Dynamic Shear Analysis of Fetal Membrane

These experiments aimed to develop a protocol for testing fetal membranes using dynamic shear analysis, which is a novel method for this tissue. As discussed in section 4.1.5, this method measures the **elasticity of a material via the elastic storage modulus (G') which is often referred to as "stiffness"**. We hypothesised that stiffer fetal membrane with a higher G' (also referred to as storage modulus or Young's modulus) would be less susceptible to rupture and would have higher integrity.

We observed a large variability of G' between the technical repeats for all conditions as can be seen with our large error bars in Figure 4.4. Similar to our study, Benson-Martin et al, (2006) reports a large variability in the Young's moduli (G') within the same amniotic membrane. This is likely due to the complexity of the tissue and is expected in delicate biological samples that may change slightly *in vitro*. Because of this variability we increased the number of technical replicates to 5 per condition. Regions of the membrane may have different properties like the weakened area above the cervix. In this study, we took samples located away from the rupture line and margin of the placenta. Every effort was made to take samples from the same area of the membrane, usually a 5 x 5 cm area was large enough for all technical repeats for one experiment.

4.6.1.1 Thickness

We found no significant difference in FM thickness between the conditions (cryopreserved, fresh, stimulated with TNFα or LPS). A previous study used uniaxial mechanical analysis to compare term and preterm membranes, they also measured no difference in thickness between term and preterm. However they observed that the thinner the amniotic membrane, the stiffer the membranes were in both term and preterm samples (Benson-Martin et al., 2006). We did not observe this correlation in our study however, higher G' may be more apparent in membranes that are thin due to being overly stretched as stretching is known to strengthen or in this case, stiffen, samples (Pandey et al., 2007). We did not sample from thinned areas of the membrane near rupture sites which is likely the reason for our similar thickness of samples across patients.

4.6.1.2 Storage at 4 °C

Initial experiments were performed on fresh membranes but, due to timing of sample collection and availability of equipment, these samples were stored at 4 °C for either 24 or 48hr. Storage of fetal membranes at 4 °C in PBS for up to 24 hours before mechanical testing has previously not been shown to have an impact on experimental results (Faturechi et al., 2015; Schober, Kusy, Whitley, et al., 1994). However, our results (Figure 4.5) show a significantly decreased G' in samples stored in PBS at 4 °C compared to those tested without storage at 4 °C. This decrease in integrity likely represents the reduced viability of the cells and tissue at temperatures that do not replicate *in vivo* conditions. The contrast to previous studies may be due to using dynamic shear analysis which is a more sensitive technique than either burst testing or uniaxial rupture testing used in previous studies. These techniques measure the force required to rupture the tissue rather than the properties of the tissue under different rates of deformation as dynamic shear analysis does.

4.6.1.3 Storage at-80°C

Due to SARS-CoV-2 restrictions, testing samples on the day of delivery became impossible due to mandatory laboratory closures between March 2020 – March 2021. During this time, where possible samples were cryopreserved for mechanical testing at a later date.

Many studies have investigated cryopreservation of fetal membrane for wound healing applications. These studies that focussed on the amnion have demonstrated that amnion cells are viable and retain their antimicrobial properties after thawing (Mao et al., 2017; Tehrani et al., 2013). Additionally, using histology, Cooke et al, (2014) demonstrated that the structure of amnion was unchanged after cryopreservation. Another study replicated this histological finding but also reported >80% cell viability after thawing, and that membranes remained viable for at least a week in culture (Duan-Arnold et al., 2015).

We demonstrated that our fetal membrane samples were still viable after thawing and culture via trypan blue staining (Figure 4.9). Therefore, the membrane samples should be able to respond to stimuli such as bacteria or cytokines after cryopreservation, which we hypothesise will alter the structure of the tissue. However, our results (Figure 4.5) show that the cryopreservation process significantly reduces the stiffness of full thickness fetal membrane. We observed similar stiffness in the untreated control compared to those treated with TNF-α or LPS (Figure 4.8a). In contrast, the fresh samples treated with TNF-α or LPS had a significantly lower G' compared to the untreated control (Figure 4.7). The cryopreservation appears to alter the samples to an extent that cellular signalling cannot further change the tissue in a measurable way using this technique.

The cryopreserved samples have similar G' to the artificially degraded samples, therefore we initially hypothesised that collagen, which is one of the main components of most layers in FM (Figure 1.6), is degraded after cryopreservation. However, previous studies do not support this hypothesis. Wagner et al, (2018) tested the effect of cryoprotectants and found that neither glycerol nor direct freezing significantly altered the epithelial or stromal structure of the Amnion. They observed that freezing methods did not influence tensile strength but, longer storage periods resulted in a significantly **increased tensile strength** for the amnion. Another study using the same rheology technique as our work, measured pulverised freeze–thawed collagen samples and observed increased G′ values with increasing freeze–thawing

cycles (Ding et al., 2015). The freeze–thawed samples had higher G′ values compared with those stored at 4°C (Ding et al., 2015). The stiffening of collagen after freezing has also been measured in other studies in various tissue such as aorta (Chow & Zhang, 2011) and arteries (Blondel et al., 2000).

This evidence suggests that it is not the collagen structure which is degraded post thawing in our fetal membrane samples as collagen has been shown to become stiffer with a higher G' after freezing. The increase in G' is speculated to be due to increased entanglement of collagen chains after freezing.

Fetal membranes have a multi-layered structure which includes collagen, cells and extracellular matrix (ECM). There are 2 main layers (Figure 1.6) the amnion which provides most of the structural strength of the membrane (Arikat et al., 2006), and the chorion which adheres to the uterine decidual tissue but can easily be pulled apart after delivery. Separation of the amnion and chorion is the first step in the weakening of the fetal membranes (Arikat et al., 2006). These 2 layers are connected by a spongey layer of connective tissue made up of proteoglycans, hyaluronan, glycoproteins and collagen types 1, 3 and 5 (Strauss, 2013). Degradation of both proteoglycans and hyaluronan found in connective layers and ECM will contribute to fetal membrane weakening by preventing the two layers working in parallel (Meinert et al., 2014).

Therefore, we believe that the connective layers within the membrane are susceptible to degradation during cryopreservation and are the likely cause for decreased G' values. If the amnion and chorion are able to move independently, the rheometer is not properly able to measure the shear strain and give insight into the viscoelastic properties of the whole tissue but is actually measuring the stiffness of the degraded connective tissue. Because of this, we recommend that only samples with no separation of the amnion and chorion should be tested using dynamic shear analysis.

4.6.1.4 Stimulation with infection associated molecules

We were able to pool fresh sample data from 3 patients for analysis. Additional fresh tissue was processed in the lab. However, these samples could not be used due to separation of the membrane layers or obvious discolouration, both indicating chorioamnionitis. We observed that the sample with confirmed infection had a decreased G' compared to the other fresh samples, likely because the membrane has a slimy consistency, and the amnion separates from the chorion causing the material to slip as it is tested as discussed above.

After treatment with 100 ng/mL TNF-α or 1000 ng/mL LPS we observed a significant decrease in fetal membrane stiffness (Figure 4.7). Other studies have observed the same weakening induced by TNF-α (Kumar et al., 2015) and LPS (Fortunato et al., 2001). Fortunato et al. also observed increased MMP-9 after LPS treatment which is the likely cause of weakening. Similar to the weakening after cryopreservation, degradation of the ECM is a likely contributor to the reduced stiffness we measured. The tissues response to the experimental stimuli e.g., increased inflammatory signalling will likely result in overall weakening of the membrane layers however the ECM contains hyaluronan, glycoproteins which are more easily degraded than collagen. Supporting this, (Garcia-Lopez et al., 2007) observed ECM degradation and structural disarrangement via histology after LPS stimulation.

Other cytokines have also been shown to produce a weakening effect, Kumar et al, (2005) exposed samples to 0-100 ng/mL of TNF-α or IL-1β, and measured a decrease in membrane strength in a dose and time dependent manner. Both IL-1β and TNF-α also induced MMP-9 and inhibited TIMP-3. TNF-α, IL-1β and MMPs naturally increase in the amniotic fluid at later gestations and are thought to contribute to membrane weakening at term (Hulboy et al., 1997). If these molecules are induced prematurely by bacteria, membrane weakening usually seen at term may appear at earlier gestations.

One study has demonstrated a potential treatment for cytokine induced weakening. Moore et al, (2009) hypothesised that the antioxidant α-lipoic acid would inhibit TNF-α weakening. They reported TNF-α decreased the rupture strength of the FM fragments by more than 50% and increased MMP-9 release. α-lipoic acid was found to inhibit all TNF-induced effects.

α-lipoic acid is a good candidate for future treatment however extensive *in vitro* studies are required to confirm the efficacy. Our novel technique for characterising the mechanical

properties of fetal membrane could be used to compare untreated vs cytokine stimulated vs cytokine + α-lipoic acid. However, as with treatments such as antibiotics, any treatment would likely need to be administered before irreversible inflammation occurs.

4.6.1.5 Stimulation with bacteria-associated metabolites

In addition to TNF-α and LPS, we treated the membranes with bacteria-associated metabolites: L-Lactate, D-Lactate, and acetate (Figure 4.8b). However, we observed minimal difference between the conditions. This is unsurprising, as previously discussed, the structure and therefore elasticity of the cryopreserved membrane samples are altered and unlikely to change further after stimulation. Unfortunately, due to the challenges of acquiring fresh uninfected tissue on the morning of an experiment during the pandemic, there was sufficient time to test only 3-4 conditions and so it was not possible to test both metabolites and TNF-α/LPS in one experiment, as was done with the cryopreserved samples. We chose to focus on TNF- α and LPS and so do not have data on the effect of metabolites on fresh fetal membrane. However, now this novel technique has been optimised this work can be continued in future studies. As demonstrated by infection associated molecules, bacterial stimuli are able to influence the mechanical properties of fetal membrane tissue. Experiments with live bacteria would encompass the tissues response to both the presence of bacteria and also bacterial products.

4.6.1.6 Stiffness & Microbiome

The microbiota have been implicated in ECM changes leading to altered stiffness in relation to cancer (Martinez-Vidal et al., 2021) therefore we hypothesise that the vaginal microbiome is able to influence the nearby gestation tissues i.e. the cervix and fetal membranes.

Bacteria have been shown to mimic endogenous membrane-bound proteinases (Vollmer et al., 1996) which can breakdown the ECM. Additionally, *Candida albicans and Streptococcus pneumoniae* are known to produce hyaluronidase which is able to breakdown hyaluronic acid, hyaluronan present in fetal membrane (Hynes & Walton, 2000). Tissue stiffness may also be influenced by LOX which is an extracellular enzyme able to catalyse the crosslinking of collagen and elastin; genes for has been identified in Bacteroidetes, Actinobacteria and Proteobacteria (Martinez-Vidal et al., 2021).

Therefore, the community profile of vaginal microbiome could greatly influence the structure of fetal membrane. Dysbiosis may lead to impaired tissue homeostasis due to the altered response from the host, bacterial products and pH (see Figure 1.9) leading to measurable changes in the viscoelasticity of fetal membrane (Martinez-Vidal et al., 2021). The potential influence of the cervico-vaginal microbiome on fetal membrane integrity is worth investigating in future experiments.

4.6.1.7 Tissue Relaxation

In addition to G', we are also able to measure the normal force (N) from the tissue samples as it is compressed and tested. Each sample is compressed to a normal force of 0.2 N and as the tissue relaxes, the normal force decreases (Figure 4.10). Conditions in the first repeat appeared to have a visible difference in relaxation times, with the untreated condition relaxing at a decreased rate compared to the treated conditions. However, after pooling the experiments there was no significant difference between conditions. Despite not finding significant differences, this should be investigated further at in future studies as the relaxation time gives us more information regarding the properties of fetal membrane and can provide insight into the likelihood of rupture. This data includes samples from 3 patients and so a larger sample size may reveal interesting results.

4.6.2 Collagenase & Gelatinase Assay

We aimed to investigate the collagen degradation abilities of vaginal bacteria in order to assess which organisms are likely to play a role in fetal membrane degradation and subsequent PPROM. To do this, we used a fluorescent gelatin assay which allows us to measure degradation from a range of enzymes that may play a role in PPROM including collagenase and gelatinase. This DQ™ gelatin assay has previously been tested using a range of MMP-9 enzymes including those implicated in PPROM and was found to be useful in studies of MMPs (Vandooren, 2011). Bacterial proteases are beneficial for microorganisms and allow better colonisation of the host, access into deeper tissues, evasion of host immune response and access to required amnio acids for growth (Duarte et al., 2016).

4.6.2.1 Gelatin degradation from live bacteria

The pilot experiment tested PBS (clear, low opacity) and MRS (dark brown, high opacity). The live bacteria were resuspended in either PBS or MRS at 10^7 CFU/mL based on the *in vivo* concentration of bacteria in CVF (Li et al., 2019). The PBS condition is ideal for fluorescence readings as it will not obscure fluorescence released by the gelatin. However, as PBS does not contain the required nutrients for bacterial growth, over 20 hours the bacteria may no longer be viable and may not degrade the gelatin. The MRS condition provides the correct nutrients for the bacteria, but obscures fluorescence released from the gelatin as it degrades. Both conditions were tested at 1:1, 1:10, 1:100 dilution with PBS to reduce the concentration of bacteria but also the opacity of the MRS broth.

As expected, we found that the raw fluorescence readings for PBS vs MRS were significantly different due to the opacity (Figure 7.1). Therefore, each condition was normalised to the first time point to show the change in fluorescence throughout the experiment rather than raw fluorescence values. We observed fluorescence decreased rapidly over time for all bacterial species suspended in either PBS or MRS compared to the PBS control which decreased steadily over time (Figure 7.2). It is likely that bacterial growth throughout the duration of the assay obscures fluorescence readings as cells multiply. As often observed in bacterial culture, growth leads to “cloudiness” in liquid cultures (Faidy & Ali-Shtayeh, 2000).

The reduction in fluorescence during the experiment due to bacterial growth is supported by the dose dependent pattern of the fluorescence decrease (Figure 7.2c). The 1:1 dilution

containing the most bacteria decreased immediately, the 1:10 dilution after 30 min and the 1:100 dilution after 1hr 45min. We successfully recovered live lactobacilli from all live bacteria conditions after 20 hours in aerobic conditions on plate and can therefore say that the bacteria were viable and able to multiply during the assay.

Arguably, bacterial growth will be reduced as these organisms are anaerobic and the plate reader is exposed to aerobic environment. However, as they are grown anaerobically, they are in peak condition and are able to survive short periods in aerobic conditions as observed in other studies testing anaerobes using this assay (Lithgow et al., 2022).

However, to remove the challenges of live bacteria obscuring the fluorescence reading we focussed on bacterial products only.

4.6.2.2 Gelatin degradation from bacterial products

In order to investigate the enzyme activity of bacterial products, 3 species of commensal vaginal bacteria were grown in optimal conditions. After which, bacterial cultures were normalised by the addition of fresh MRS broth, centrifuged and the supernatant was filtered to remove all bacteria. We successfully verified the absence of bacteria by culturing the conditioned media and confirming no bacterial growth for 7 days.

We observed that the opacity of the non-diluted conditioned media appears too high for fluorescence readings as there is no visible increase in fluorescence compared to the 1:10 dilution which rapidly increases (Figure 7.3). The 1:10 media diluted with PBS appears to allow fluorescence to be detected without diluting the enzymes to an extent that they no longer degrade the gelatin. The 1:100 dilution appears to give a small increase in fluorescence but is a lot smaller compared to the 1:10. This cannot be due to opacity as the 1:100 has x10 more PBS and so has a lower opacity (Figure 7.3). Therefore, the 1:100 dilution appears to dilute enzymes to a concentration where they do not degrade the gelatin. As this was a pilot experiment, there was only one repeat but, based on this result, we subsequently focussed on conditioned cell-free media diluted 1:10 and 1:20 in PBS.

In subsequent experiments, we observed a significant increase in fluorescence in the bacterial conditioned media compared to negative controls (p=0.0010, Figure 4.12). This indicates that these bacteria, although considered commensal, may have the capacity to degrade FM and contribute to PPROM. However, we found no significant difference between species of

Lactobacillus. This is expected as these organisms are closely related despite some e.g. *L. iners* being associated with preterm birth (Verstraelen et al., 2009). In order to better assess the degrading capacities of these species we would need to compare their activity to. a known collagen degrading organism such as GBS which has been shown to efficiently cleave type IV collagen as well as fibronectin, albumin, fibrinogen and laminin (Soares et al., 2008). However, the mechanism by which each organism contributes to PTB should be considered. For some *Lactobacillus* species, co-existence with more pathogenic organisms such as *Gardnerella vaginalis* is thought to be the reason they are associated with PTB (Verstraelen et al., 2009). Therefore, it is important to consider the interactions between species as well as individual degradation abilities.

In contrast to our findings, Lithgow et al, (2022) used the DQ™ collagen type IV from human placenta assay to investigate *Porphyromonas* species in relation to FM rupture and used *L. crispatus* as a comparison. They detected no significant collagenase activity from *L. crispatus* (Lithgow et al., 2022). This result could be due to a much smaller collagen degradation ability in *L. crispatus* relative to *Porphyromonas.* Additionally, this study used a more specific collagen IV substrate compared to our study which used gelatin that can be degraded by a wider range of enzymes.

Vaginal bacteria such as GBS or *Ureaplasma parvum* are known to induce MMP production via inflammation (Potts et al., 2016; Flores-Herrera et al., 2012). However, very few bacterial species are able to produce true collagenases that can cleave the triple helix structure of a collagen fibre (Duarte et al., 2016). MMP-1 is able to degrade collagen type I, II, III, gelatins and other components of the ECM but not type IV (Lu et al., 2011). Hence, by using a type IV specific assay, some degradation abilities may be missed. *In vivo* bacterial presence and products interact with the host cells and can cause a cascade of inflammatory signals and release of MMPs. For example, MMP-9 which is able to degrade collagen type III, IV, V, gelatin and elastin is activated by MMP-2, 3, 10, and 13 (Lu et al., 2011).

Unfortunately, due to time constraints, we were not able to analyse the conditioned media containing the bacteria products to identify which enzymes were present. The conditioned media is stored and can be analysed in future experiments. As this is a non-specific gelatinase assay, a range of enzymes could contribute to the degradation we observed. However, we

can draw some conclusions based on the relative fluorescence compared to the collagenase positive controls.

4.6.2.3 Enzyme concentration

We found in the pilot experiment that 0.5 U/mL and 0.05 U/mL collagenase produced significantly higher fluorescence than the bacterial products. Subsequently, we reduced this to 0.005 and 0.001 U/mL but fluorescence was still significantly higher than the conditioned media. This allows us to speculate that the concentration of enzymes in the conditioned media was fairly low, definitely lower than 0.001 U/mL collagenase. However, the conditioned media may not contain collagenase which rapidly breaks down gelatinase, other enzymes able to degrade gelatin such as MMPs are more commonly produced by bacteria, and these may degrade gelatin at a slower rate (Goldberg, 1972).

Ideally, bacterial products could be analysed using a non-targeted approach such as mass spectrometry or enzyme arrays to measure metabolites and enzymes. Alternatively, using the methods from this chapter, serial dilutions of collagenase and other enzymes could be performed to identify a similar fluorescence curve more accurately and therefore concentration of enzymes present using this method.

4.6.2.4 Collagenase inhibition from bacterial products

To test bacterial collagenase inhibition which is thought to be a desirable property of commensal bacteria associated with term birth, we compared conditioned media, conditioned media + collagenase, and MRS + collagenase. The bacterial conditioned media was not statistically different from the controls (Figure 4.14) however, we did observe a lower rate of fluorescence increase for the conditioned media + collagenase compared to MRS + collagenase (Figure 4.13). Despite the lack of statistical significance, this trend should be investigated in future research. Key health promoting bacteria such as *Lactobacillus* may be able to protect from PPROM associated MMPs and bacteria.

Our finding, although not significant, is supported by other studies that found that *Lactobacillus* species down regulated MMP-1, MMP-2, MMP-3, MMP-9, MMP-10 and up regulated TIMP-1 and TIMP-2 which would decrease degradation and reduce the chance of PPROM (Shirzad et al., 2018). From these results it seems likely that commensal organisms such as *L. crispatus* may produce some MMPs able to degrade gelatin but play a bigger role

in inhibiting degradation of fetal membranes from other bacterial products produced by more pathogenic organisms. Further work to compare common organisms within the VMB are needed to understand the relative degradation and inhibition abilities.

In summary, we observed significantly increased degradation of gelatin by *Lactobacillus* products compared to the control. This finding demonstrates that commensal bacteria could play a role in the weakening of FM prior to PPROM. However, we did not compare *Lactobacillus* with organisms known to produce collagenases so the degradation abilities may be relatively small in comparison.

Despite the likely low collagen degradation ability of some species, it is important to study both commensal bacteria and those associated with PPROM in order to understand the interactions between bacteria. This point is highlighted by our second finding, although not significant, which showed that *Lactobacillus* could play a role in collagenase inhibition and potentially protect FM from weakening. Ultimately, weakening of FM will depend on the balance of enzymes and inhibitors produced by the host and the cervico-vaginal microbiome which can include a range of organisms. Consequently, it is vital to study all vaginal species not just those most associated with PPROM.

4.7 Summary & Future Work

To summarise, this work is part of a limited number of studies that investigate mechanical and molecular properties of FM and normal vaginal microbiota. We were able to use a novel technique (DSA) to test the mechanical properties of the fetal membrane after exposure to infection and inflammation associated signals. We found that tissue that had been exposed to these signals had a lower G' compared to controls, indicating reduced tissue viscoelasticity and increased susceptibility to rupture/PPROM.

It was observed that after purposeful degradation, the samples showed reduced G'. However, we also saw this after cryopreservation where the amnion is known to become stiffer and G' increases. We conclude that it is the connective layers between the amnion and chorion that are most susceptible to degradation and cryopreservation. Once the connection between layers is weakened the 2 layers are then able to move more independently and so our method which includes rotation, is unable to measure the tissue accurately. This method would still be useful for the amnion alone but cannot measure multiple layers that can move independently. This data gives us insight into the mechanical structure of full thickness membrane, which is less studied than amnion alone but better reflects the complexity of the tissue.

Experiments using a novel technique were successfully optimised, these methods and preliminary data can be taken forward in future work. Additionally, the protocol for tissue collection, dissection, stimulation and transportation to the natural materials lab could potentially be used for a different novel technique.

A degradation assessment assay for bacterial products was also optimised, this can be used in future work for a range of bacteria to assess their ability to weaken fetal membranes and contribute to PPROM. Or, to inhibit collagenase and prevent membrane weakening.

We found that bacterial products degraded gelatin and so increased fluorescence more than the controls. We also observed a non-significant trend which should be further investigated as it indicated that *Lactobacillus* species may be able to inhibit collagenase and play a role in inhibiting PPROM. We have not yet compared a pathogenic organism so cannot compare the relative gelatin-degrading abilities of the commensal organisms.

Commensal bacteria may play a larger role in PTB and PPROM than originally thought. These organisms are understudied as most research focussed on GBS. However, it is important to study the vaginal community as a whole. Future models should aim to encompass multiple species to capture the interactions between bacteria in the niche. We know that collagenase contributes to PPROM, and it is likely that bacteria play a role in PPROM. However more research is needed to gain more insight.

4.7.1 Future Work

Unfortunately, due to SARS-CoV-2 restrictions, rheology experiments containing live vaginal bacteria could not be completed. However, this work serves as a great starting point for future studies. One significant factor that was previously not studied is the transportation and storage temperatures of the tissue between labs. Our recommendation for future studies is to keep tissue samples at biological temperature whenever possible to reduce structural changes. Periods of room temperature is significantly better than 4°C or cryopreservation as shown by our data and supported by (Chow & Zhang, 2011) who demonstrated increased stress and strain in aorta after cold storage.

Firstly, vaginal organisms can be individually tested on FM and assessed using DSA technique as optimised in this study. The impact of organisms on membrane viscoelasticity can be compared to assess which organisms play a role in membrane weakening. In addition to mechanical assessment, cytokine analysis of culture media using an ELISA would allow us to measure the inflammatory response of the tissue and correlate that with the mechanical data. Similarly, MMP secretion in the culture media could be assessed and compared. Media from experiments outlined in this chapter is currently stored at -80 ℃ and can be analysed to add to the data presented here.

Secondly, once individual vaginal bacteria have been assessed, combinations of bacteria could be investigated to observe the interactions between species. Ideally, mixed infection models which replicate common vaginal communities dominated by *Lactobacillus* species or anaerobes would be tested.

Thirdly, in relation to biomarker identification, metabolomic analysis of the FM infection model can be performed. This would aid the identification of microbial-associated metabolite biomarkers as discussed in chapter 3.

Regarding the gelatin model, we tested 3 species of *Lactobacillus*, additional species should be tested to compare the degradation abilities of a range of common commensal bacteria. Similar to the tissue model, these experiments could provide valuable data on the interactions of vaginal bacteria in combination and in mock communities. Importantly these mock communities should be reflective of women from a range of settings and can be based on the future microbiome data following on from chapter 4.

Additionally, we used a fluorescent gelatin model to capture a broad view of the degradation abilities of bacteria. However, this assay is also available with type I and IV collagen from human placenta. While degradation of gelatin will demonstrate bacterial capacity to degrade the ECM, degradation of type I and IV collagen would demonstrate significant ability to degrade human fetal membranes.

To estimate the degradation capacities of bacteria, future work could include a serial dilution of collagenase, gelatinase, MMPs along with the bacterial products to find an enzyme fluorescence profile that more closely matches the bacterial fluorescence profile.

The proof-of-concept experiments presented in this chapter outlines two useful models for studying fetal membrane-microbial interactions. Now that the methods have been optimised additional biological repeats would provide more robust data and provide more insight into the mechanisms of PPROM.

5 Chapter 5 - Conclusions and Future work

PTB is a complex multifactorial issue (Ansari et al., 2021; Georgiou et al., 2015). Some aspects are relatively well understood such as multiple births, pre-eclampsia and IUGR (Goldenberg et al., 2008). However, sPTL and PPROM is less well understood. While it is established that infection and inflammation play a big role in many sPTB (Gilman-Sachs et al., 2018; Mitchell & Marrazzo, 2014; Bastek et al., 2011) the mechanisms of how this occurs are well studied but still not completely understood.

The aims of this study were:

1. To optimise a standardised CVF microbiome protocol that can be used consistently for partner country samples (chapter 2)
2. To investigate prediction methods of PTB and PPROM utilising CVF metabolites and microbiome (chapter 3)
3. To investigate how microorganisms in the VMB may contribute to PPROM (chapter 4)

5.1 Oxford Nanopore Sequencing of the Vaginal microbiome

The VMB has been associated with PTB and PPROM however, not all studies found significant associations. Some studies found no association between the VMB and PTB (Stout et al., 2017; Nelson et al., 2016) whilst others found that abundance of specific organisms e.g *Gardnerella vaginalis* and *Lactobacillus crispatus* were associated with preterm outcomes (Hyman et al., 2014). *Lactobacillus gasseri* and *Lactobacillus crispatus* are thought to promote term birth. Whilst *Gardnerella*, *Lactobacillus iners* and *Lactobacillus jenseni* are thought to contribute to PTB (Callahan et al., 2017; Stafford et al., 2017; DiGiulio et al., 2015). Identification of vaginal microbial communities which increased the risk of PTB would allow patients to be triaged prior to them developing preterm labour symptoms. This would allow time for interventions such as tocolytics or steroids which may improve neonatal outcomes.

My study optimised the bacterial DNA extraction method of CVF swabs which had been stored at -80 C for use with Oxford Nanopore sequencing. Due to the length of sample storage, bacterial cells had lysed exposing their DNA. This meant that conventional protocols that include a host DNA depletion step or prolonged heating degraded the DNA we were aiming to extract. Therefore, I optimised the DNA isolation protocol which allowed for the most bacterial DNA to be extracted from these sub-optimal samples.

I also identified that the universal primer pair commonly used for 16s sequencing was not able to amplify many vaginal species. I developed a novel universal primer pair which is able to amplify the majority of key vaginal species including *Gardnerella* species which are notoriously underrepresented in sequencing studies.

Furthermore, I identified bacterial species present in the samples and compared results from term, preterm and PPROM delivered patients to identify potential vaginal organism biomarkers associated with PTB from UK samples. Unfortunately, we had insufficient sample number to investigate the predictive value of VMB for PPROM. Future studies could aim to target PPROM patients to increase numbers.

We hypothesised that women who give birth prematurely will have a significantly different VMB profile that can be used to identify those at a higher risk of PTB. We identified three bacteria that were able to predict PTB *(Atopobium vaginae* and *Gardnerella vaginalis* at GTP1 and *Rhizobium rhizogenes* at GTP 1 + 2 + 3) in addition to ratio of *G. vaginalis* and *L. iners.*

These findings support previous research that identified high levels of *A. vaginae* and *G. vaginalis* resulted in higher risk of PTB in high risk patients (Bretelle et al., 2015).

Additional clinical samples from the UK cohort are needed to increase sample size and draw more confident predictive models, particularly for the PPROM subgroup. Of note, we observed no significant findings at GTP2, this is likely due to the smaller sample sizes. Future studies should ensure sufficient samples for statistical significance after the exclusion of samples which did not meet the quality criteria.

Where possible we should aim to remove steps that introduce bias. Primer design and amplification steps presents a big opportunity to introduce bias (Sirichoat et al., 2021). Therefore, where possible we should aim to collect a large volume of starting material to remove the need for amplification. This can be done using collection methods such as a vaginal cup as done in previous studies (Moncla et al., 2016) which would provide more CVF and remove the need of an elution buffer like PBS which can interfere with the analysis.

The field of vaginal microbiome research would benefit from more standardised protocols between studies. Every step in the microbiome methodology has the potential to introduce variation and bias into the results which is likely the reason for so many conflicting studies and lack of repeatable findings.

Based on my analysis I recommend further research into bacterial species that were significantly implicated in preterm birth. These are: *Coriobacteriales bacterium, Megasphaera* species, *R. rhizogenes, L. rhamnosus, G. vaginalis,* and *A. vaginae.* Future research should perform targeted analysis of these species while still investigating the vaginal community as a whole. This study characterised the VMB of women from the UK, current work is utilising the methods I developed to analyse samples from Bangladesh and South Africa in order to compare microbial community composition in a range of settings. This will allow us to ensure that PTB prediction methods are applicable to women around the globe and positively impact the greatest number of patients.

5.2 Prediction of Preterm Birth using Microbial Metabolites in Cervico-vaginal Fluid

The CVF metabolome provides a snapshot of the vaginal ecosystem which includes host epithelial cells, microbiota, and microbial transformation of host derived products. Bacterial diversity has been shown to strongly influence the metabolite composition of the CVF (McMillan et al., 2015). Up to 40% of all PTBs can be linked to infection (Romero & Dey., 2014; Goldenberg et al., 2008). Therefore, microbial associated metabolites may be able to indicate the presence of dysbiosis and/or infection and consequently the risk of PTB. Identification of CVF metabolome profiles which increase the risk of PTB would again allow patients to be triaged prior to them developing preterm labour symptoms. This would allow time for interventions such as tocolytics or steroids which may improve neonatal outcomes.

I identified metabolites that were able to predict PTB including pantothenate, phytoene, adenosine, dehydrosafynol, giganin, nonacosane and urate. These metabolites should be further studied to better understand the relationship between the CVF metabolome and PTB. In particular, the pantothenate and cholesterol pathway should be investigated further. We found that pantothenate significantly increased and lanosterol significantly decreased in preterm patients. These metabolite pathways both have links to preterm birth (Ghartey et al., 2017; Menon et al., 2014) and *Lactobacillus* species (Yao et al., 2018).

The CVF metabolome is able to provide information on the microbiome, host and their interactions. However, the metabolism of common vaginal bacteria is understudied.

My results provide further evidence that that women who give birth prematurely have a significantly different metabolite profile than their term counterparts. In the future that can be used to identify a higher risk of PTB.

I identified metabolites which have predictive value for PTB including pantothenate, phytoene, adenosine, dehydrosafynol, giganin, nonacosane and urate. Further research is required to assess the role of these metabolites in the initiation of PTB. More research is needed to characterise the metabolism of common vaginal bacteria, alone and in a representative community to better understand the metabolic pathway of the VMB and host.

5.3 Do Vaginal Microbes Contribute to Preterm Prelabour Rupture of Fetal Membranes?

Currently, efforts to predict PPROM have been limited in accuracy (Romero et al., 2019; Gomez-Lopez et al., 2018; Choi et al., 2012). A key part of our hypothesis was that vaginal bacteria contribute to PPROM through degradation of the FM. *In vitro* experiments in chapter 2 investigated the mechanical properties of human FM and the ability of vaginal bacteria to degrade analogues of the membrane, specifically a fluorescently labelled gelatin substrate. The mechanism of membrane weakening prior to PPROM is well studied but the initiating factors are still unknown. Briefly, membrane weakening is thought to arise in response to factors including inflammatory cytokines (Gomez-Lopez et al., 2018; Kumar et al., 2005; Menon & Fortunato, 2004), collagen remodelling (Dixon et al., 2018; Lannon et al., 2014) and EMT (Janzen et al., 2017; Assumpcao et al., 2016). The VMB is a dynamic ecosystem which interacts with host cells in the vagina, cervix and FM overlying the cervix (Smith & Ravel, 2017; Petrova et al., 2015; Moloney et al., 2014). Many pathogens including GBS (Lannon et al., 2014; Krzyaściak et al., 2013) are well studied in relation to PTB and PPROM however, there are very few studies investigating non-pathogenic vaginal organisms in relation to PPROM, and inflammation. Commensal vaginal bacteria have also been hypothesised to contribute to the weakening of the FM and therefore the onset of PPROM. This could occur through their release of proteases that degrade the structural protein components of the membrane. I attempted to determine the effects of several common commensal bacterial species, found in the vagina, on the degradation of a gelatin substrate.

I investigated the degradation and inhibition capacity of *Lactobacillus* species. Conditioned bacterial media increased gelatin degradation compared to controls which indicates some ability for bacterial products degrade FM. These products are likely proteases, but further work is needed to elucidate the precise identity of the proteases responsible for this effect. A proteome profiler array would allow the conditioned bacterial media to be screened for a wide range of proteases. The proteases with greatest abundance could then be further characterised by (enzyme-linked immunosorbent assay) ELISA or Western blotting to quantify levels and determine activity. Following identification of several active proteases, specific inhibitors could be co-incubated along with conditioned media in an attempt to abolish the

effect I observed and confirm the degradation was a result of the proteases identified. This would further support the role of proteases in the degradation of the FM.

In addition to the degradation abilities of *Lactobacillus* species, we also investigated the degradation **inhibition** abilities using bacterial conditioned media and collagenase. Although the results did not reach statistical significance the trend in the data suggests that *Lactobacillus* species reduced degradation. *Lactobacillus* species are considered to be health promoting (Amabebe & Anumba, 2018). We hypothesise that these organisms are able to mitigate the effects of other potentially more pathogenic organisms by inhibiting other bacterial enzymes capable of weakening FM. Future research is required to support this hypothesis. Reduced levels of *Lactobacillus* in vaginal samples is the most consistent finding that is able to indicate increased risk of PTB (Arena & Daccò, 2021; Brown et al., 2019; Callahan et al., 2017; DiGiulio et al., 2015; Usui et al., 2002).

We compared three closely related species: *L. gasseri, L. jensenii* and *L. crispatus* which did not allow us to investigate the relative degradation abilities of a range of organisms as has been done in (Lithgow et al., 2022). Lithgow's investigation demonstrated a low degradation ability of *L. crispatus* compared to *Porphyromonas* species. Degradation by *L. gasseri, L. jensenii* and *L. crispatus* was similar and all relatively low compared to the collagenase control. However, it was not possible determine if these levels were low compared to other bacteria. To determine this, we require additional organisms which have been associated with PPROM such as *G. vaginalis* to determine how the bacteria in our study compare allowing us to properly investigate the contribution of commensal vaginal bacteria to FM degradation.

I also investigated the viscoelastic property or "stiffness" of FM and found that after exposure to the inflammatory cytokine TNF-α, FM was significantly less stiff compared to the untreated. This finding supports previous studies which showed, using different methods, that cytokines were able to weaken FM (Kumar et al., 2005). This could be due to apoptosis which was found to be induced by cytokines (Uchide et al., 2012). It also confirms that this novel technique can be utilised to investigate factors (such as vaginal bacterial community) on membrane viscoelasticity which provides insight into the likelihood of PPROM. Organisms of interest identified in chapter 4 including: *Coriobacteriales bacterium, Megasphaera* species, *R. rhizogenes, L. rhamnosus, G. vaginalis,* and *A. vaginae* should be tested in future studies using

the methods developed in this chapter. This would provide insight into the ability of each bacterium to influence the risk of PTB.

Together these results provide evidence of the possibility that the VMB could play a role in PPROM via inflammatory signalling and bacterial products.

In conclusion, my work has developed methods which will become the foundation of many future studies. These include: the novel rheology technique dynamic shear analysis, fluorescent degradation assays optimised for bacterial products, isolation and validation of hAEC, novel primer pair 319F/MCRevA, extraction of bacterial DNA from CVF swabs stored at -80 C and, 16s sequencing of CVF swabs. Additionally, we identified vaginal bacteria and metabolites that may increase the risk of PTB. These should be investigated further in vitro to better understand the mechanisms which lead to the initiation of sPTL. In parallel, further clinical studies should be performed to validate these findings on a larger UK cohort. Further to UK samples, CVF samples from partner countries should be analysed to elucidate differences in the microbiome based on setting. There remains a need for a minimally invasive, widely available predictive test for PTB which is able to stratify risk of pregnant women.

6 References

Ahrens, P., O'Brien Andersen, L., Lilje, B., Johannesen, T. B., Dahl, E. G., Baig, S., Jensen, J. S. & Falk, L. (2020). Changes in the Vaginal Microbiota Following Antibiotic Treatment for Mycoplasma Genitalium, Chlamydia Trachomatis and Bacterial Vaginosis. *PLoS ONE*, 15(7 July).

Al-Zaid, N. S., Bou-Resli, M. N. & Goldspink, G. (1980). BURSTING PRESSURE AND COLLAGEN CONTENT OF FETAL MEMBRANES AND THEIR RELATION TO PREMATURE RUPTURE OF THE MEMBRANES. *BJOG: An International Journal of Obstetrics & Gynaecology*, 87(3), 227–229.

Alcaraz, A., Mrowiec, A., Insausti, C. L., García-Vizcaíno, E. M., Ruiz-Canada, C., López-Martínez, M. C., Moraleda, J. M. & Nicolás, F. J. (2013). Autocrine TGF-β Induces Epithelial to Mesenchymal Transition in Human Amniotic Epithelial Cells. *Cell Transplantation*, 22(8), 1351–1367.

Aldunate, M., Srbinovski, D., Hearps, A. C., Latham, C. F., Ramsland, P. A., Gugasyan, R., Cone, R. A. & Tachedjian, G. (2015). Antimicrobial and Immune Modulatory Effects of Lactic Acid and Short Chain Fatty Acids Produced by Vaginal Microbiota Associated with Eubiosis and Bacterial Vaginosis. *Frontiers in Physiology*, 6(JUN), 164.

Alessandrello, M. J., Parellada, E. A., Juárez Tomás, M. S., Neske, A., Vullo, D. L. & Ferrero, M. A. (2017). Polycyclic Aromatic Hydrocarbons Removal by Immobilized Bacterial Cells Using Annonaceous Acetogenins for Biofilm Formation Stimulation on Polyurethane Foam. *Journal of Environmental Chemical Engineering*, 5(1), 189–195.

Alisoltani, A. et al. (2020). Microbial Function and Genital Inflammation in Young South African Women at High Risk of HIV Infection. *Microbiome*, 8(1), 1–21.

Alvares, Cruz, Brandão, R. and M. (2010). *The Role of Cytokines in Immune Regulation of Female Reproductive Physiology*. Universidade Federal Fluminense, Faculdade de Veterinária.

Amabebe, E. & Anumba, D. O. C. (2020). Female Gut and Genital Tract Microbiota-Induced Crosstalk and Differential Effects of Short-Chain Fatty Acids on Immune Sequelae. *Frontiers in Immunology*, 11.

Amabebe, E. & Anumba, D. O. C. C. (2018). The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Frontiers in Medicine*, 5(June), 1–11.

Amabebe, E., Bhatnagar, N., Kamble, N., Reynolds, S. & Anumba, D. O. (2022). Exploring the Antimicrobial Properties of Vaginal Lactobacillus Crispatus against Preterm Birth-Associated Bacteria. *Reproduction & fertility*, 3(3), L6–L8.

Amabebe, E., Chapman, D. R., Stern, V. L., Stafford, G. & Anumba, D. O. C. (2018). Mid-Gestational Changes in Cervicovaginal Fluid Cytokine Levels in Asymptomatic Pregnant Women Are Predictive Markers of Inflammation-Associated Spontaneous Preterm Birth. *Journal of Reproductive Immunology*, 126(January), 1–10.

Amabebe, E., Reynolds, S. & Anumba, D. O. C. (2021). Spectral Binning of Cervicovaginal Fluid Metabolites Improves Prediction of Spontaneous Preterm Birth and Lactobacillus Species Dominance. *Reproduction and Fertility*, 2(4), L4–L6.

Amabebe, E., Reynolds, S., He, X., Wood, R., Stern, V. & Anumba, D. O. C. C. (2019). Infection/Inflammation-Associated Preterm Delivery within 14 Days of Presentation with Symptoms of Preterm Labour: A Multivariate Predictive Model (L. Giannella, Ed.). *PLOS ONE*, 14(9), e0222455.

Amabebe, E., Reynolds, S., Stern, V. L., Parker, J. L., Stafford, G. P., Paley, M. N. & Anumba, D. O. C. (2016). Identifying Metabolite Markers for Preterm Birth in Cervicovaginal Fluid by Magnetic Resonance Spectroscopy. *Metabolomics*, 12(4), 67.

Amabebe, E., Reynolds, S., Stern, V., Stafford, G., Paley, M. & Anumba, D. O. C. C. (2016). Cervicovaginal Fluid Acetate: A Metabolite Marker of Preterm Birth in Symptomatic Pregnant Women. *Frontiers in Medicine*, 3(OCT), 48.

Amar, S., Avni, Y. S., O'Rourke, N. & Michael, T. (2022). Prevalence of Common Infectious Diseases after COVID-19 Vaccination and Easing of Pandemic Restrictions in Israel. *JAMA Network Open*, 5(2), e2146175–e2146175.

Amarasekare, P. (2003). Competitive Coexistence in Spatially Structured Environments: A Synthesis. *Ecology Letters*, 6(12), 1109–1122.

Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C. S., Eschenbach, D. & Holmes, K. K. (1983). Nonspecific Vaginitis. Diagnostic Criteria and Microbial and Epidemiologic Associations. *American Journal of Medicine*, 74(1), 14–22.

Anahtar, M. N., Byrne, E. H., Doherty, K. E., Bowman, B. A., Yamamoto, H. S., Soumillon, M., Padavattan, N., Ismail, N., Moodley, A., Sabatini, M. E., Ghebremichael, M. S., Nusbaum, C., Huttenhower, C., Virgin, H. W.,

Ndung'u, T., Dong, K. L., Walker, B. D., Fichorova, R. N. & Kwon, D. S. (2015). Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract. *Immunity*, 42(5), 965–976.

Anahtar, M. N., Gootenberg, D. B., Mitchell, C. M. & Kwon, D. S. (2018). Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host & Microbe*, 23(2), 159–168.

Andersson, S., Minjarez, D., Yost, N. P. & Word, R. A. (2008). Estrogen and Progesterone Metabolism in the Cervix during Pregnancy and Parturition. *The Journal of Clinical Endocrinology & Metabolism*, 93(6), 2366–2374.

Andrews, W. W., Goldenberg, R. L. & Hauth, J. C. (1995). Preterm Labor: Emerging Role of Genital Tract Infections. *Infectious Agents and Disease*, 4(4), 196–211.

Ansari, A., Bose, S., You, Y., Park, S. & Kim, Y. (2021). Molecular Mechanism of Microbiota Metabolites in Preterm Birth: Pathological and Therapeutic Insights. *International Journal of Molecular Sciences*, 22(15), 8145.

Ansari, A., Lee, H., You, Y. A., Jung, Y., Park, S., Kim, S. M., Hwang, G. S. & Kim, Y. J. (2020). Identification of Potential Biomarkers in the Cervicovaginal Fluid by Metabolic Profiling for Preterm Birth. *Metabolites*, 10(9), 1–13.

Anukam, K., Agbakoba, N., Okoli, A. & Oguejiofor, C. (2019). Vaginal Bacteriome of Nigerian Women in Health and Disease: A Study with 16S RRNA Metagenomics. *Tropical Journal of Obstetrics and Gynaecology*, 36(1), 96.

Anukam, K. C., Osazuwa, E. O., Ahonkhai, I. & Reid, G. (2006). Lactobacillus Vaginal Microbiota of Women Attending a Reproductive Health Care Service in Benin City, Nigeria. *Sexually Transmitted Diseases*, 33(1), 59–62.

Anumba, D. O. C., Stern, V., Healey, J. T., Dixon, S. & Brown, B. H. (2021). Value of Cervical Electrical Impedance Spectroscopy to Predict Spontaneous Preterm Delivery in Asymptomatic Women: The ECCLIPPx Prospective Cohort Study. *Ultrasound in Obstetrics and Gynecology*, 58(2), 293–302.

Araldi, E., Fernández-Fuertes, M., Canfrán-Duque, A., Tang, W., Cline, G. W., Madrigal-Matute, J., Pober, J. S., Lasunción, M. A., Wu, D., Fernández-Hernando, C. & Suárez, Y. (2017). Lanosterol Modulates TLR4-Mediated Innate Immune Responses in Macrophages. *Cell Reports*, 19(13), 2743–2755.

Arena, B. & Daccò, M. D. (2021). Evaluation of Vaginal Microbiota in Women Admitted to the Hospital for Premature Labour. *Acta Biomedica*, 92(5), 2021292.

Argentini, C., Fontana, F., Alessandri, G., Lugli, G. A., Mancabelli, L., Ossiprandi, M. C., van Sinderen, D., Ventura, M., Milani, C. & Turroni, F. (2022). Evaluation of Modulatory Activities of Lactobacillus Crispatus Strains in the Context of the Vaginal Microbiota. *Microbiology Spectrum*, 10(2).

Arikat, S., Novince, R. W., Mercer, B. M., Kumar, D., Fox, J. M., Mansour, J. M. & Moore, J. J. (2006). Separation of Amnion from Choriodecidua Is an Integral Event to the Rupture of Normal Term Fetal Membranes and Constitutes a Significant Component of the Work Required. *American Journal of Obstetrics and Gynecology*, 194(1), 211–217.

Armstrong, R. A. (2019). Should Pearson's Correlation Coefficient Be Avoided? *Ophthalmic and Physiological Optics*, 39(5), 316–327.

Arroyo-Moreno, S., Cummings, M., Corcoran, D. B., Coffey, A. & McCarthy, R. R. (2022). Identification and Characterization of Novel Endolysins Targeting Gardnerella Vaginalis Biofilms to Treat Bacterial Vaginosis. *npj Biofilms and Microbiomes*, 8(1), 1–12.

Aslam, S., Sayeed Saraf, V., Saleem, S., Saeed, S., Javed, S., Junjua, M. & Bokhari, H. (2022). Lactobacillus Species Signature in Association with Term and Preterm Births from Low-Income Group of Pakistan. *Journal of Maternal-Fetal and Neonatal Medicine*, 35(15), 2843–2852.

Assumpcao, M. G., Lei, M. Y. Y., Sen, S., Challis, J., Chaudhuri, G. & Janzen, C. (2016). 246: The Role of Epithelial to Mesenchymal Transition (EMT) in Amniotic Membrane Rupture. *American Journal of Obstetrics and Gynecology*, 214(1), S145.

Athayde, N., Edwin, S. S., Romero, R., Gomez, R., Maymon, E., Pacora, P. & Menon, R. (1998). A Role for Matrix Metalloproteinase-9 in Spontaneous Rupture of the Fetal Membranes, in: *American Journal of Obstetrics and Gynecology*, (pp. 1248–1253). Mosby.

Aujoulat, F., Marchandin, H., Zorgniotti, I., Masnou, A. & Jumas-Bilak, E. (2015). Rhizobium Pusense Is the Main Human Pathogen in the Genus Agrobacterium/Rhizobium. *Clinical Microbiology and Infection*, 21(5), 472.e1-472.e5.

Azouzi, F., Chahed, C., Marzouk, M., Ferjani, A., Hannechi, N., Fekih, M., Ben Salem, Y. & Boukadida, J. (2015). Chorioamnionitis Due to Lactococcus Lactis Cremoris: A Case Report. *Case Reports in Women's Health*, 7, 1–2.

Bai, G., Gajer, P., Nandy, M., Ma, B., Yang, H., Sakamoto, J., Blanchard, M. H., Ravel, J. & Brotman, R. M. (2012). Comparison of Storage Conditions for Human Vaginal Microbiome Studies (A. J. Ratner, Ed.). *PLoS ONE*, 7(5), e36934.

Balakirski, G., Gerdes, S., Beissert, S., Ochsendorf, F., von Kiedrowski, R. & Wilsmann-Theis, D. (2022). Therapy of Psoriasis during Pregnancy and Breast-Feeding. *JDDG - Journal of the German Society of Dermatology*, 20(5), 653–683.

Baldwin, E. A., Walther-Antonio, M., MacLean, A. M., Gohl, D. M., Beckman, K. B., Chen, J., White, B., Creedon, D. J. & Chia, N. (2015). Persistent Microbial Dysbiosis in Preterm Premature Rupture of Membranes from Onset until Delivery. *PeerJ*, 3, e1398.

Barberini, L., Palmas, F., Fais, M. F., Mereu, R., Noto, A., Fattuoni, C., Mais, V., Chiodo, A. & Meloni, A. (2019). Urinary Profiles Associated with Bacterial Metabolites from Asymptomatic Pregnant Women with at Term or Preterm Premature Rupture of Membranes: A Pilot Study. *Journal of Maternal-Fetal and Neonatal Medicine*.

Barinov, S. V., Tirskaya, Y. I., Kadsyna, T. V., Lazareva, O. V., Medyannikova, I. V. & Tshulovski, Y. I. (2022). Pregnancy and Delivery in Women with a High Risk of Infection in Pregnancy. *Journal of Maternal-Fetal and Neonatal Medicine*, 35(11), 2122–2127.

Barnes, H. A., Hutton, J. F. & Kenneth, W. (2001). *An Introduction to Rheology*.

Baskaradoss, J. K., Geevarghese, A. & Kutty, V. R. (2011). Maternal Periodontal Status and Preterm Delivery: A Hospital Based Case-Control Study. *Journal of Periodontal Research*, 46(5), 542–549.

Bassler, B. L. & Losick, R. (2006). Bacterially Speaking. *Cell*, 125(2), 237–246.

Bastek, J. A., Gómez, L. M. & Elovitz, M. A. (2011). The Role of Inflammation and Infection in Preterm Birth. *Clinics in Perinatology*, 38(3), 385–406.

Bayigga, L., Nabatanzi, R., Ssekagiri, A., Kateete, D. P., Sekikubo, M., Anderson, D. J., Xu, J., Kwon, D. S. & Nakanjako, D. (2020). Diverse Vaginal Microbiome Was Associated with Pro-Inflammatory Vaginal Milieu among Pregnant Women in Uganda. *Human Microbiome Journal*, 18, 100076.

Begum, J., Samal, S. K., Ghose, S. & Niranjan, G. (2017). Vaginal Fluid Urea and Creatinine in the Diagnosis of Premature Rupture of Membranes in Resource Limited Community Settings. *Journal of family & reproductive health*, 11(1), 43–49.

Behnia, F., Sheller, S. & Menon, R. (2016). Mechanistic Differences Leading to Infectious and Sterile Inflammation. *American Journal of Reproductive Immunology*, 75(5), 505–518.

Bellad, M. B., Hoffman, M. K., Mallapur, A. A., Charantimath, U. S., Katageri, G. M., Ganachari, M. S., Kavi, A., Ramdurg, U. Y., Bannale, S. G., Revankar, A. P., Sloan, N. L., Kodkany, B. S., Goudar, S. S. & Derman, R. J. (2018). Clindamycin to Reduce Preterm Birth in a Low Resource Setting: A Randomised Placebo-Controlled Clinical Trial. *BJOG: An International Journal of Obstetrics and Gynaecology*, 125(12), 1601–1609.

Benson-Martin, J., Zammaretti, P., Bilic, G., Schweizer, T., Portmann-Lanz, B., Burkhardt, T., Zimmermann, R. & Ochsenbein-Kölble, N. (2006). The Young's Modulus of Fetal Preterm and Term Amniotic Membranes. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, 128(1–2), 103–107.

Bergeron, J., Gerges, N., Guiraut, C., Grbic, D., Allard, M. J., Fortier, L. C., Vaillancourt, C. & Sébire, G. (2016). Activation of the IL-1β/CXCL1/MMP-10 Axis in Chorioamnionitis Induced by Inactivated Group B Streptococcus. *Placenta*, 47, 116–123.

Berghella, V., Palacio, M., Ness, A., Alfirevic, Z., Nicolaides, K. H. & Saccone, G. (2017). Cervical Length Screening for Prevention of Preterm Birth in Singleton Pregnancy with Threatened Preterm Labor: Systematic Review and Meta-Analysis of Randomized Controlled Trials Using Individual Patient-Level Data. *Ultrasound in Obstetrics and Gynecology*, 49(3), 322–329.

Bhat, R., Rai, Y., Suresh, A. & Rajesh, A. (2009). Cytolytic Vaginosis: A Review. *Indian Journal of Sexually Transmitted Diseases and AIDS*, 30(1), 48.

Bilic, G., Zeisberger, S. M., Mallik, A. S., Zimmermann, R. & Zisch, A. H. (2008). Comparative Characterization of Cultured Human Term Amnion Epithelial and Mesenchymal Stromal Cells for Application in Cell Therapy. *Cell Transplantation*.

Bircher, K., Ehret, A. E., Spiess, D., Ehrbar, M., Simões-Wüst, A. P., Ochsenbein-Kölble, N., Zimmermann, R. & Mazza, E. (2019). On the Defect Tolerance of Fetal Membranes. *Interface Focus*, 9(5).

Bitew, A., Mengist, A., Belew, H., Aschale, Y. & Reta, A. (2021). The Prevalence, Antibiotic Resistance Pattern, and Associated Factors of Bacterial Vaginosis among Women of the Reproductive Age Group from Felege Hiwot Referral Hospital, Ethiopia. *Infection and Drug Resistance*, 14, 2685–2696.

Blondel, W. C. P. M., Lehalle, B., Maurice, G., Wang, X. & Stoltz, J. F. (2000). Rheological Properties of Fresh and Cryopreserved Human Arteries Tested in Vitro. *Rheologica Acta*, 39(5), 461–468.

Blostein, F., Gelaye, B., Sanchez, S. E., Williams, M. A. & Foxman, B. (2020). Vaginal Microbiome Diversity and Preterm Birth: Results of a Nested Case–Control Study in Peru. *Annals of Epidemiology*, 41, 28–34.

Boersma, Y. L., Newman, J., Adams, T. E., Cowieson, N., Krippner, G., Bozaoglu, K. & Peat, T. S. (2014). The Structure of Vanin 1: A Key Enzyme Linking Metabolic Disease and Inflammation. *Acta Crystallographica*

Section D: Biological Crystallography, 70(12), 3320–3329.
Bogavac, M., Brkic, S., Simin, N. & Celic, D. (2012). Mid-Pregnancy Interleukins Levels in Serum and Amniotic Fluid as Predictors of Preterm Delivery. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians*, 1–13.
Boldenow, E., Hogan, K. A., Chames, M. C., Aronoff, D. M., Xi, C. & Loch-Caruso, R. (2015). Role of Cytokine Signaling in Group B Streptococcus-Stimulated Expression of Human Beta Defensin-2 in Human Extraplacental Membranes. *American Journal of Reproductive Immunology*, 73(3), 263–272.
Borgdorff, H., Tsivtsivadze, E., Verhelst, R., Marzorati, M., Jurriaans, S., Ndayisaba, G. F., Schuren, F. H. & Van De Wijgert, J. H. H. M. (2014). Lactobacillus-Dominated Cervicovaginal Microbiota Associated with Reduced HIV/STI Prevalence and Genital HIV Viral Load in African Women. *ISME Journal*, 8(9), 1781–1793.
Boris, S. & Barbés, C. (2000). Role Played by Lactobacilli in Controlling the Population of Vaginal Pathogens. *Microbes and Infection*, 2(5), 543–546.
Bourne, G. L. & Lacy, D. (1960). Ultra-Structure of Human Amnion and Its Possible Relation to the Circulation of Amniotic Fluid. *Nature*, 186(4729), 952–954.
Bradshaw, C. S. & Sobel, J. D. (2016). Current Treatment of Bacterial Vaginosis-Limitations and Need for Innovation. *Journal of Infectious Diseases*, 214(S1), S14–S20.
Braveman, P., Heck, K., Egerter, S., Dominguez, T. P., Rinki, C., Marchi, K. S. & Curtis, M. (2017). Worry about Racial Discrimination: A Missing Piece of the Puzzle of Black-White Disparities in Preterm Birth? *PLoS ONE*, 12(10), e0186151.
Bretelle, F., Rozenberg, P., Pascal, A., Favre, R., Bohec, C., Loundou, A., Senat, M. V., Aissi, G., Lesavre, N., Brunet, J., Heckenroth, H., Luton, D., Raoult, D. & Fenollar, F. (2015). High Atopobium Vaginae and Gardnerella Vaginalis Vaginal Loads Are Associated with Preterm Birth. *Clinical Infectious Diseases*, 60(6), 860–867.
Brik, M., Hernández, A. I. M., Pedraz, C. C. & Perales, A. (2010). Phosphorylated Insulin-like Growth Factor Binding Protein-1 and Cervical Measurement in Women with Threatening Preterm Birth. *Acta Obstetricia et Gynecologica Scandinavica*, 89(2), 268–274.
Brilian, A. I. & Virginia, D. M. (2017). Effect of Calcium Supplementation during Pregnancy in Maternal Patient to Preterm Birth in One of Private Hospitals in Yogyakarta. *Indonesian Journal of Clinical Pharmacy*, 6(3), 153–163.
Brocklehurst, P., Gordon, A., Heatley, E. & Milan, S. J. (2013). Antibiotics for Treating Bacterial Vaginosis in Pregnancy. *Cochrane Database of Systematic Reviews*, 2013(1), 262.
Brown, R. G., Al-Memar, M., Marchesi, J. R., Lee, Y. S., Smith, A., Chan, D., Lewis, H., Kindinger, L., Terzidou, V., Bourne, T., Bennett, P. R. & MacIntyre, D. A. (2019). Establishment of Vaginal Microbiota Composition in Early Pregnancy and Its Association with Subsequent Preterm Prelabor Rupture of the Fetal Membranes. *Translational Research*, 207, 30–43.
Brown, R. G., Marchesi, J. R., Lee, Y. S., Smith, A., Lehne, B., Kindinger, L. M., Terzidou, V., Holmes, E., Nicholson, J. K., Bennett, P. R. & MacIntyre, D. A. (2018). Vaginal Dysbiosis Increases Risk of Preterm Fetal Membrane Rupture, Neonatal Sepsis and Is Exacerbated by Erythromycin. *BMC Medicine*, 16(1), 9.
Brueggemann, A. B. et al. (2021). Changes in the Incidence of Invasive Disease Due to Streptococcus Pneumoniae, Haemophilus Influenzae, and Neisseria Meningitidis during the COVID-19 Pandemic in 26 Countries and Territories in the Invasive Respiratory Infection Surveillance Initiative: A . *The Lancet Digital Health*, 3(6), e360–e370.
Bryant-Greenwood, G. D. (1998). The Extracellular Matrix of the Human Fetal Membranes: Structure and Function. *Placenta*, 19(1), 1–11.
Buerzle, W. & Mazza, E. (2013). On the Deformation Behavior of Human Amnion. *Journal of Biomechanics*, 46(11), 1777–1783.
Burhouse, A., Lea, C., Ray, S., Bailey, H., Davies, R., Harding, H., Howard, R., Jordan, S., Menzies, N., White, S., Phillips, K. & Luyt, K. (2017). Preventing Cerebral Palsy in Preterm Labour: A Multiorganisational Quality Improvement Approach to the Adoption and Spread of Magnesium Sulphate for Neuroprotection. *BMJ Open Quality*, 6(2), e000189.
Burris, H. H., Wright, C. J., Kirpalani, H., Collins, J. W., Lorch, S. A., Elovitz, M. A. & Hwang, S. S. (2020). The Promise and Pitfalls of Precision Medicine to Resolve Black–White Racial Disparities in Preterm Birth. *Pediatric Research*, 87(2), 221–226.
Byrns, M. C. (2014). Regulation of Progesterone Signaling during Pregnancy: Implications for the Use of Progestins for the Prevention of Preterm Birth. *Journal of Steroid Biochemistry and Molecular Biology*, 139, 173–181.
Callahan, B. J. et al. (2017). Replication and Refinement of a Vaginal Microbial Signature of Preterm Birth in Two

Racially Distinct Cohorts of US Women. *Proceedings of the National Academy of Sciences of the United States of America*, 114(37), 9966–9971.

Calonghi, N., Parolin, C., Sartor, G., Verardi, L., Giordani, B., Frisco, G., Marangoni, A. & Vitali, B. (2017). Interaction of Vaginal Lactobacillus Strains with HeLa Cells Plasma Membrane. *Beneficial Microbes*, 8(4), 625–633.

Campisciano, G., Florian, F., D'Eustacchio, A., Stanković, D., Ricci, G., De Seta, F. & Comar, M. (2017). Subclinical Alteration of the Cervical–Vaginal Microbiome in Women with Idiopathic Infertility. *Journal of Cellular Physiology*, 232(7), 1681–1688.

Cappelletti, M., Della Bella, S., Ferrazzi, E., Mavilio, D. & Divanovic, S. (2016). Inflammation and Preterm Birth. *Journal of Leukocyte Biology*, 99(1), 67–78.

Carducci, B., Keats, E. C. & Bhutta, Z. A. (2021). Zinc Supplementation for Improving Pregnancy and Infant Outcome. *Cochrane Database of Systematic Reviews*, 2021(3).

Carnrot, C. (2006). Bacterial Enzymes in Thymidylate Synthesis.

Carter, R. A., Pan, K., Harville, E. W., McRitchie, S. & Sumner, S. (2019). Metabolomics to Reveal Biomarkers and Pathways of Preterm Birth: A Systematic Review and Epidemiologic Perspective. *Metabolomics*, 15(9).

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., Ong, W. K., Paley, S., Subhraveti, P. & Karp, P. D. (2020). The MetaCyc Database of Metabolic Pathways and Enzymes-a 2019 Update. *Nucleic Acids Research*, 48(D1), D455–D453.

Castro, J., Machado, D. & Cerca, N. (2019). Unveiling the Role of Gardnerella Vaginalis in Polymicrobial Bacterial Vaginosis Biofilms: The Impact of Other Vaginal Pathogens Living as Neighbors. *ISME Journal*, 13(5), 1306–1317.

Castro, J., Rosca, A. S., Muzny, C. A. & Cerca, N. (2021). Atopobium Vaginae and Prevotella Bivia Are Able to Incorporate and Influence Gene Expression in a Pre-Formed Gardnerella Vaginalis Biofilm. *Pathogens*, 10(2), 1–16.

Challis, J. R. G., Matthews, S. G., Gibb, W. & Lye, S. J. (1997). Prostaglandins and Parturition. *Annals of the New York Academy of Sciences*, 21(1 Uterus, The), 514–550.

Chaudhury, S., Holland, C., Vollrath, F. & Carr, A. J. (2011). Comparing Normal and Torn Rotator Cuff Tendons Using Dynamic Shear Analysis. *Journal of Bone and Joint Surgery - Series B*, 93 B(7), 942–948.

Chawanpaiboon, S., Vogel, J. P., Moller, A.-B., Lumbiganon, P., Petzold, M., Hogan, D., Landoulsi, S., Jampathong, N., Kongwattanakul, K., Laopaiboon, M., Lewis, C., Rattanakanokchai, S., Teng, D. N., Thinkhamrop, J., Watananirun, K., Zhang, J., Zhou, W. & Gülmezoglu, A. M. (2019). Global, Regional, and National Estimates of Levels of Preterm Birth in 2014: A Systematic Review and Modelling Analysis. *The Lancet Global Health*, 7(1), e37–e46.

Choi, S. J., Park, S. D., Jang, I. H., Uh, Y. & Lee, A. (2012). The Prevalence of Vaginal Microorganisms in Pregnant Women with Preterm Labor and Preterm Birth. *Annals of Laboratory Medicine*, 32(3), 194–200.

Choudhary, R. K. & Capuco, A. V. (2012). In Vitro Expansion of the Mammary Stem/Progenitor Cell Population by Xanthosine Treatment. *BMC Cell Biology*, 13(1), 1–8.

Chow, M. J. & Zhang, Y. (2011). Changes in the Mechanical and Biochemical Properties of Aortic Tissue Due to Cold Storage. *Journal of Surgical Research*, 171(2), 434–442.

Chung, P., Buhimschi, I. A., Han, Y. W., Buhimschi, C. S., Shen, T., Chung, P., Buhimschi, I. A. & Buhimschi, C. S. (2009). Uncultivated Bacteria as Etiologic Agents of Intra-Amniotic Inflammation Leading to Preterm Birth. *Journal of Clinical Microbiology*, 47(1), 38–47.

Cobo, T., Palacio, M., Martínez-Terrón, M., Navarro-Sastre, A., Bosch, J., Filella, X. & Gratacós, E. (2011). Clinical and Inflammatory Markers in Amniotic Fluid as Predictors of Adverse Outcomes in Preterm Premature Rupture of Membranes. *American Journal of Obstetrics and Gynecology*, 205(2), 126.e1-126.e8.

Coles, R. & Kharasch, E. D. (2007). Stereoselective Analysis of Bupropion and Hydroxybupropion in Human Plasma and Urine by LC/MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 857(1), 67–75.

Conde-Agudelo, A. & Romero, R. (2015). Predictive Accuracy of Changes in Transvaginal Sonographic Cervical Length over Time for Preterm Birth: A Systematic Review and Metaanalysis. *American journal of obstetrics and gynecology*, 213(6), 789–801.

Cooke, M., Tan, E. K., Mandrycky, C., He, H., O'Connell, J. & Tseng, S. C. G. (2014). Comparison of Cryopreserved Amniotic Membrane and Umbilical Cord Tissue with Dehydrated Amniotic Membrane/Chorion Tissue. *Journal of Wound Care*, 23(10), 465–476.

Cools, P. (2017). The Role of Escherichia Coli in Reproductive Health: State of the Art. *Research in Microbiology*, 168(9–10), 892–901.

Costa, M. A. (2016). The Endocrine Function of Human Placenta: An Overview. *Reproductive BioMedicine Online*,

32(1), 14–43.
Coutsoudis, A., Pillay, K., Spooner, E., Kuhn, L. & Coovadia, H. M. (1999). Randomized Trial Testing the Effect of Vitamin A Supplementation on Pregnancy Outcomes and Early Mother-to-Child HIV-1 Transmission in Durban, South Africa. *AIDS*, 13(12), 1517–1524.
Cselovszky, J., Wolf, G. & Hammes, W. P. (1992). Production of Formate, Acetate, and Succinate by Anaerobic Fermentation of Lactobacillus Pentosus in the Presence of Citrate. *Applied Microbiology and Biotechnology*, 37(1), 94–97.
Cuperlovic-Culf, M. (2018). Machine Learning Methods for Analysis of Metabolic Data and Metabolic Pathway Modeling. *Metabolites*, 8(1), 4.
Daise, D. T. A. (2018). Maternal Hyperuricemia and Birth Outcome in Normotensive Singleton Pregnancy: A Prospective Cohort Study. *Journal of Medical Science And clinical Research*, 6(10).
Dang, Y., Li, W., Tran, V. & Khalil, R. A. (2013). EMMPRIN-Mediated Induction of Uterine and Vascular Matrix Metalloproteinases during Pregnancy and in Response to Estrogen and Progesterone. *Biochemical Pharmacology*, 86(6), 734–747.
Davenne, T., Bridgeman, A., Rigby, R. E. & Rehwinkel, J. (2020). Deoxyguanosine Is a TLR7 Agonist. *European Journal of Immunology*, 50(1), 56–62.
Delahaye, C. & Nicolas, J. (2021). Sequencing DNA with Nanopores: Troubles and Biases. *PLoS ONE*, 16(10 October), e0257521.
Devuyst, O. & Igarashi, T. (2018). Renal Fanconi Syndrome, Dent Disease, and Bartter Syndrome, in: *Genetics of Bone Biology and Skeletal Disease: Second Edition*, (pp. 783–799). Academic Press.
Diaz-Dinamarca, D. A., Hernandez, C., Escobar, D. F., Soto, D. A., Muñoz, G. A., Badilla, J. F., Manzo, R. A., Carrión, F., Kalergis, A. M. & Vasquez, A. E. (2020). Mucosal Vaccination with Lactococcus Lactis-Secreting Surface Immunological Protein Induces Humoral and Cellular Immune Protection against Group b Streptococcus in a Murine Model. *Vaccines*, 8(2), 146.
DiGiulio, D. B., Callahan, B. J., McMurdie, P. J., Costello, E. K., Lyell, D. J., Robaczewska, A., Sun, C. L., Goltsman, D. S. A., Wong, R. J., Shaw, G., Stevenson, D. K., Holmes, S. P. & Relman, D. A. (2015). Temporal and Spatial Variation of the Human Microbiota during Pregnancy. *Proceedings of the National Academy of Sciences*, 112(35), 11060–11065.
DiGiulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M., Gotsch, F., Kim, C. J., Erez, O., Edwin, S. & Relman, D. A. (2008). Microbial Prevalence, Diversity and Abundance in Amniotic Fluid during Preterm Labor: A Molecular and Culture-Based Investigation (N. M. Fisk, Ed.). *PLoS ONE*, 3(8), 3056.
Ding, C., Zhang, M. & Li, G. (2015). Effect of Cyclic Freeze-Thawing Process on the Structure and Properties of Collagen. *International Journal of Biological Macromolecules*, 80, 317–323.
Diop, K., Dufour, J. C., Levasseur, A. & Fenollar, F. (2019). Exhaustive Repertoire of Human Vaginal Microbiota. *Human Microbiome Journal*, 11, 100051.
Dixon, C. L., Richardson, L., Sheller-Miller, S., Saade, G. & Menon, R. (2018). A Distinct Mechanism of Senescence Activation in Amnion Epithelial Cells by Infection, Inflammation, and Oxidative Stress. *American Journal of Reproductive Immunology*, 79(3), e12790.
Donders, G. G., Van Calsteren, K., Bellen, G., Reybrouck, R., Van Den Bosch, T., Riphagen, I. & Van Lierde, S. (2009). Predictive Value for Preterm Birth of Abnormal Vaginal Flora, Bacterial Vaginosis and Aerobic Vaginitis during the First Trimester of Pregnancy. *BJOG: An International Journal of Obstetrics and Gynaecology*, 116(10), 1315–1324.
Donders, G. G. G., Vereecken, A., Bosmans, E. & Spitz, B. (2003). Vaginal Cytokines in Normal Pregnancy. *American Journal of Obstetrics and Gynecology*, 189(5), 1433–1438.
Doster, R. S., Kirk, L. A., Tetz, L. M., Rogers, L. M., Aronoff, D. M. & Gaddy, J. A. (2017). Staphylococcus Aureus Infection of Human Gestational Membranes Induces Bacterial Biofilm Formation and Host Production of Cytokines. *Journal of Infectious Diseases*, 215(4), 653–657.
Duan-Arnold, Y., Gyurdieva, A., Johnson, A., Uveges, T. E., Jacobstein, D. A. & Danilkovitch, A. (2015). Retention of Endogenous Viable Cells Enhances the Anti-Inflammatory Activity of Cryopreserved Amnion. *Advances in Wound Care*, 4(9), 523–533.
Duarte, A. S., Correia, A. & Esteves, A. C. (2016). Bacterial Collagenases-A Review. *Critical Reviews in Microbiology*, 42(1), 106–126.
Dude, C. M., Saylany, A., Brown, A., Elovitz, M. & Anton, L. (2020). Microbial Supernatants from Mobiluncus Mulieris, a Bacteria Strongly Associated with Spontaneous Preterm Birth, Disrupts the Cervical Epithelial Barrier through Inflammatory and MiRNA Mediated Mechanisms. *Anaerobe*, 61.
Dunlop, A. L., Satten, G. A., Hu, Y. J., Knight, A. K., Hill, C. C., Wright, M. L., Smith, A. K., Read, T. D., Pearce, B. D. & Corwin, E. J. (2021). Vaginal Microbiome Composition in Early Pregnancy and Risk of Spontaneous

Preterm and Early Term Birth Among African American Women. *Frontiers in Cellular and Infection Microbiology*, 11, 321.

Durack, J. & Lynch, S. V. (2019). The Gut Microbiome: Relationships with Disease and Opportunities for Therapy. *Journal of Experimental Medicine*, 216(1), 20–40.

Dutta, E. H., Behnia, F., Boldogh, I., Saade, G. R., Taylor, B. D., Kacerovský, M. & Menon, R. (2016). Oxidative Stress Damage-Associated Molecular Signaling Pathways Differentiate Spontaneous Preterm Birth and Preterm Premature Rupture of the Membranes. *Molecular Human Reproduction*, 22(2), 143–157.

El-Aneed, A., Cohen, A. & Banoub, J. (2009). Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. *Applied Spectroscopy Reviews*, 44(3), 210–230.

El-Khier, N. T. A., Megahed, K. F. A. E. & Shokier, M. A. (2014). Detection and Biovar Discrimination of Ureaplasma Urealyticum Colonization in Preterm Neonates under Ventilation and Correlation with Bronchopulmonary Dysplasia. *African Journal of Microbiology Research*, 8(27), 2631–2638.

El-Khwad, M., Stetzer, B., Moore, R. M., Kumar, D., Mercer, B., Arikat, S., Redline, R. W., Mansour, J. M. & Moore, J. J. (2005). Term Human Fetal Membranes Have a Weak Zone Overlying the Lower Uterine Pole and Cervix Before Onset of Labor. *Biology of Reproduction*, 72(3), 720–726.

El-Shazly, S., Makhseed, M., Azizieh, F. & Raghupathy, R. (2004). Increased Expression of Pro-Inflammatory Cytokines in Placentas of Women Undergoing Spontaneous Preterm Delivery or Premature Rupture of Membranes. *American Journal of Reproductive Immunology*, 52(1), 45–52.

Elovitz, M. A., Brown, A. G., Anton, L., Gilstrop, M., Heiser, L. & Bastek, J. (2014). Distinct Cervical MicroRNA Profiles Are Present in Women Destined to Have a Preterm Birth. *American Journal of Obstetrics and Gynecology*, 210(3), 221.e1-221.e11.

Elovitz, M. A., Gajer, P., Riis, V., Brown, A. G., Humphrys, M. S., Holm, J. B. & Ravel, J. (2019). Cervicovaginal Microbiota and Local Immune Response Modulate the Risk of Spontaneous Preterm Delivery. *Nature Communications*, 10(1), 1305.

Eltzschig, H. K. (2009). Adenosine: An Old Drug Newly Discovered. *Anesthesiology*, 111(4), 904–915.

Faidy, Y. & Ali-Shtayeh, M. (2000). *Laboratory Experiments in General Microbiology*.

Fang, X. ping, Song, R., Gu, Z. ming, Rieser, M. J., Miesbauer, L. R., Smith, D. L. & McLaughlin, J. L. (1993). A New Type of Cytotoxic Annonaceous Acetogenin: Giganin from Goniothalamus Giganteus. *Bioorganic and Medicinal Chemistry Letters*, 3(6), 1153–1156.

Faturechi, R., Hashemi, A. & Fatouraee, N. (2015). Do Mechanical Properties of Human Fetal Membrane Depend on Strain Rate. *Journal of Obstetrics and Gynaecology Research*, 41(1), 84–91.

Feng, L., Allen, T. K., Marinello, W. P. & Murtha, A. P. (2018). Infection-Induced Thrombin Production: A Potential Novel Mechanism for Preterm Premature Rupture of Membranes (PPROM). *American Journal of Obstetrics and Gynecology*, 219(1), 101.e1-101.e12.

Fernández-Arche, A., Marquez-Martín, A., Vazquez, R. de la P., Perona, J. S., Terencio, C., Perez-Camino, C. & Ruiz-Gutierrez, V. (2009). Long-Chain Fatty Alcohols from Pomace Olive Oil Modulate the Release of Proinflammatory Mediators. *Journal of Nutritional Biochemistry*, 20(3), 155–162.

Fettweis, J. M. et al. (2019). The Vaginal Microbiome and Preterm Birth. *Nature Medicine*, 25(6), 1012–1021.

Fichorova, R. N., Yamamoto, H. S., Delaney, M. L., Onderdonk, A. B. & Doncel, G. F. (2011). Novel Vaginal Microflora Colonization Model Providing New Insight into Microbicide Mechanism of Action. *mBio*, 2(6).

Figueroa, D., Mancuso, M. S., Szychowski, J. M., Paden, M. M. & Owen, J. (2011). Does Midtrimester Nugent Score or High Vaginal PH Predict Gestational Age at Delivery in Women at Risk for Recurrent Preterm Birth? *American Journal of Obstetrics and Gynecology*, 204(1), 46.e1-46.e4.

Flores-Espinosa, P., Pineda-Torres, M., Vega-Sánchez, R., Estrada-Gutiérrez, G., Espejel-Nuñez, A., Flores-Pliego, A., Maida-Claros, R., Paredes-Vivas, Y., Morales-Méndez, I., Sosa-González, I., Chávez-Mendoza, A. & Zaga-Clavellina, V. (2014). Progesterone Elicits an Inhibitory Effect upon LPS-Induced Innate Immune Response in Pre-Labor Human Amniotic Epithelium. *American Journal of Reproductive Immunology*, 71(1), 61–72.

Flores-Herrera, H., García-López, G., Díaz, N. F., Molina-Hernández, A., Osorio-Caballero, M., Soriano-Becerril, D. & Zaga-Clavellina, V. (2012). An Experimental Mixed Bacterial Infection Induced Differential Secretion of Proinflammatory Cytokines (IL-1β, TNFα) and ProMMP-9 in Human Fetal Membranes. *Placenta*, 33(4), 271–277.

Fokina, V. M., West, H., Oncken, C., Clark, S. M., Ahmed, M. S., Hankins, G. D. V. & Nanovskaya, T. N. (2016). Bupropion Therapy during Pregnancy: The Drug and Its Major Metabolites in Umbilical Cord Plasma and Amniotic Fluid. *American Journal of Obstetrics and Gynecology*, 215(4), 497.e1-497.e7.

Foley, D. A., Yeoh, D. K., Minney-Smith, C. A., Martin, A. C., Mace, A. O., Sikazwe, C. T., Le, H., Levy, A., Moore, H. C. & Blyth, C. C. (2021). The Interseasonal Resurgence of Respiratory Syncytial Virus in Australian Children Following the Reduction of Coronavirus Disease 2019-Related Public Health Measures. *Clinical*

Infectious Diseases, 73(9), E2829–E2830.
Forney, L. J., Gajer, P., Williams, C. J., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., Karlebach, S., Brotman, R. M., Davis, C. C., Ault, K. & Ravel, J. (2010). Comparison of Self-Collected and Physician-Collected Vaginal Swabs for Microbiome Analysis. *Journal of Clinical Microbiology*, 48(5), 1741–1748.
Fortner, K. B., Grotegut, C. A., Ransom, C. E., Bentley, R. C., Feng, L., Lan, L., Heine, R. P., Seed, P. C. & Murtha, A. P. (2014). Bacteria Localization and Chorion Thinning among Preterm Premature Rupture of Membranes (K. Sun, Ed.). *PLoS ONE*, 9(1), e83338.
Fortunato, S. J. & Menon, R. (2001). Distinct Molecular Events Suggest Different Pathways for Preterm Labor and Premature Rupture of Membranes, in: *American Journal of Obstetrics and Gynecology*, (pp. 1399–1406). Mosby.
Fortunato, S. J., Menon, R. & Lombardi, S. J. (1997). Collagenolytic Enzymes (Gelatinases) and Their Inhibitors in Human Amniochorionic Membrane. *American Journal of Obstetrics and Gynecology*, 177(4), 731–741.
Fortunato, S. J., Menon, R. & Lombardi, S. J. (2002). Role of Tumor Necrosis Factor-α in the Premature Rupture of Membranes and Preterm Labor Pathways. *American Journal of Obstetrics and Gynecology*, 187(5), 1159–1162.
Fox, G. E., Magrum, L. J. & Balch, W. E. (1977). Classification of Methanogenic Bacteria by 16S Ribosomal RNA Characterization. *Proceedings of the National Academy of Sciences of the United States of America*, 74(10), 4537–4541.
Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A. & Olsen, G. J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S RRNA Genes. *Applied and Environmental Microbiology*, 74(8), 2461–2470.
Freitas, A. C., Chaban, B., Bocking, A., Rocco, M., Yang, S., Hill, J. E., Money, D. M., Hemmingsen, S., Reid, G., Dumonceaux, T., Gloor, G., Links, M., O'Doherty, K., Tang, P., Van Schalkwyk, J. & Yudin, M. (2017). The Vaginal Microbiome of Pregnant Women Is Less Rich and Diverse, with Lower Prevalence of Mollicutes, Compared to Non-Pregnant Women. *Scientific Reports*, 7(1).
French, J. (2005). In Vitro Reduction in Chorioamniotic Membrane Strength and Elasticity Induced by Collagenase Producing Microorganisms and Neutrophils. *Journal of Nurse-Midwifery*, 33(1), 40.
Fukuda, K., Ogawa, M., Taniguchi, H. & Saito, M. (2016). Molecular Approaches to Studying Microbial Communities: Targeting the 16S Ribosomal RNA Gene. *Journal of UOEH*, 38(3), 223–232.
Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M., Zhong, X., Koenig, S. S., Fu, L., Ma, Z., Zhou, X., Abdo, Z., Forney, L. J. & Ravel, J. (2012). Temporal Dynamics of the Human Vaginal Microbiota. *Science Translational Medicine*, 4(132), 132–52.
Gao, Y., Lu, Y., Teng, K. L., Chen, M. L., Zheng, H. J., Zhu, Y. Q. & Zhong, J. (2011). Complete Genome Sequence of Lactococcus Lactis Subsp. Lactis CV56, a Probiotic Strain Isolated from the Vaginas of Healthy Women. *Journal of Bacteriology*, 193(11), 2886–2887.
Garcia-Lopez, G., Vadillo-Ortega, F., Merchant-Larios, H., Maida-Claros, R., Osorio, M., Soriano-Becerril, D., Flores-Herrera, H., Beltran-Montoya, J., Garfias-Becerra, Y. & Zaga-Clavellina, V. (2007). Evidence of in Vitro Differential Secretion of 72 and 92 KDa Type IV Collagenases after Selective Exposure to Lipopolysaccharide in Human Fetal Membranes. *Molecular Human Reproduction*, 13(6), 409–418.
Garza, D. R. & Dutilh, B. E. (2015). From Cultured to Uncultured Genome Sequences: Metagenomics and Modeling Microbial Ecosystems. *Cellular and Molecular Life Sciences*, 72(22), 4287–4308.
Genaro-Mattos, T. C., Klingelsmith, K. B., Allen, L. B., Anderson, A., Tallman, K. A., Porter, N. A., Korade, Z. & Mirnics, K. (2021). Sterol Biosynthesis Inhibition in Pregnant Women Taking Prescription Medications. *ACS Pharmacology and Translational Science*, 4(2), 848–857.
Genc, M. R., Vardhana, S., Delaney, M. L., Onderdonk, A., Tuomala, R., Norwitz, E. & Witkin, S. S. (2004). Relationship between a Toll-like Receptor-4 Gene Polymorphism, Bacterial Vaginosis-Related Flora and Vaginal Cytokine Responses in Pregnant Women. *European Journal of Obstetrics Gynecology and Reproductive Biology*, 116(2), 152–156.
Georgiou, H. M., Di Quinzio, M. K. W., Permezel, M. & Brennecke, S. P. (2015). Predicting Preterm Labour: Current Status and Future Prospects. *Disease Markers*, 2015(July), 1–9.
Ghartey, J., Anglim, L., Romero, J., Brown, A. G., Elovitz, M. A., Bastek, J. A., Brown, A. G., Anglim, L. & Elovitz, M. A. (2017). Women with Symptomatic Preterm Birth Have a Distinct Cervicovaginal Metabolome. *American Journal of Perinatology*, 34(11), 1078–1083.
Ghartey, J., Bastek, J. A., Brown, A. G., Anglim, L. & Elovitz, M. A. (2015). Women with Preterm Birth Have a Distinct Cervicovaginal Metabolome. *American Journal of Obstetrics and Gynecology*, 212(6), 776.e1-776.e12.
Gill, C., van de Wijgert, J. H. H. M. H. M., Blow, F. & Darby, A. C. (2016). Evaluation of Lysis Methods for the

Extraction of Bacterial DNA for Analysis of the Vaginal Microbiota (P. E. Larsen, Ed.). *PLoS ONE*, 11(9), e0163148.

Gilman-Sachs, A., Dambaeva, S., Salazar Garcia, M. D., Hussein, Y., Kwak-Kim, J. & Beaman, K. (2018). Inflammation Induced Preterm Labor and Birth. *Journal of Reproductive Immunology*, 129, 53–58.

Gimeno-Molina, B., Muller, I., Kropf, P. & Sykes, L. (2022). The Role of Neutrophils in Pregnancy, Term and Preterm Labour. *Life*, 12(10), 1512.

Glascock, A. L., Jimenez, N. R., Boundy, S., Koparde, V. N., Brooks, J. P., Edwards, D. J., Strauss, J. F., Jefferson, K. K., Serrano, M. G., Buck, G. A. & Fettweis, J. M. (2021). Unique Roles of Vaginal Megasphaera Phylotypes in Reproductive Health. *Microbial Genomics*, 7(12), 526.

Goepfert, A. R., Varner, M., Ward, K., Macpherson, C., Klebanoff, M., Goldenberg, R. L., Mercer, B., Meis, P., Iams, J., Moawad, A., Carey, J. C., Leveno, K., Wapner, R., Caritis, S. N., Miodovnik, M., Sorokin, Y., O'Sullivan, M. J., Van Dorsten, J. P. & Langer, O. (2005). Differences in Inflammatory Cytokine and Toll-like Receptor Genes and Bacterial Vaginosis in Pregnancy. *American Journal of Obstetrics and Gynecology*, 193(4), 1478–1485.

Goldberg, A. L. (1972). Correlation between Rates of Degradation of Bacterial Proteins in Vivo and Their Sensitivity to Proteases. *Proceedings of the National Academy of Sciences of the United States of America*, 69(9), 2640–2644.

Goldenberg, R. L. & Culhane, J. F. (2003). Infection as a Cause of Preterm Birth. *Clinics in Perinatology*, 30(4), 677–700.

Goldenberg, R. L., Culhane, J. F., Iams, J. D. & Romero, R. (2008). Epidemiology and Causes of Preterm Birth. *The Lancet*, 371(9606), 75–84.

Goldenberg, R. L., Iams, J. D., Mercer, B. M., Meis, P. J., Moawad, A. H., Copper, R. L., Das, A., Thom, E., Johnson, F., McNellis, D., Miodovnik, M., Van Dorsten, J. P., Caritis, S. N., Thurnau, G. R. & Bottoms, S. F. (1998). The Preterm Prediction Study: The Value of New vs Standard Risk Factors in Predicting Early and All Spontaneous Preterm Births. *American Journal of Public Health*, 88(2), 233–238.

Gomez-Lopez, N., Romero, R., Panaitescu, B., Leng, Y., Xu, Y., Tarca, A. L., Faro, J., Pacora, P., Hassan, S. S. & Hsu, C. D. (2018). Inflammasome Activation during Spontaneous Preterm Labor with Intra-Amniotic Infection or Sterile Intra-Amniotic Inflammation. *American Journal of Reproductive Immunology*, 80(5), e13049.

Gomez-Lopez, N., StLouis, D., Lehr, M. A., Sanchez-Rodriguez, E. N. & Arenas-Hernandez, M. (2014). Immune Cells in Term and Preterm Labor. *Cellular and Molecular Immunology*, 11(6), 571–581.

Gonzalez, J. M., Franzke, C. W., Yang, F., Romero, R. & Girardi, G. (2011). Complement Activation Triggers Metalloproteinases Release Inducing Cervical Remodeling and Preterm Birth in Mice. *American Journal of Pathology*, 179(2), 838–849.

Goodman, A. H. (2000). Why Genes Don't Count (for Racial Differences in Health). *American Journal of Public Health*, 90(11), 1699–1702.

Gosmann, C. et al. (2017). Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity*, 46(1), 29–37.

Gottipamula, S. & Sridhar, K. N. (2018). Large-Scale Isolation, Expansion and Characterization of Human Amniotic Epithelial Cells. *International Journal of Stem Cells*, 11(1), 87–95.

Greenough, A. (2012). Long Term Respiratory Outcomes of Very Premature Birth (<32 Weeks). *Seminars in Fetal and Neonatal Medicine*, 17(2), 73–76.

Guaschino, S., De Seta, F., Piccoli, M., Maso, G. & Alberico, S. (2006). Aetiology of Preterm Labour: Bacterial Vaginosis. *BJOG: An International Journal of Obstetrics & Gynaecology*, 113(SUPPL. 3), 46–51.

Gudnadottir, U., Debelius, J. W., Du, J., Hugerth, L. W., Danielsson, H., Schuppe-Koistinen, I., Fransson, E. & Brusselaers, N. (2022). The Vaginal Microbiome and the Risk of Preterm Birth: A Systematic Review and Network Meta-Analysis. *Scientific Reports*, 12(1), 1–8.

Gupta, R. S. (2021). Eggerthellaceae, in: *Bergey's Manual of Systematics of Archaea and Bacteria*, (pp. 1–11). John Wiley & Sons, Ltd.

Haahr, T., Jensen, J. S., Thomsen, L., Duus, L., Rygaard, K. & Humaidan, P. (2016). Abnormal Vaginal Microbiota May Be Associated with Poor Reproductive Outcomes: A Prospective Study in IVF Patients. *Human Reproduction*, 31(4), 795–803.

Hadley, E. E., Richardson, L. S., Torloni, M. R. & Menon, R. (2018). Gestational Tissue Inflammatory Biomarkers at Term Labor: A Systematic Review of Literature. *American Journal of Reproductive Immunology*, 79(2), e12776.

Hajhashemi, V. & Khanjani, P. (2014). Analgesic and Anti-Inflammatory Activities of Bupropion in Animal Models. *Research in Pharmaceutical Sciences*, 9(4), 251–257.

Hanley, M., Sayres, L., Reiff, E. S., Wood, A., Grotegut, C. A. & Kuller, J. A. (2019). Tocolysis: A Review of the

Literature. *Obstetrical and Gynecological Survey*, 74(1), 50–55.
Hantoushzadeh, S., Aliabad, R. A. & Norooznezhad, A. H. (2020). Antibiotics, Inflammation, and Preterm Labor: A Missed Conclusion. *Journal of Inflammation Research*, 13, 245–254.
Haque, M. M., Merchant, M., Kumar, P. N., Dutta, A. & Mande, S. S. (2017). First-Trimester Vaginal Microbiome Diversity: A Potential Indicator of Preterm Delivery Risk. *Scientific Reports*, 7(1), 1–10.
Hargreaves, D. C. & Medzhitov, R. (2005). Innate Sensors of Microbial Infection. *Journal of Clinical Immunology*, 25(6), 503–510.
Hargrove, J. L., Greenspan, P. & Hartle, D. K. (2004). Nutritional Significance and Metabolism of Very Long Chain Fatty Alcohols and Acids from Dietary Waxes. *Experimental Biology and Medicine*, 229(3), 215–226.
Harrington, D. J. (1996). Bacterial Collagenases and Collagen-Degrading Enzymes and Their Potential Role in Human Disease. *Infection and Immunity*, 64(6), 1885–1891.
Hatanaka, A. R., Franca, M. S., Hamamoto, T. E. N. K., Rolo, L. C., Mattar, R. & Moron, A. F. (2019). Antibiotic Treatment for Patients with Amniotic Fluid "Sludge" to Prevent Spontaneous Preterm Birth: A Historically Controlled Observational Study. *Acta Obstetricia et Gynecologica Scandinavica*, 98(9), 1157–1163.
Hearps, A. C., Tyssen, D., Srbinovski, D., Bayigga, L., Diaz, D. J. D., Aldunate, M., Cone, R. A., Gugasyan, R., Anderson, D. J. & Tachedjian, G. (2017). Vaginal Lactic Acid Elicits an Anti-Inflammatory Response from Human Cervicovaginal Epithelial Cells and Inhibits Production of pro-Inflammatory Mediators Associated with HIV Acquisition. *Mucosal Immunology*, 10(6), 1480–1490.
Helmig, R., Oxlund, H., Petersen, L. K. & Uldbjerg, N. (1993). Different Biomechanical Properties of Human Fetal Membranes Obtained before and after Delivery. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 48(3), 183–189.
Henderson, J., Carson, C. & Redshaw, M. (2016). Impact of Preterm Birth on Maternal Well-Being and Women's Perceptions of Their Baby: A Population-Based Survey. *BMJ Open*, 6(10), e012676.
Henriques, A., Cereija, T., MacHado, A. & Cerca, N. (2012). In Silico vs in Vitro Analysis of Primer Specificity for the Detection of Gardnerella Vaginalis, Atopobium Vaginae and Lactobacillus Spp. *BMC Research Notes*, 5(1), 1–4.
Hill, G. B. (1998). Preterm Birth: Associations with Genital and Possibly Oral Microflora. *Annals of periodontology / the American Academy of Periodontology*, 3(1), 222–232.
Hillier, S. L., Nugent, R. P., Eschenbach, D. A., Krohn, M. A., Gibbs, R. S., Martin, D. H., Cotch, M. F., Edelman, R., Pastorek, J. G., Rao, A. V., Mcnellis, D., Regan, J. A., Carey, J. C. & Klebanoff, M. A. (1995). Association between Bacterial Vaginosis and Preterm Delivery of a Low-Birth-Weight Infant. *New England Journal of Medicine*, 333(26), 1737–1742.
Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., Ng, K. F., Suen, M. W. M. & Tai, H. L. (2003). Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *The Clinical biochemist. Reviews*, 24(1), 3–12.
Hočevar, K., Maver, A., Vidmar Šimic, M., Hodžić, A., Haslberger, A., Premru Seršen, T. & Peterlin, B. (2019). Vaginal Microbiome Signature Is Associated With Spontaneous Preterm Delivery. *Frontiers in Medicine*, 6, 201.
Holland, C., Vollrath, F. & Gill, H. S. (2014). Horses and Cows Might Teach Us about Human Knees. *Naturwissenschaften*, 101(4), 351–354.
Holst, R. M., Hagberg, H., Wennerholm, U. B., Skogstrand, K., Thorsen, P. & Jacobsson, B. (2011). Prediction of Microbial Invasion of the Amniotic Cavity in Women with Preterm Labour: Analysis of Multiple Proteins in Amniotic and Cervical Fluids. *BJOG: An International Journal of Obstetrics and Gynaecology*, 118(2), 240–249.
Horton, A. L. & Boggess, K. A. (2012). Periodontal Disease and Preterm Birth. *Contemporary Ob/Gyn*, 57(12), 44–46.
Hoshino, Y. & Gaucher, E. A. (2021). Evolution of Bacterial Steroid Biosynthesis and Its Impact on Eukaryogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 118(25), e2101276118.
Hsieh, M. W., Chen, H. Y. & Tsai, C. C. (2021). Screening and Evaluation of Purine-Nucleoside-Degrading Lactic Acid Bacteria Isolated from Winemaking Byproducts in Vitro and Their Uric Acid-Lowering Effects in Vivo. *Fermentation*, 7(2).
Hug, L. A., Baker, B. J., Anantharaman, K., Brown, C. T., Probst, A. J., Castelle, C. J., Butterfield, C. N., Hernsdorf, A. W., Amano, Y., Ise, K., Suzuki, Y., Dudek, N., Relman, D. A., Finstad, K. M., Amundson, R., Thomas, B. C. & Banfield, J. F. (2016). A New View of the Tree of Life. *Nature Microbiology*, 1(5), 1–6.
Hugerth, L. W., Pereira, M., Zha, Y., Seifert, M., Kaldhusdal, V., Boulund, F., Krog, M. C., Bashir, Z., Hamsten, M., Fransson, E., Nielsen, H. S., Schuppe-Koistinen, I. & Engstrand, L. (2020). Assessment of In Vitro and In Silico Protocols for Sequence-Based Characterization of the Human Vaginal Microbiome. *mSphere*, 5(6).

Hulboy, D. L., Rudolph, L. A. & Matrisian, L. M. (1997). Matrix Metalloproteinases as Mediators of Reproductive Function. *Molecular Human Reproduction*, 3(1), 27–45.

Hyman, R. W., Fukushima, M., Jiang, H., Fung, E., Rand, L., Johnson, B., Vo, K. C., Caughey, A. B., Hilton, J. F., Davis, R. W. & Giudice, L. C. (2014). Diversity of the Vaginal Microbiome Correlates with Preterm Birth. *Reproductive Sciences*, 21(1), 32–40.

Hynes, W. L. & Walton, S. L. (2000). Hyaluronidases of Gram-Positive Bacteria. *FEMS Microbiology Letters*, 183(2), 201–207.

Ivan Fernandez Vega, B. G. (2014). The Role of Heparan Sulfate Proteoglycans in Bacterial Infections. *Journal of Medical Microbiology & Diagnosis*, 03(04).

Jacobsson, B., Aaltonen, R., Rantakokko-Jalava, K., Morken, N. H. & Alanen, A. (2009). Quantification of Ureaplasma Urealyticum DNA in the Amniotic Fluid from Patients in PTL and PPROM and Its Relation to Inflammatory Cytokine Levels. *Acta Obstetricia et Gynecologica Scandinavica*, 88(1), 63–70.

Janmey, P. A., Georges, P. C. & Hvidt, S. (2007). Basic Rheology for Biologists. *Methods in Cell Biology*, 83, 1–27.

Janzen, C., Sen, S., Lei, M. Y. Y., De Assumpcao, M. G., Challis, J. & Chaudhuri, G. (2017). The Role of Epithelial to Mesenchymal Transition in Human Amniotic Membrane Rupture. *Journal of Clinical Endocrinology and Metabolism*, 102(4), 1261–1269.

Jarde, A., Lutsiv, O., Beyene, J. & McDonald, S. (2019). Vaginal Progesterone, Oral Progesterone, 17-OHPC, Cerclage, and Pessary for Preventing Preterm Birth in At-risk Singleton Pregnancies: An Updated Systematic Review and Network Meta-analysis. *BJOG: An International Journal of Obstetrics & Gynaecology*, 126(5), 556–567.

Jayaprakash, T. P., Wagner, E. C., Van Schalkwyk, J., Albert, A. Y. K., Hill, J. E., Money, D. M., Hemmingsen, S. M., Castillo, E. & Janssen, P. A. (2016). High Diversity and Variability in the Vaginal Microbiome in Women Following Preterm Premature Rupture of Membranes (PPROM): A Prospective Cohort Study (M. Ciccozzi, Ed.). *PLoS ONE*, 11(11), e0166794.

Jayes, F. L., Liu, B., Moutos, F. T., Kuchibhatla, M., Guilak, F. & Leppert, P. C. (2016). Loss of Stiffness in Collagen-Rich Uterine Fibroids after Digestion with Purified Collagenase Clostridium Histolyticum. *American Journal of Obstetrics and Gynecology*, 215(5), 596.e1-596.e8.

Jespers, V. et al. (2014). Prevalence and Correlates of Bacterial Vaginosis in Different Sub-Populations of Women in Sub-Saharan Africa: A Cross-Sectional Study (D. N. Fredricks, Ed.). *PLoS ONE*, 9(10), e109670.

Jespers, V., Kyongo, J., Joseph, S., Hardy, L., Cools, P., Crucitti, T., Mwaura, M., Ndayisaba, G., Delany-Moretlwe, S., Buyze, J., Vanham, G. & Van De Wijgert, J. H. H. M. (2017). A Longitudinal Analysis of the Vaginal Microbiota and Vaginal Immune Mediators in Women from Sub-Saharan Africa. *Scientific Reports*, 7(1), 11974.

Jespers, V., van de Wijgert, J., Cools, P., Verhelst, R., Verstraelen, H., Delany-Moretlwe, S., Mwaura, M., Ndayisaba, G. F., Mandaliya, K., Menten, J., Hardy, L. & Crucitti, T. (2015). The Significance of Lactobacillus Crispatus and L. Vaginalis for Vaginal Health and the Negative Effect of Recent Sex: A Cross-Sectional Descriptive Study across Groups of African Women. *BMC Infectious Diseases*, 15(1), 115.

Joesoef, M. R., Hillier, S. L., Wiknjosastro, G., Sumampouw, H., Linnan, M., Norojono, W., Idajadi, A. & Utomo, B. (1995). Intravaginal Clindamycin Treatment for Bacterial Vaginosis: Effects on Preterm Delivery and Low Birth Weight. *American Journal of Obstetrics & Gynecology*, 173(5), 1527–1531.

Jones, M. B., Highlander, S. K., Anderson, E. L., Li, W., Dayrit, M., Klitgord, N., Fabani, M. M., Seguritan, V., Green, J., Pride, D. T., Yooseph, S., Biggs, W., Nelson, K. E. & Craig Venter, J. (2015). Library Preparation Methodology Can Influence Genomic and Functional Predictions in Human Microbiome Research. *Proceedings of the National Academy of Sciences of the United States of America*, 112(45), 14024–14029.

Jung, E. Y., Park, J. W., Ryu, A., Lee, S. Y., Cho, S. H. & Park, K. H. (2016). Prediction of Impending Preterm Delivery Based on Sonographic Cervical Length and Different Cytokine Levels in Cervicovaginal Fluid in Preterm Labor. *Journal of Obstetrics and Gynaecology Research*, 42(2), 158–165.

Kacerovsky, M., Pliskova, L., Bolehovska, R., Lesko, D., Gerychova, R., Janku, P., Matlak, P., Simetka, O., Stranik, J., Faist, T., Mls, J., Vescicik, P., Jacobsson, B. & Musilova, I. (2021). Cervical Gardnerella Vaginalis in Women with Preterm Prelabor Rupture of Membranes. *PLoS ONE*, 16(1 January), e0245937.

Kacerovsky, M., Vrbacky, F., Kutova, R., Pliskova, L., Andrys, C., Musilova, I., Menon, R., Lamont, R. & Nekvindova, J. (2015). Cervical Microbiota in Women with Preterm Prelabor Rupture of Membranes (C. Kanellopoulos-Langevin, Ed.). *PLoS ONE*, 10(5), e0126884.

Kagan, K. O. & Sonek, J. (2015). How to Measure Cervical Length. *Ultrasound in Obstetrics and Gynecology*, 45(3), 358–362.

Karadağ, C., Akar, B., Gönenç, G., Aslancan, R., Yılmaz, N. & Çalışkan, E. (2020). Aspirin, Low Molecular Weight Heparin, or Both in Preventing Pregnancy Complications in Women with Recurrent Pregnancy Loss and

Factor V Leiden Mutation. *Journal of Maternal-Fetal and Neonatal Medicine*, 33(11), 1934–1939.
Kataoka, S., Yamada, T., Chou, K., Nishida, R., Morikawa, M., Minami, M., Yamada, H., Sakuragi, N. & Minakami, H. (2006). Association between Preterm Birth and Vaginal Colonization by Mycoplasmas in Early Pregnancy. *Journal of Clinical Microbiology*, 44(1), 51–55.
Kaur, H., Merchant, M., Haque, M. M. & Mande, S. S. (2020). Crosstalk Between Female Gonadal Hormones and Vaginal Microbiota Across Various Phases of Women's Gynecological Lifecycle. *Frontiers in Microbiology*, 11.
Keelan, J. A. (2018). Intrauterine Inflammatory Activation, Functional Progesterone Withdrawal, and the Timing of Term and Preterm Birth. *Journal of Reproductive Immunology*, 125, 89–99.
Kenyon, C., Colebunders, R. & Crucitti, T. (2013). The Global Epidemiology of Bacterial Vaginosis: A Systematic Review. *American Journal of Obstetrics and Gynecology*, 209(6), 505–523.
Kerry-Barnard, S., Zhou, L., Phillips, L., Furegato, M., Witney, A. A., Sadiq, S. T. & Oakeshott, P. (2022). Vaginal Microbiota in Ethnically Diverse Young Women Who Did or Did Not Develop Pelvic Inflammatory Disease: Community-Based Prospective Study. *Sexually Transmitted Infections*, 0, sextrans-2021-055260.
Khan, K. A., Petrou, S., Dritsaki, M., Johnson, S. J., Manktelow, B., Draper, E. S., Smith, L. K., Seaton, S. E., Marlow, N., Dorling, J., Field, D. J. & Boyle, E. M. (2015). Economic Costs Associated with Moderate and Late Preterm Birth: A Prospective Population-Based Study. *BJOG: An International Journal of Obstetrics and Gynaecology*, 122(11), 1495–1505.
Khan, S., Al-Sweih, N., Othman, A. H. & Dhar, R. (2014). Bacteremia Caused by Rhizobium Radiobacter in a Preterm Neonate. *Indian Journal of Pediatrics*, 81(2), 191–192.
El Khwad, M. et al. (2006). Fetal Membranes From Term Vaginal Deliveries Have a Zone of Weakness Exhibiting Characteristics of Apoptosis and Remodeling. *Journal of the Society for Gynecologic Investigation*, 13(3), 191–195.
Kim, C. J., Chaemsaithong, P., Chaiyasit, N., Yoon, B. H. & Kim, Y. M. (2015). Acute Chorioamnionitis and Funisitis: Definition, Pathologic Features, and Clinical Significance. *American Journal of Obstetrics and Gynecology*, 213(4), S29–S52.
Kim, J., Durai, P., Jeon, D., Jung, I. D., Lee, S. J., Park, Y. M. & Kim, Y. (2018). Phloretin as a Potent Natural TLR2/1 Inhibitor Suppresses TLR2-Induced Inflammation. *Nutrients*, 10(7), 868.
Kim, Y. M., Romero, R., Chaiworapongsa, T., Kim, G. J., Kim, M. R., Kuivaniemi, H., Tromp, G., Espinoza, J., Bujold, E., Abrahams, V. M. & Mor, G. (2004). Toll-like Receptor-2 and -4 in the Chorioamniotic Membranes in Spontaneous Labor at Term and in Preterm Parturition That Are Associated with Chorioamnionitis, in: *American Journal of Obstetrics and Gynecology*, (pp. 1346–1355).
Klein, L. L. & Gibbs, R. S. (2005). Infection and Preterm Birth. *Obstetrics and Gynecology Clinics of North America*, 32(3), 397–410.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F. O. (2013). Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Research*, 41(1), e1–e1.
Knudsen, B. F. & Kaack, K. V. (2015). A Review of Human Health and Disease Claims for Elderberry (Sambucus Nigra) Fruit. *Acta Horticulturae*, 1061, 121–132.
Komiya, S., Matsuo, Y., Nakagawa, S., Morimoto, Y., Kryukov, K., Okada, H. & Hirota, K. (2021). MinION™, a Portable Long-Read Sequencer, Enables Rapid Vaginal Microbiota Analysis in a Clinical Setting. *bioRxiv*, 2021.01.07.425663.
Kovachev, S. (2018). Defence Factors of Vaginal Lactobacilli. *Critical Reviews in Microbiology*, 44(1), 31–39.
Krikun, G., Lockwood, C. J., Abrahams, V. M., Mor, G., Paidas, M. & Guller, S. (2007). Expression of Toll-like Receptors in the Human Decidua. *Histology and Histopathology*, 22(7–9), 847–854.
Krzyaściak, W., Pluskwa, K. K., Jurczak, A. & Koaścielniak, D. (2013). The Pathogenicity of the Streptococcus Genus. *European Journal of Clinical Microbiology and Infectious Diseases*, 32(11), 1361–1376.
Kumar, D., Fung, W., Moore, R. M., Pandey, V., Fox, J., Stetzer, B., Mansour, J. M., Mercer, B. M., Redline, R. W., Moore, J. J., Redline, R. W., Moore, R. M., Mansour, J. M., Mercer, B. M., Fung, W., Fox, J., Kumar, D. & Pandey, V. (2005). Proinflammatory Cytokines Found in Amniotic Fluid Induce Collagen Remodeling, Apoptosis, and Biophysical Weakening of Cultured Human Fetal Membranes1. *Biology of Reproduction*, 74(1), 29–34.
Kumar, D., Springel, E., Moore, R. M., Mercer, B. M., Philipson, E., Mansour, J. M., Mesiano, S., Schatz, F., Lockwood, C. J. & Moore, J. J. (2015). Progesterone Inhibits in Vitro Fetal Membrane Weakening. *American Journal of Obstetrics and Gynecology*, 213(4), 520.e1-520.e9.
Kumar, S., Kumari, N., Talukdar, D., Kothidar, A., Sarkar, M., Mehta, O., Kshetrapal, P., Wadhwa, N., Thiruvengadam, R., Desiraju, B. K., Nair, G. B., Bhatnagar, S., Mukherjee, S. & Das, B. (2021). The Vaginal

Microbial Signatures of Preterm Birth Delivery in Indian Women. *Frontiers in Cellular and Infection Microbiology*, 11, 402.

Kunzier, N. B., Kinzler, W. L., Chavez, M. R., Adams, T. M., Brand, D. A. & Vintzileos, A. M. (2016). The Use of Cervical Sonography to Differentiate True from False Labor in Term Patients Presenting for Labor Check. *American Journal of Obstetrics and Gynecology*, 215(3), 372.e1-372.e5.

Kusanovic, J. P., Espinoza, J., Romero, R., Gonçalves, L. F., Nien, J. K., Soto, E., Khalek, N., Camacho, N., Hendler, I., Mittal, P., Friel, L. A., Gotsch, F., Erez, O., Than, N. G., Mazaki-Tovi, S., Schoen, M. L. & Hassan, S. S. (2007). Clinical Significance of the Presence of Amniotic Fluid 'sludge' in Asymptomatic Patients at High Risk for Spontaneous Preterm Delivery. *Ultrasound in Obstetrics and Gynecology*, 30(5), 706–714.

Kwak, D.-W., Hwang, H.-S., Kwon, J.-Y., Park, Y.-W. & Kim, Y.-H. (2014). Co-Infection with Vaginal Ureaplasma Urealyticum and Mycoplasma Hominis Increases Adverse Pregnancy Outcomes in Patients with Preterm Labor or Preterm Premature Rupture of Membranes. *The Journal of Maternal-Fetal & Neonatal Medicine*, 27(4), 333–337.

Kwak, W., Han, Y. H., Seol, D., Kim, Hyaekang, Ahn, H., Jeong, M., Kang, J., Kim, Heebal & Kim, T. H. (2020). Complete Genome of Lactobacillus Iners KY Using Flongle Provides Insight Into the Genetic Background of Optimal Adaption to Vaginal Econiche. *Frontiers in Microbiology*, 11, 1048.

Lai, C. C., Teng, L. J., Hsueh, P. R., Yuan, A., Tsai, K. C., Tang, J. L. & Tien, H. F. (2004). Clinical and Microbiological Characteristics of Rhizobium Radiobacter Infections. *Clinical Infectious Diseases*, 38(1), 149–153.

Lajos, G. J., Passini Junior, R., Nomura, M. L., Amaral, E., Pereira, B. G., Milanez, H. & Parpinelli, M. Â. (2008). Colonização Bacteriana Do Canal Cervical Em Gestantes Com Trabalho de Parto Prematuro Ou Ruptura Prematura de Membranas. *Revista Brasileira de Ginecologia e Obstetrícia*, 30(8).

Lamichhane, S., Sen, P., Dickens, A. M., Hyötyläinen, T. & Orešič, M. (2018). An Overview of Metabolomics Data Analysis: Current Tools and Future Perspectives, in: *Comprehensive Analytical Chemistry*, (pp. 387–413). Elsevier.

Lamont, R. F. (2015). Advances in the Prevention of Infection-Related Preterm Birth. *Frontiers in Immunology*, 6(NOV), 566.

Lannon, S. M. R., Vanderhoeven, J. P., Eschenbach, D. A., Gravett, M. G. & Waldorf, K. M. A. (2014). Synergy and Interactions among Biological Pathways Leading to Preterm Premature Rupture of Membranes. *Reproductive Sciences*, 21(10), 1215–1227.

Laronha, H. & Caldeira, J. (2020). Structure and Function of Human Matrix Metalloproteinases. *Cells*, 9(5), 1076.

Larsen, J. W. & Sever, J. L. (2008). Group B Streptococcus and Pregnancy: A Review. *American Journal of Obstetrics and Gynecology*, 198(4), 440–450.

Laursen, T., Borch, J., Knudsen, C., Bavishi, K., Torta, F., Martens, H. J., Silvestro, D., Hatzakis, N. S., Wenk, M. R., Dafforn, T. R., Olsen, C. E., Motawia, M. S., Hamberger, B., Møller, B. L. & Bassard, J. E. (2016). Characterization of a Dynamic Metabolon Producing the Defense Compound Dhurrin in Sorghum. *Science*, 354(6314), 890–893.

Lavery, J. P. & Miller, C. E. (1979). Deformation and Creep in the Human Chorioamniotic Sac. *American Journal of Obstetrics and Gynecology*, 134(4), 366–375.

Lavery, J. P., Miller, C. E. & Knight, R. D. (1982). The Effect of Labor on the Rheologic Response of Chorioamniotic Membranes. *Obstetrics and Gynecology*, 60(1), 87–92.

Lee, S. Bin, Lee, W. S., Shin, J. S., Jang, D. S. & Lee, K. T. (2017). Xanthotoxin Suppresses LPS-Induced Expression of INOS, COX-2, TNF-α, and IL-6 via AP-1, NF-KB, and JAK-STAT Inactivation in RAW 264.7 Macrophages. *International Immunopharmacology*, 49, 21–29.

Lee, S. E., Romero, R., Kim, E. C. & Yoon, B. H. (2009). A High Nugent Score but Not a Positive Culture for Genital Mycoplasmas Is a Risk Factor for Spontaneous Preterm Birth. *Journal of Maternal-Fetal and Neonatal Medicine*, 22(3), 212–217.

Leikina, E., Mertts, M. V., Kuznetsova, N. & Leikin, S. (2002). Type I Collagen Is Thermally Unstable at Body Temperature. *Proceedings of the National Academy of Sciences of the United States of America*, 99(3), 1314–1318.

Leitich, H. & Kiss, H. (2007). Asymptomatic Bacterial Vaginosis and Intermediate Flora as Risk Factors for Adverse Pregnancy Outcome. *Best Practice and Research: Clinical Obstetrics and Gynaecology*, 21(3), 375–390.

Lennard, K., Dabee, S., Barnabas, S. L., Havyarimana, E., Blakney, A., Jaumdally, S. Z., Botha, G., Mkhize, N. N., Bekker, L. G., Lewis, D. A., Gray, G., Mulder, N., Passmore, J. A. S. & Jaspana, H. B. (2018). Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infection and Immunity*, 86(1), e00410-17.

Levy, M., Thaiss, C. A. & Elinav, E. (2016). Metabolites: Messengers between the Microbiota and the Immune System. *Genes and Development*, 30(14), 1589–1597.

Lewiń-ska, M., Zelenko, U., Merzel, F., Grdadolnik, S. G., Murray, J. C. & Rozman, D. (2013). Polymorphisms of CYP51A1 from Cholesterol Synthesis: Associations with Birth Weight and Maternal Lipid Levels and Impact on CYP51 Protein Structure. *PLoS ONE*, 8(12), e82554.

Lewis, F. M. T., Bernstein, K. T. & Aral, S. O. (2017). Vaginal Microbiome and Its Relationship to Behavior, Sexual Health, and Sexually Transmitted Diseases. *Obstetrics and Gynecology*, 129(4), 643–654.

Li, T., Liu, Z. H., Li, K. & Bai, H. H. (2019). Evaluation of the Vaginal Microbiome in Clinical Diagnosis and Management of Vaginal Infectious Diseases. *Chinese Medical Journal*, 132(9), 1100.

Li, W., Alfaidy, N. & Challis, J. R. G. (2004). Expression of Extracellular Matrix Metalloproteinase Inducer in Human Placenta and Fetal Membranes at Term Labor. *Journal of Clinical Endocrinology and Metabolism*, 89(6), 2897–2904.

Li, W., Narice, B. F., Anumba, D. O. & Matcher, S. J. (2019). Polarization-Sensitive Optical Coherence Tomography with a Conical Beam Scan for the Investigation of Birefringence and Collagen Alignment in the Human Cervix. *Biomedical Optics Express*, 10(8), 4190.

Li, W., Zhao, X., Li, S., Chen, X., Cui, H., Chang, Y. & Zhang, R. (2020). Upregulation of TNF-α and IL-6 Induces Preterm Premature Rupture of Membranes by Activation of ADAMTS-9 in Embryonic Membrane Cells. *Life Sciences*, 260, 118237.

Liang, H., Xie, Z., Liu, B., Song, X. & Zhao, G. (2019). A Routine Urine Test Has Partial Predictive Value in Premature Rupture of the Membranes. *Journal of International Medical Research*, 47(6), 030006051984116.

Lim, R., Barker, G., Wall, C. A. & Lappas, M. (2013). Dietary Phytophenols Curcumin, Naringenin and Apigenin Reduce Infection-Induced Inflammatory and Contractile Pathways in Human Placenta, Foetal Membranes and Myometrium. *Molecular Human Reproduction*, 19(7), 451–462.

Lithgow, K. V., Buchholz, V. C. H., Ku, E., Konschuh, S., D'Aubeterre, A. & Sycuro, L. K. (2022). Protease Activities of Vaginal Porphyromonas Species Disrupt Coagulation and Extracellular Matrix in the Cervicovaginal Niche. *bioRxiv*, 2021.07.07.447795.

Long, J. C. & Kittles, R. A. (2009). Human Genetic Diversity and the Nonexistence of Biological Races. *Human Biology*, 81(5–6), 777–798.

Lopes dos Santos Santiago, G., Tency, I., Verstraelen, H., Verhelst, R., Trog, M., Temmerman, M., Vancoillie, L., Decat, E., Cools, P. & Vaneechoutte, M. (2012). Longitudinal QPCR Study of the Dynamics of L. Crispatus, L. Iners, A. Vaginae, (Sialidase Positive) G. Vaginalis, and P. Bivia in the Vagina. *PLoS ONE*, 7(9), e45281.

Loriaux, D. L., Ruder, H. J., Knab, D. R. & Lipsett, M. B. (1972). Estrone Sulfate, Estrone, Estradiol and Estriol Plasma Levels in Human Pregnancy. *Journal of Clinical Endocrinology and Metabolism*, 35(6), 887–891.

Lovereen, S., Khanum, A., Nargis, N., Begum, S. & Afroze, R. (2018). Maternal and Neonatal Outcome in Premature Rupture of Membranes. *Bangladesh Journal of Medical Science*, 17(3), 479–483.

Lu, P., Takai, K., Weaver, V. M. & Werb, Z. (2011). Extracellular Matrix Degradation and Remodeling in Development and Disease. *Cold Spring Harbor Perspectives in Biology*, 3(12).

Lumley, S. F., Richens, N., Lees, E., Cregan, J., Kalimeris, E., Oakley, S., Morgan, M., Segal, S., Dawson, M., Walker, A. S., Eyre, D. W., Crook, D. W., Beer, S., Novak, A., Stoesser, N. E. & Matthews, P. C. (2022). Changes in Paediatric Respiratory Infections at a UK Teaching Hospital 2016–2021; Impact of the SARS-CoV-2 Pandemic. *Journal of Infection*, 84(1), 40–47.

Luo, G., Abrahams, V. M., Tadesse, S., Funai, E. F., Hodgson, E. J., Gao, J. & Norwitz, E. R. (2010). Progesterone Inhibits Basal and Tnf-α-Induced Apoptosis in Fetal Membranes: A Novel Mechanism to Explain Progesterone-Mediated Prevention of Preterm Birth. *Reproductive Sciences*, 17(6), 532–539.

M. Harirah, H., A. Borahay, M., Zaman, W., S. Ahmed, M., G. Hager, I. & D. V. Hankins, G. (2012). Increased Apoptosis in Chorionic Trophoblasts of Human Fetal Membranes with Labor at Term. *International Journal of Clinical Medicine*, 03(02), 136–142.

MacIntyre, D. A., Chandiramani, M., Lee, Y. S., Kindinger, L., Smith, A., Angelopoulos, N., Lehne, B., Arulkumaran, S., Brown, R., Teoh, T. G., Holmes, E., Nicoholson, J. K., Marchesi, J. R. & Bennett, P. R. (2015). The Vaginal Microbiome during Pregnancy and the Postpartum Period in a European Population. *Scientific Reports*, 5.

Macklaim, J. M., Gloor, G. B., Anukam, K. C., Cribby, S. & Reid, G. (2011). At the Crossroads of Vaginal Health and Disease, the Genome Sequence of Lactobacillus Iners AB-1. *Proceedings of the National Academy of Sciences of the United States of America*, 108(SUPPL. 1), 4688–4695.

Magon, N. & Kumar, P. (2012). Hormones in Pregnancy. *Nigerian Medical Journal*, 53(4), 179.

Maia, M. C., Nomura, R., Mendonça, F., Rios, L. & Moron, A. (2020). Is Cervical Length Evaluated by Transvaginal Ultrasonography Helpful in Detecting True Preterm Labor? *Journal of Maternal-Fetal and Neonatal Medicine*, 33(17), 2902–2908.

Malak, T. M., Ockleford, C. D., Bell, S. C., Dalgleish, R., Bright, N. & Macvicar, J. (1993). Confocal Immunofluorescence Localization of Collagen Types I, III, IV, V and VI and Their Ultrastructural

Organization in Term Human Fetal Membranes. *Placenta*, 14(4), 385–406.
Mangham, L. J., Petrou, S., Doyle, L. W., Draper, E. S. & Marlow, N. (2009). The Cost of Preterm Birth throughout Childhood in England and Wales. *Pediatrics*, 123(2), e312–e327.
Mao, Y., Hoffman, T., Singh-Varma, A., Duan-Arnold, Y., Moorman, M., Danilkovitch, A. & Kohn, J. (2017). Antimicrobial Peptides Secreted from Human Cryopreserved Viable Amniotic Membrane Contribute to Its Antibacterial Activity. *Scientific Reports*, 7(1).
Marquet, M., Zöllkau, J., Pastuschek, J., Viehweger, A., Schleußner, E., Makarewicz, O., Pletz, M. W., Ehricht, R. & Brandt, C. (2022). Evaluation of Microbiome Enrichment and Host DNA Depletion in Human Vaginal Samples Using Oxford Nanopore's Adaptive Sequencing. *Scientific Reports*, 12(1), 1–10.
Martinez-Porchas, M., Villalpando-Canchola, E., Suarez, L. E. O. & Vargas-Albores, F. (2017). How Conserved Are the Conserved 16S-RRNA Regions? *PeerJ*, 2017(2), e3036.
Martínez-Reyes, C. P., Manjarrez-Reyna, A. N., Méndez-García, L. A., Aguayo-Guerrero, J. A., Aguirre-Sierra, B., Villalobos-Molina, R., López-Vidal, Y., Bobadilla, K. & Escobedo, G. (2020). Uric Acid Has Direct Proinflammatory Effects on Human Macrophages by Increasing Proinflammatory Mediators and Bacterial Phagocytosis Probably via URAT1. *Biomolecules*, 10(4), 576.
Martinez-Vidal, L., Murdica, V., Venegoni, C., Pederzoli, F., Bandini, M., Necchi, A., Salonia, A. & Alfano, M. (2021). Causal Contributors to Tissue Stiffness and Clinical Relevance in Urology. *Communications Biology*, 4(1).
Mathews, C. K. (2014). Deoxyribonucleotides as Genetic and Metabolic Regulators. *The FASEB Journal*, 28(9), 3832.
Mattei, V., Murugesan, S., Al Hashmi, M., Mathew, R., James, N., Singh, P., Kumar, M., Prasath Lakshmanan, A., Terranegra, A., Al Khodor, S. & Tomei, S. (2019). Evaluation of Methods for the Extraction of Microbial DNA from Vaginal Swabs Used for Microbiome Studies. *Frontiers in Cellular and Infection Microbiology*, 9(JUN), 197.
Mauri, A., Perrini, M., Ehret, A. E., De Focatiis, D. S. A. & Mazza, E. (2015). Time-Dependent Mechanical Behavior of Human Amnion: Macroscopic and Microscopic Characterization. *Acta Biomaterialia*, 11(1), 314–323.
Mazor, M., Hershkovitz, R., Ghezzi, F., Maymon, E., Horowitz, S. & Leiberman, J. R. (1996). Intraamniotic Infection in Patients with Preterm Labor and Twin Pregnancies. *Acta Obstetricia et Gynecologica Scandinavica*, 75(7), 624–627.
Mcelrath, T., Cantonwine, D. E., Jeyabalan, A., Doss, R., Page, G., Roberts, J. M., Brohman, B., Rosenblatt, K. P., Downes, K. L., Ravel, J., Gajer, P. & Elovitz, M. A. (2018). 15: Gardnerella Vaginalis and Spontaneous Preterm Birth: New Insights. *American Journal of Obstetrics & Gynecology*, 218(1), S12–S13.
McGregor, J. A., French, J. I., Lawellin, D., Franco-Buff, A., Smith, C. & Todd, J. K. (1987). Bacterial Protease-Induced Reduction of Chorioamniotic Membrane Strength and Elasticity. *Obstetrics and Gynecology*, 69(2), 167–74.
McGregor, J. A., French, J. I., Lawellin, D. & Todd, J. K. (1988). Editorial: Preterm Birth and Infection: Pathogenic Possibilities. *American Journal of Reproductive Immunology and Microbiology*, 16(3), 123–132.
McKenzie, R., Maarsingh, J. D., Łaniewski, P. & Herbst-Kralovetz, M. M. (2021). Immunometabolic Analysis of Mobiluncus Mulieris and Eggerthella Sp. Reveals Novel Insights Into Their Pathogenic Contributions to the Hallmarks of Bacterial Vaginosis. *Frontiers in Cellular and Infection Microbiology*, 11, 1320.
McLaren, J., Malak, T. M. & Bell, S. C. (1999). Structural Characteristics of Term Human Fetal Membranes Prior to Labour: Identification of an Area of Altered Morphology Overlying the Cervix. *Human Reproduction*, 14(1), 237–241.
McMillan, A., Rulisa, S., Sumarah, M., Macklaim, J. M., Renaud, J., Bisanz, J. E., Gloor, G. B. & Reid, G. (2015). A Multi-Platform Metabolomics Approach Identifies Highly Specific Biomarkers of Bacterial Diversity in the Vagina of Pregnant and Non-Pregnant Women. *Scientific Reports*, 5(1), 1–14.
McParland, P. C. & Bell, S. C. (2004). The Fetal Membranes and Mechanisms Underlying Their Labour-Associated and Pre-Labour Rupture during Pregnancy. *Fetal and Maternal Medicine Review*, 15(1), 73–108.
Mehta, O. et al. (2020). Vaginal Microbiome of Pregnant Indian Women: Insights into the Genome of Dominant Lactobacillus Species. *Microbial Ecology*, 80(2), 487–499.
Meinert, M., Malmström, A., Petersen, A. C., Eriksen, G. V. & Uldbjerg, N. (2014). Chorioamniontis in Preterm Delivery Is Associated with Degradation of Decorin and Biglycan and Depletion of Hyaluronan in Fetal Membranes. *Placenta*, 35(8), 546–551.
Menon, R. & Fortunato, S. J. (2004). Fetal Membrane Inflammatory Cytokines: A Switching Mechanism between the Preterm Premature Rupture of the Membranes and Preterm Labor Pathways. *Journal of Perinatal Medicine*, 32(5), 391–399.
Menon, R., Jones, J., Gunst, P. R., Kacerovsky, M., Fortunato, S. J., Saade, G. R. & Basraon, S. (2014). Amniotic Fluid Metabolomic Analysis in Spontaneous Preterm Birth. *Reproductive Sciences*, 21(6), 791–803.

Menon, R., Peltier, M. R., Eckardt, J. & Fortunato, S. J. (2009). Diversity in Cytokine Response to Bacteria Associated with Preterm Birth by Fetal Membranes. *American Journal of Obstetrics and Gynecology*, 201(3), 306.e1-306.e6.

Mercer, B. M. (2003). Preterm Premature Rupture of the Membranes. *The American College of Obstetricians and Gynecologists. Published by Wolters Kluwer Health, Inc*, 101(1), 178–193.

Mersha, T. B. & Abebe, T. (2015). Self-Reported Race/Ethnicity in the Age of Genomic Research: Its Potential Impact on Understanding Health Disparities. *Human Genomics*, 9(1), 1–15.

Miao, L., Nielsen, M., Thewalt, J., Ipsen, J. H., Bloom, M., Zuckermann, M. J. & Mouritsen, O. G. (2002). From Lanosterol to Cholesterol: Structural Evolution and Differential Effects on Lipid Bilayers. *Biophysical Journal*, 82(3), 1429–1444.

Millar, L. K., Stollberg, J., DeBuque, L. & Bryant-Greenwood, G. (2000). Fetal Membrane Distention: Determination of the Intrauterine Surface Area and Distention of the Fetal Membranes Preterm and at Term. *American Journal of Obstetrics and Gynecology*, 182(1 I), 128–134.

Miller, C. E., Lavery, J. P. & Donnelly, T. A. (1979). Determination of Elastic Parameters For Human Fetal Membranes. *Citation: Journal of Rheology*, 23, 57.

Mirmonsef, P., Zariffard, M. R., Gilbert, D., Makinde, H., Landay, A. L. & Spear, G. T. (2012). Short-Chain Fatty Acids Induce Pro-Inflammatory Cytokine Production Alone and in Combination with Toll-Like Receptor Ligands. *American Journal of Reproductive Immunology*, 67(5), 391–400.

Mirsaleh-Kohan, N., Robertson, W. D. & Compton, R. N. (2008). Electron Ionization Time-of-Flight Mass Spectrometry: Historical Review and Current Applications. *Mass Spectrometry Reviews*, 27(3), 237–285.

Mitchell, C. & Marrazzo, J. (2014). Bacterial Vaginosis and the Cervicovaginal Immune Response. *American Journal of Reproductive Immunology*, 71(6), 555–563.

Moloney, R. D., Desbonnet, L., Clarke, G., Dinan, T. G. & Cryan, J. F. (2014). The Microbiome: Stress, Health and Disease. *Mammalian Genome*, 25(1–2), 49–74.

Moncla, B. J., Chappell, C. A., Debo, B. M. & Meyn, L. A. (2016). The Effects of Hormones and Vaginal Microflora on the Glycome of the Female Genital Tract: Cervical-Vaginal Fluid. *PLoS ONE*, 11(7), e0158687.

Monsivais, L. A., Sheller-Miller, S., Russell, W., Saade, G. R., Dixon, C. L., Urrabaz-Garza, R. & Menon, R. (2020). Fetal Membrane Extracellular Vesicle Profiling Reveals Distinct Pathways Induced by Infection and Inflammation in Vitro. *American Journal of Reproductive Immunology*, 84(3), e13282.

Moore, R. M., Novak, J. B., Kumar, D., Mansour, J. M., Mercer, B. M. & Moore, J. J. (2009). Alpha-Lipoic Acid Inhibits Tumor Necrosis Factor-Induced Remodeling and Weakening of Human Fetal Membranes. *Biology of Reproduction*, 80(4), 781–787.

Moosa, Y., Kwon, D., de Oliveira, T. & Wong, E. B. (2020). Determinants of Vaginal Microbiota Composition. *Frontiers in Cellular and Infection Microbiology*, 10, 467.

Morales, S. E. & Holben, W. E. (2009). Empirical Testing of 16S RRNA Gene PCR Primer Pairs Reveals Variance in Target Specificity and Efficacy Not Suggested by in Silico Analysis. *Applied and Environmental Microbiology*, 75(9), 2677–2683.

Motedayyen, H., Esmaeil, N., Tajik, N., Khadem, F., Ghotloo, S., Khani, B. & Rezaei, A. (2017). Method and Key Points for Isolation of Human Amniotic Epithelial Cells with High Yield, Viability and Purity. *BMC Research Notes*, 10(1).

Murtha, A. P. & Edwards, J. M. (2014). The Role of Mycoplasma and Ureaplasma in Adverse Pregnancy Outcomes. *Obstetrics and Gynecology Clinics of North America*, 41(4), 615–627.

Muzny, C. A., Łaniewski, P., Schwebke, J. R. & Herbst-Kralovetz, M. M. (2020). Host-Vaginal Microbiota Interactions in the Pathogenesis of Bacterial Vaginosis. *Current Opinion in Infectious Diseases*, 33(1), 59–65.

Myllykallio, H., Skouloubris, S., Grosjean, H. & Liebl, U. (2009). Folate-Dependent Ihymidylate-Forming Enzymes: Parallels between DNA and RNA Metabolic Enzymes and Evolutionary Implications, in: *DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution*, (pp. 275–289). Landes Bioscience.

Nelson, D. B., Hanlon, A., Nachamkin, I., Haggerty, C., Mastrogiannis, D. S., Liu, C. & Fredricks, D. N. (2014). Early Pregnancy Changes in Bacterial Vaginosis-Associated Bacteria and Preterm Delivery. *Paediatric and Perinatal Epidemiology*, 28(2), 88–96.

Nelson, D., Shin, H., Wu, J. & Dominguez-Bello, M. (2016). The Gestational Vaginal Microbiome and Spontaneous Preterm Birth among Nulliparous African American Women. *American Journal of Perinatology*, 33(09), 887–893.

Neske, A., Ruiz Hidalgo, J., Cabedo, N. & Cortes, D. (2020). Acetogenins from Annonaceae Family. Their Potential Biological Applications. *Phytochemistry*, 174, 112332.

NICE. (2019). *Recommendations | Preterm Labour and Birth | Guidance | NICE*. NICE.

NIH. (2013). Periodontal (Gum) Disease: Causes, Symptoms, and Treatments. *National Institute of Dental and Craniofacial Research*, 13(1142), 1–12.

Nitto, T. & Onodera, K. (2013). *The Linkage between Coenzyme A Metabolism and Inflammation: Roles of Pantetheinase*. Elsevier.

Noda-Nicolau, N. M., Polettini, J., Peltier, M. R., da Silva, M. G. & Menon, R. (2016). Combinations and Loads of Bacteria Affect the Cytokine Production by Fetal Membranes: An in Vitro Study. *American Journal of Reproductive Immunology*, 76(6), 504–511.

Novy, M. J., Duffy, L., Axthelm, M. K., Sadowsky, D. W., Witkin, S. S., Gravett, M. G., Cassell, G. H. & Waites, K. B. (2009). Ureaplasma Parvum or Mycoplasma Hominis as Sole Pathogens Cause Chorioamnionitis, Preterm Delivery, and Fetal Pneumonia in Rhesus Macaques. *Reproductive Sciences*, 16(1), 56–70.

Nugent, R. P., Krohn, M. A. & Hillier, S. L. (1991). Reliability of Diagnosing Bacterial Vaginosis Is Improved by a Standardized Method of Gram Stain Interpretation. *Journal of clinical microbiology*, 29(2), 297–301.

O'Connor, A. R., Wilson, C. M. & Fielder, A. R. (2007). Ophthalmological Problems Associated with Preterm Birth. *Eye*, 21(10), 1254–1260.

O'Hanlon, D. E., Lanier, B. R., Moench, T. R. & Cone, R. A. (2010). Cervicovaginal Fluid and Semen Block the Microbicidal Activity of Hydrogen Peroxide Produced by Vaginal Lactobacilli. *BMC Infectious Diseases*, 10(1), 120.

O'Hanlon, D. E., Moench, T. R. & Cone, R. A. (2011). In Vaginal Fluid, Bacteria Associated with Bacterial Vaginosis Can Be Suppressed with Lactic Acid but Not Hydrogen Peroxide. *BMC Infectious Diseases*, 11(1), 200.

O'Hanlon, D. E., Moench, T. R. & Cone, R. A. (2013). Vaginal PH and Microbicidal Lactic Acid When Lactobacilli Dominate the Microbiota (A. Landay, Ed.). *PLoS ONE*, 8(11), e80074.

Odogwu, N. M., Chen, J., Onebunne, C. A., Jeraldo, P., Yang, L., Johnson, S., Ayeni, F. A., Walther-Antonio, M. R. S., Olayemi, O. O., Chia, N. & Omigbodun, A. O. (2021). Predominance of Atopobium Vaginae at Midtrimester: A Potential Indicator of Preterm Birth Risk in a Nigerian Cohort. *mSphere*, 6(1).

Odogwu, N. M., Onebunne, C. A., Chen, J., Ayeni, F. A., Walther-Antonio, M. R. S., Olayemi, O. O., Chia, N. & Omigbodun, A. O. (2021). Lactobacillus Crispatus Thrives in Pregnancy Hormonal Milieu in a Nigerian Patient Cohort. *Scientific Reports*, 11(1), 1–19.

Oduah, E. I., Linhardt, R. J. & Sharfstein, S. T. (2016). Heparin: Past, Present, and Future. *Pharmaceuticals*, 9(3), 38.

Oh, K. J., Romero, R., Park, J. Y., Kang, J., Hong, J. S. & Yoon, B. H. (2019). A High Concentration of Fetal Fibronectin in Cervical Secretions Increases the Risk of Intra-Amniotic Infection and Inflammation in Patients with Preterm Labor and Intact Membranes. *Journal of Perinatal Medicine*, 47(3), 288–303.

Olson, S., Berg, K., Bonham, V., Boyer, J., Brody, L., Brooks, L., Collins, F., Guttmacher, A., McEwen, J., Muenke, M., Olson, S., Wang, V. O., Rodriguez, L. L., Vydelingum, N. & Warshauer-Baker, E. (2005). The Use of Racial, Ethnic, and Ancestral Categories in Human Genetics Research. *American Journal of Human Genetics*, 77(4), 519–532.

Onderdonk, A. B., Delaney, M. L. & Fichorova, R. N. (2016). The Human Microbiome during Bacterial Vaginosis. *Clinical Microbiology Reviews*, 29(2), 223–238.

Osman, I., Young, A., Ledingham, M. A., Thomson, A. J., Jordan, F., Greer, I. A. & Norman, J. E. (2003). Leukocyte Density and Pro-Inflammatory Cytokine Expression in Human Fetal Membranes, Decidua, Cervix and Myometrium before and during Labour at Term. *Molecular Human Reproduction*, 9(1), 41–45.

Ouseph, M. M., Krigman, H. & He, M. (2019). Streptococcus Pneumoniae–An Uncommon but Noteworthy Cause of Intrauterine Fetal Demise and Acute Necrotizing Funisitis. *Fetal and Pediatric Pathology*, 38(4), 352–358.

Overy, S. A., Walker, H. J., Malone, S., Howard, T. P., Baxter, C. J., Sweetlove, L. J., Hill, S. A. & Quick, W. P. (2005). Application of Metabolite Profiling to the Identification of Traits in a Population of Tomato Introgression Lines. *Journal of Experimental Botany*, 56(410), 287–296.

Oyarzun, E., Yamamoto, M., Kato, S., Gomez, R., Lizama, L. & Moenne, A. (1998). Specific Detection of 16 Micro-Organisms in Amniotic Fluid by Polymerase Chain Reaction and Its Correlation with Preterm Delivery Occurrence. *American Journal of Obstetrics and Gynecology*, 179(5), 1115–1119.

Oyen, M. L., Cook, R. F. & Calvin, S. E. (2004). Mechanical Failure of Human Fetal Membrane Tissues. *Journal of Materials Science: Materials in Medicine*, 15(6), 651–658.

Padron, J. G., Saito Reis, C. A. & Kendal-Wright, C. E. (2020). The Role of Danger Associated Molecular Patterns in Human Fetal Membrane Weakening. *Frontiers in Physiology*, 11, 602.

Pandey, V., Jaremko, K., Moore, R. M., Mercer, B. M., Stetzer, B., Kumar, D., Fox, J. M., Mansour, J. M. & Moore, J. J. (2007). The Force Required to Rupture Fetal Membranes Paradoxically Increases with Acute in Vitro Repeated Stretching. *American Journal of Obstetrics and Gynecology*, 196(2), 165.e1-165.e7.

Park, S., You, Y.-A., Kim, Y.-H., Kwon, E., Ansari, A., Kim, S. M., Lee, G., Hur, Y. M., Jung, Y. J., Kim, K. & Kim, Y. J.

(2022). Ureaplasma and Prevotella Colonization with Lactobacillus Abundance during Pregnancy Facilitates Term Birth. *Scientific Reports*, 12(1).

Parnell, L. A., Briggs, C. M. & Mysorekar, I. U. (2017). *Maternal Microbiomes in Preterm Birth: Recent Progress and Analytical Pipelines*. W.B. Saunders.

Passos, M., Jayaram, A. & Witkin, S. S. (2011). Ureaplasma Urealyticum and Mycoplasma Hominis in Preterm Labor. *J bras Doenças Sex Transm*, 23(3), 138–141.

Payne, M. S., Newnham, J. P., Doherty, D. A., Furfaro, L. L., Pendal, N. L., Loh, D. E. & Keelan, J. A. (2021). A Specific Bacterial DNA Signature in the Vagina of Australian Women in Midpregnancy Predicts High Risk of Spontaneous Preterm Birth (the Predict1000 Study). *American Journal of Obstetrics and Gynecology*, 224(2), 206.e1-206.e23.

Pearlman, M. & Almandoz, J. (2020). *Digestion and Absorption of Micronutrients*.

Peebles, K., Velloza, J., Balkus, J. E., McClelland, R. S. & Barnabas, R. V. (2019). High Global Burden and Costs of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *Sexually Transmitted Diseases*, 46(5), 304–311.

Peelen, M. J., Luef, B. M., Lamont, R. F., de Milliano, I., Jensen, J. S., Limpens, J., Hajenius, P. J., Jørgensen, J. S. & Menon, R. (2019). The Influence of the Vaginal Microbiota on Preterm Birth: A Systematic Review and Recommendations for a Minimum Dataset for Future Research. *Placenta*, 79, 30–39.

Peltier, M. R. (2003). Immunology of Term and Preterm Labor. *Reproductive biology and endocrinology : RB&E*, 1(1), 122.

Perrini, M., Bürzle, W., Haller, C., Ochsenbein-Kölble, N., Deprest, J., Zimmermann, R., Mazza, E. & Ehrbar, M. (2013). Contractions, a Risk for Premature Rupture of Fetal Membranes: A New Protocol with Cyclic Biaxial Tension. *Medical Engineering and Physics*, 35(6), 846–851.

Petricevic, L., Domig, K. J., Nierscher, F. J., Sandhofer, M. J., Fidesser, M., Krondorfer, I., Husslein, P., Kneifel, W. & Kiss, H. (2014). Characterisation of the Vaginal Lactobacillus Microbiota Associated with Preterm Delivery. *Scientific Reports*, 4(1), 5136.

Petrova, M. I., Lievens, E., Malik, S., Imholz, N. & Lebeer, S. (2015). Lactobacillus Species as Biomarkers and Agents That Can Promote Various Aspects of Vaginal Health. *Frontiers in Physiology*, 6(MAR), 81.

Petrova, M. I., Reid, G., Vaneechoutte, M. & Lebeer, S. (2017). Lactobacillus Iners: Friend or Foe? *Trends in Microbiology*, 25(3), 182–191.

Petrunin, D. D., Gryaznova, I. M., Petrunina, Y. A. & Tatarinov, Y. S. (1976). Immunochemical Identification of Organ-Specific Human Placental Ain2-Globulin and Its Content in the Amniotic Fluid. *Bulletin of Experimental Biology and Medicine*, 82(1), 994–996.

Pirjani, R., Moini, A., Almasi-Hashiani, A., Farid Mojtahedi, M., Vesali, S., Hosseini, L. & Sepidarkish, M. (2021). Placental Alpha Microglobulin-1 (PartoSure) Test for the Prediction of Preterm Birth: A Systematic Review and Meta-Analysis. *Journal of Maternal-Fetal and Neonatal Medicine*, 34(20), 3445–3457.

Plummer, E. L., Vodstrcil, L. A., Doyle, M., Danielewski, J. A., Murray, G. L., Fehler, G., Fairley, C. K., Bulach, D. M., Garland, S. M., Chow, E. P. F., Hocking, J. S. & Bradshaw, C. S. (2021). A Prospective, Open-Label Pilot Study of Concurrent Male Partner Treatment for Bacterial Vaginosis. *mBio*, 12(5).

Van Der Pol, W. J., Kumar, R., Morrow, C. D., Blanchard, E. E., Taylor, C. M., Martin, D. H., Lefkowitz, E. J. & Muzny, C. A. (2019). In Silico and Experimental Evaluation of Primer Sets for Species-Level Resolution of the Vaginal Microbiota Using 16S Ribosomal RNA Gene Sequencing. *The Journal of Infectious Diseases*, 219(2), 305–314.

Potts, L. C., Feng, L., Seed, P. C., Jayes, F. L., Kuchibhatla, M., Antczak, B., Nazzal, M. K. & Murtha, A. P. (2016). Inflammatory Response of Human Gestational Membranes to Ureaplasma Parvum Using a Novel Dual-Chamber Tissue Explant System. *Biology of Reproduction*, 94(5).

Pressman, E. K., Cavanaugh, J. L. & Woods, J. R. (2002). Physical Properties of the Chorioamnion throughout Gestation. *American Journal of Obstetrics and Gynecology*, 187(3), 672–675.

Prevost, T. P., Suresh, S., House, M. D. & Socrate, S. (2006). Biomechanics of the Human Chorioamnion. *Journal of Biomechanics*, 39, S343.

Püschl, I. C., Bonde, L., Reading, I. C., Maguire, P., Macklon, N. S. & Van Rijn, B. B. (2020). Salivary Uric Acid as a Predictive Test of Preeclampsia, Pregnancy-Induced Hypertension and Preterm Delivery: A Pilot Study. *Acta Obstetricia et Gynecologica Scandinavica*, 99(10), 1339–1345.

Quetglas-Llabrés, M. M. et al. (2022). Pharmacological Properties of Bergapten: Mechanistic and Therapeutic Aspects. *Oxidative Medicine and Cellular Longevity*, 2022.

Quinto, E. J., Jiménez, P., Caro, I., Tejero, J., Mateo, J. & Girbés, T. (2014). Probiotic Lactic Acid Bacteria: A Review. *Food and Nutrition Sciences*, 05(18), 1765–1775.

Raba, G. & Tabarkiewicz, J. (2018). Cytokines in Preterm Delivery: Proposal of a New Diagnostic Algorithm.

Journal of Immunology Research, 2018.

Radan, A. P., Aleksandra Polowy, J., Heverhagen, A., Simillion, C., Baumann, M., Raio, L., Schleussner, E., Mueller, M. & Surbek, D. (2020). Cervico-Vaginal Placental α-Macroglobulin-1 Combined with Cervical Length for the Prediction of Preterm Birth in Women with Threatened Preterm Labor. *Acta Obstetricia et Gynecologica Scandinavica*, 99(3), 357–363.

Rangaswamy, N., Kumar, D., Moore, R. M., Mercer, B. M., Mansour, J. M., Redline, R. & Moore, J. J. (2012). Weakening and Rupture of Human Fetal Membranes – Biochemistry and Biomechanics, in: *Preterm Birth - Mother and Child*, (pp. 151–182). InTech.

Rasheed, Z., Akhtar, N., Khan, A., Khan, K. A. & Haqqi, T. M. (2010). Butrin, Isobutrin, and Butein from Medicinal Plant Butea Monosperma Selectively Inhibit Nuclear Factor-KB in Activated Human Mast Cells: Suppression of Tumor Necrosis Factor-α, Interleukin (IL)-6, and IL-8. *Journal of Pharmacology and Experimental Therapeutics*, 333(2), 354–363.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L. & Forney, L. J. (2011). Vaginal Microbiome of Reproductive-Age Women. *Proceedings of the National Academy of Sciences*, 108(Supplement_1), 4680–4687.

Reisenberger, K., Egarter, C., Schiebel, I., Obermair, A., Kiss, H. & Lehner, R. (1997). In Vitro Cytokine and Prostaglandin Production by Amnion Cells in the Presence of Bacteria. *American Journal of Obstetrics and Gynecology*, 176(5), 981–984.

Ren, J., Yue, B., Wang, H., Zhang, B., Luo, X., Yu, Z., Zhang, J., Ren, Y., Mani, S., Wang, Z. & Dou, W. (2021). Acacetin Ameliorates Experimental Colitis in Mice via Inhibiting Macrophage Inflammatory Response and Regulating the Composition of Gut Microbiota. *Frontiers in Physiology*, 11, 1858.

Ries, C. (2014). Cytokine Functions of TIMP-1. *Cellular and Molecular Life Sciences*, 71(4), 659–672.

Riley, P. S. & Weaver, R. E. (1977). Comparison of Thirty Seven Strains of Vd 3 Bacteria with Agrobacterium Radiobacter: Morphological and Physiological Observations. *Journal of Clinical Microbiology*, 5(2), 172–177.

Ringborg, A., Berg, J., Norman, M., Westgren, M. & Jönsson, B. (2006). Preterm Birth in Sweden: What Are the Average Lengths of Hospital Stay and the Associated Inpatient Costs? *Acta Paediatrica, International Journal of Paediatrics*, 95(12), 1550–1555.

Rodríguez-Pérez, H., Ciuffreda, L. & Flores, C. (2021). NanoCLUST: A Species-Level Analysis of 16S RRNA Nanopore Sequencing Data. *Bioinformatics (Oxford, England)*, 37(11), 1600–1601.

Romero, R. & Chaiworapongsa, T. (2002). Preterm Labor, Intrauterine Infection, and the Fetal Inflammatory Response Syndrome. *American Academy of Pediatrics*, 3(5), 73–85.

Romero, R., Chaiworapongsa, T., Alpay Savasan, Z., Xu, Y., Hussein, Y., Dong, Z., Kusanovic, J. P., Kim, C. J. & Hassan, S. S. (2011). Damage-Associated Molecular Patterns (DAMPs) in Preterm Labor with Intact Membranes and Preterm PROM: A Study of the Alarmin HMGB1. *Journal of Maternal-Fetal and Neonatal Medicine*, 24(12), 1444–1455.

Romero, R., Dey, S. K. & Fisher, S. J. (2014). Preterm Labor: One Syndrome, Many Causes. *Science*, 345(6198), 760–765.

Romero, R., Gomez-Lopez, N., Winters, A. D., Jung, E., Shaman, M., Bieda, J., Panaitescu, B., Pacora, P., Erez, O., Greenberg, J. M., Ahmad, M. M., Hsu, C. D. & Theis, K. R. (2019). Evidence That Intra-Amniotic Infections Are Often the Result of an Ascending Invasion - A Molecular Microbiological Study. *Journal of Perinatal Medicine*, 47(9), 915–931.

Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Bieda, J., Chaemsaithong, P., Miranda, J., Chaiworapongsa, T. & Ravel, J. (2014). The Vaginal Microbiota of Pregnant Women Who Subsequently Have Spontaneous Preterm Labor and Delivery and Those with a Normal Delivery at Term. *Microbiome*, 2(1), 1–15.

Romero, R., Kusanovic, J. P., Espinoza, J., Gotsch, F., Nhan-Chang, C. L., Erez, O., Kim, C. J., Khalek, N., Mittal, P., Goncalves, L. F., Schaudinn, C., Hassan, S. S. & Costerton, J. W. (2007). What Is Amniotic Fluid 'Sludge'? *Ultrasound in Obstetrics and Gynecology*, 30(5), 793–798.

Romero, R., Mazaki-Tovi, S., Vaisbuch, E., Kusanovic, J. P., Chaiworapongsa, T., Gomez, R., Nien, J. K., Yoon, B. H., Mazor, M., Luo, J., Banks, D., Ryals, J. & Beecher, C. (2010). Metabolomics in Premature Labor: A Novel Approach to Identify Patients at Risk for Preterm Delivery. *Journal of Maternal-Fetal and Neonatal Medicine*, 23(12), 1344–1359.

Romero, R., Sirtori, M., Oyarzun, E., Avila, C., Mazor, M., Callahan, R., Sabo, V., Athanassiadis, A. P. & Hobbins, J. C. (1989). Infection and Labor. V. Prevalence, Microbiology, and Clinical Significance of Intraamniotic Infection in Women with Preterm Labor and Intact Membranes. *American Journal of Obstetrics and*

Gynecology, 161(3), 817–824.
Rosca, A. S., Castro, J., Sousa, L. G. V., França, A., Vaneechoutte, M. & Cerca, N. (2022). In Vitro Interactions within a Biofilm Containing Three Species Found in Bacterial Vaginosis (BV) Support the Higher Antimicrobial Tolerance Associated with BV Recurrence. *Journal of Antimicrobial Chemotherapy*, 77(8), 2183–2190.
Ruma, M. S., Bittner, K. C. & Soh, C. B. (2017). Current Perspectives on the Use of Fetal Fibronectin Testing in Preterm Labor Diagnosis and Management. *The American journal of managed care*, 23(19), S356–S362.
Salsoso, R., Farías, M., Gutiérrez, J., Pardo, F., Chiarello, D. I., Toledo, F., Leiva, A., Mate, A., Vázquez, C. M. & Sobrevia, L. (2017). Adenosine and Preeclampsia. *Molecular Aspects of Medicine*, 55, 126–139.
Sandhir, R. (2014). Metabolic Pathways: Lipid Metabolism, in: *Encyclopedia of Food Microbiology: Second Edition*, (pp. 520–534). Academic Press.
Sandu, C., Folescu, R., Pop, E. & Motoc, A. G. M. (2013). Hematogenous Placental Infection in Acute Respiratory Infections. *Romanian Journal of Morphology and Embryology*, 54(1), 157–161.
Saunders, S., Bocking, A., Challis, J. & Reid, G. (2007). Effect of Lactobacillus Challenge on Gardnerella Vaginalis Biofilms. *Colloids and Surfaces B: Biointerfaces*, 55(2), 138–142.
Sbarra, A. J., Thomas, G. B. & Cetrulo, C. L. (1987). Effect of Bacterial Growth on the Bursting Pressure of Fetal Membranes in Vitro. *Obstetrics and Gynecology*, 70(1), 107–110.
Schinkovitz, A., Gibbons, S., Stavri, M., Cocksedge, M. J. & Bucar, F. (2003). Ostruthin: An Antimycobacterial Coumarin from the Roots of Peucedanum Ostruthium. *Planta Medica*, 69(4), 369–371.
Schmitz, T. (2016). Prévention Des Complications de La Prématurité Par l'administration Anténatale de Corticoïdes. *Journal de Gynecologie Obstetrique et Biologie de la Reproduction*, 45(10), 1399–1417.
Schober, E. A., Kusy, R. P. & Savitz, D. A. (1994). Resistance of Fetal Membranes to Concentrated Force Applications and Reconciliation of Puncture and Burst Testing. *Annals of Biomedical Engineering*, 22(5), 540–548.
Schober, E. A., Kusy, R. P., Whitley, J. Q. & Savitz, D. A. (1994). Effect of Thickness on the Fracture Characteristics of Fetal Membranes. *Journal of Materials Science: Materials in Medicine*, 5(3), 130–137.
Schoonmaker, J. N., Lawellin, D. W., Lunt, B. & McGregor, J. A. (1989). Bacteria and Inflammatory Cells Reduce Chorioamniotic Membrane Integrity and Tensile Strength. *Obstetrics and gynecology*, 74(4), 590–6.
Seeger, C., Poulsen, C. & Dandanell, G. (1995). Identification and Characterization of Genes (XapA, XapB, and XapR) Involved in Xanthosine Catabolism in Escherichia Coli. *Journal of Bacteriology*, 177(19), 5506–5516.
Sennstrom, M. B. (2000). Human Cervical Ripening, an Inflammatory Process Mediated by Cytokines. *Molecular Human Reproduction*, 6(4), 375–381.
Shannon, C. E. (1948). A Mathematical Theory of Communication. *Bell System Technical Journal*, 27(3), 379–423.
Sharma, N., Angural, S., Rana, M., Puri, N., Kondepudi, K. K. & Gupta, N. (2020). Phytase Producing Lactic Acid Bacteria: Cell Factories for Enhancing Micronutrient Bioavailability of Phytate Rich Foods. *Trends in Food Science & Technology*, 96, 1–12.
Sharma, R., Maimanuku, L. R., Morse, Z. & Pack, A. R. (2007). Preterm Low Birth Weights Associated with Periodontal Disease in the Fiji Islands. *International Dental Journal*, 57(4), 257–260.
Shimaoka, M., Yo, Y., Doh, K., Kotani, Y., Suzuki, A., Tsuji, I., Mandai, M. & Matsumura, N. (2019). Association between Preterm Delivery and Bacterial Vaginosis with or without Treatment. *Scientific Reports*, 9(1).
Shiozaki, A., Yoneda, S., Yoneda, N., Yonezawa, R., Matsubayashi, T., Seo, G. & Saito, S. (2014). Intestinal Microbiota Is Different in Women with Preterm Birth: Results from Terminal Restriction Fragment Length Polymorphism Analysis (A. C. Zenclussen, Ed.). *PLoS ONE*, 9(11), e111374.
Shirzad, M., Hamedi, J., Motevaseli, E. & Modarressi, M. H. (2018). Anti-Elastase and Anti-Collagenase Potential of Lactobacilli Exopolysaccharides on Human Fibroblast. *Artificial Cells, Nanomedicine and Biotechnology*, 46(sup1), 1051–1061.
Short, C. S., Quinlan, R., Bennett, P., Shattock, R. J. & Taylor, G. P. (2018). Optimising the Collection of Female Genital Tract Fluid for Cytokine Analysis in Pregnant Women. *Journal of Immunological Methods*, 458, 15–20.
Shreffler, J. & Huecker, M. R. (2020). *Diagnostic Testing Accuracy: Sensitivity, Specificity, Predictive Values and Likelihood Ratios*. StatPearls Publishing.
Silva, M. G., Peraçoli, J. C., Sadatsune, T., Abreu, E. S. & Peraçoli, M. T. S. (2003). Cervical Lactobacillus and Leukocyte Infiltration in Preterm Premature Rupture of Membranes. *International Journal of Gynecology and Obstetrics*, 81(2), 175–182.
Simhan, H. N. & Canavan, T. P. (2005). Preterm Premature Rupture of Membranes: Diagnosis, Evaluation and Management Strategies. *BJOG: An International Journal of Obstetrics and Gynaecology*, 112(SUPPL. 1), 32–37.

Simpson, K. L., Keelan, J. A. & Mitchell, M. D. (1999). Labour-Associated Changes in the Regulation of Production of Immunomodulators in Human Amnion by Glucocorticoids, Bacterial Lipopolysaccharide and pro-Inflammatory Cytokines. *Journal of Reproduction and Fertility*, 166(1), 321–327.

Sirichoat, A., Sankuntaw, N., Engchanil, C., Buppasiri, P., Faksri, K., Namwat, W., Chantratita, W. & Lulitanond, V. (2021). Comparison of Different Hypervariable Regions of 16S RRNA for Taxonomic Profiling of Vaginal Microbiota Using Next-Generation Sequencing. *Archives of Microbiology*, 203(3), 1159–1166.

Skalicka-Wozniak, K., Budzynska, B., Biala, G. & Boguszewska-Czubara, A. (2018). Scopolamine-Induced Memory Impairment Is Alleviated by Xanthotoxin: Role of Acetylcholinesterase and Oxidative Stress Processes. *ACS Chemical Neuroscience*, 9(5), 1184–1194.

Slaoui, A., Benmouna, I., Zeraidi, N., Lakhdar, A., Kharbach, A. & Baydada, A. (2022). Lactococcus Lactis Cremoris Intra-Uterine Infection: About an Uncommon Case Report. *International Journal of Surgery Case Reports*, 94, 107077.

Smith, B. C., McAndrew, T., Chen, Z., Harari, A., Barris, D. M., Viswanathan, S., Rodriguez, A. C., Castle, P., Herrero, R., Schiffman, M. & Burk, R. D. (2012). The Cervical Microbiome over 7 Years and a Comparison of Methodologies for Its Characterization (A. J. Ratner, Ed.). *PLoS ONE*, 7(7), e40425.

Smith, S. B. & Ravel, J. (2017). The Vaginal Microbiota, Host Defence and Reproductive Physiology. , 595(2), 451–463.

Soares, G. C. M. T., da Silva, B. A., dos Santos, M. H. B., da Costa, A. F. E., dos Santos, A. L. S., Morandi, V. & Nagao, P. E. (2008). Metallopeptidases Produced by Group B Streptococcus: Influence of Proteolytic Inhibitors on Growth and on Interaction with Human Cell Lineages. *International Journal of Molecular Medicine*, 22(1), 119–125.

Soleimani, F., Zaheri, F. & Abdi, F. (2014). Long-Term Neurodevelopmental Outcome s After Preterm Birth. *Iranian Red Crescent Medical Journal*, 16(6), e17965.

Song, J. L., Harry, J. B., Eastman, R. T., Oliver, B. G. & White, T. C. (2004). The Candida Albicans Lanosterol 14-α-Demethylase (ERG11) Gene Promoter Is Maximally Induced after Prolonged Growth with Antifungal Drugs. *Antimicrobial Agents and Chemotherapy*, 48(4), 1136–1144.

Sotiriadis, A., Papatheodorou, S., Kavvadias, A. & Makrydimas, G. (2010). Transvaginal Cervical Length Measurement for Prediction of Preterm Birth in Women with Threatened Preterm Labor: A Meta-Analysis. *Ultrasound in Obstetrics and Gynecology*, 35(1), 54–64.

Spaans, F., De Vos, P., Bakker, W. W., Van Goor, H. & Faas, M. M. (2014). Danger Signals from ATP and Adenosine in Pregnancy and Preeclampsia. *Hypertension*, 63(6), 1154–1160.

Srinivasan, S. & Fredricks, D. N. (2008). The Human Vaginal Bacterial Biota and Bacterial Vaginosis. *Interdisciplinary Perspectives on Infectious Diseases*, 2008(22), 1–22.

Srinivasan, S., Morgan, M. T., Fiedler, T. L., Djukovic, D., Hoffman, N. G., Raftery, D., Marrazzo, J. M. & Fredricks, D. N. (2015). Metabolic Signatures of Bacterial Vaginosis. *mBio*, 6(2), 1–16.

Stafford, G. P., Parker, J. L., Amabebe, E., Kistler, J., Reynolds, S., Stern, V., Paley, M. & Anumba, D. O. C. (2017). Spontaneous Preterm Birth Is Associated with Differential Expression of Vaginal Metabolites by Lactobacilli-Dominated Microflora. *Frontiers in Physiology*, 8(AUG), 615.

Steel, J. H., Malatos, S., Kennea, N., Edwards, A. D., Miles, L., Duggan, P., Reynolds, P. R., Feldman, R. G. & Sullivan, M. H. F. (2005). Bacteria and Inflammatory Cells in Fetal Membranes Do Not Always Cause Preterm Labor. *Pediatric Research*, 57(3), 404–411.

Štimac, M., Juretić, E., Vukelić, V., Matasić, N. P., Kos, M. & Babić, D. (2014). Effect of Chorioamnionitis on Mortality, Early Onset Neonatal Sepsis and Bronchopulmonary Dysplasia in Preterm Neonates with Birth Weight of ≤ 1,500 Grams. *Collegium Antropologicum*, 38(1), 167–171.

Stout, M. J., Zhou, Y., Wylie, K. M., Tarr, P. I., Macones, G. A. & Tuuli, M. G. (2017). Early Pregnancy Vaginal Microbiome Trends and Preterm Birth. *American Journal of Obstetrics and Gynecology*, 217(3), 356.e1-356.e18.

Strauss, J. F. (2013). Extracellular Matrix Dynamics and Fetal Membrane Rupture. *Reproductive Sciences*, 20(2), 140–153.

Subramaniam, A., Kumar, R., Cliver, S. P., Zhi, D., Szychowski, J. M., Abramovici, A., Biggio, J. R., Lefkowitz, E. J., Morrow, C. & Edwards, R. K. (2016). Vaginal Microbiota in Pregnancy: Evaluation Based on Vaginal Flora, Birth Outcome, and Race. *American Journal of Perinatology*, 33(4), 401–408.

Sweeney, E. L., Dando, S. J., Kallapur, S. G. & Knox, C. L. (2017). The Human Ureaplasma Species as Causative Agents of Chorioamnionitis. *Clinical Microbiology Reviews*, 30(1), 349–379.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., Lochs, H. & Verstraelen, H. (2008). An Adherent Gardnerella Vaginalis Biofilm Persists on the Vaginal Epithelium after Standard Therapy with Oral Metronidazole. *American Journal of Obstetrics and Gynecology*, 198(1), 97.e1-97.e6.

Tabatabaei, M., Mosaffa, N., Nikoo, S., Bozorgmehr, M., Ghods, R., Kazemnejad, S., Rezania, S., Keshavarzi, B., Arefi, S., Ramezani-Tehrani, F., Mirzadegan, E. & Zarnani, A. H. (2014). Isolation and Partial Characterization of Human Amniotic Epithelial Cells: The Effect of Trypsin. *Avicenna Journal of Medical Biotechnology*, 6(1), 10–20.

Tabatabaei, N., Eren, A. M., Barreiro, L. B., Yotova, V., Dumaine, A., Allard, C. & Fraser, W. D. (2019). Vaginal Microbiome in Early Pregnancy and Subsequent Risk of Spontaneous Preterm Birth: A Case–Control Study. *BJOG: An International Journal of Obstetrics and Gynaecology*, 126(3), 349–358.

Tadamitsu, K., Tetsuya, T. & Shizuo, A. (1994). Cytokine Signal Transduction. *Cell*, 76(2), 253–262.

Talarek, S., Listos, J., Barreca, D., Tellone, E., Sureda, A., Nabavi, S. F., Braidy, N. & Nabavi, S. M. (2017). Neuroprotective Effects of Honokiol: From Chemistry to Medicine. *BioFactors*, 43(6), 760–769.

Tano, S., Ueno, T., Mayama, M., Yamada, T., Takeda, T., Uno, K., Yoshihara, M., Ukai, M., Suzuki, T., Kishigami, Y. & Oguchi, H. (2021). Relationship between Vaginal Group B Streptococcus Colonization in the Early Stage of Pregnancy and Preterm Birth: A Retrospective Cohort Study. *BMC Pregnancy and Childbirth*, 21(1), 1–9.

Tehrani, F. A., Ahmadiani, A. & Niknejad, H. (2013). The Effects of Preservation Procedures on Antibacterial Property of Amniotic Membrane. *Cryobiology*, 67(3), 293–298.

Tency, I., Verstraelen, H., Kroes, I., Holtappels, G., Verhasselt, B., Vaneechoutte, M., Verhelst, R. & Temmerman, M. (2012). Imbalances between Matrix Metalloproteinases (MMPs) and Tissue Inhibitor of Metalloproteinases (TIMPs) in Maternal Serum during Preterm Labor. *PLoS ONE*, 7(11).

Thomas, M. M., Sulek, K., McKenzie, E. J., Jones, B., Han, T. L., Villas-Boas, S. G., Kenny, L. C., McCowan, L. M. E. & Baker, P. N. (2015). Metabolite Profile of Cervicovaginal Fluids from Early Pregnancy Is Not Predictive of Spontaneous Preterm Birth. *International Journal of Molecular Sciences*, 16(11), 27741–27748.

Thorne-Lyman, A. L. & Fawzi, W. W. (2012). Vitamin A and Carotenoids during Pregnancy and Maternal, Neonatal and Infant Health Outcomes: A Systematic Review and Meta-Analysis. *Paediatric and Perinatal Epidemiology*, 26(SUPPL. 1), 36–54.

Tita, A. T. N. & Andrews, W. W. (2010). Diagnosis and Management of Clinical Chorioamnionitis. *Clinics in Perinatology*, 37(2), 339–354.

Tiwari, P., Jain, R., Kumar, K., Mishra, R. & Chandy, A. (2012). Antibacterial Activity and Physicochemical Evaluation of Roots of Butea Monosperma. *Asian Pacific Journal of Tropical Biomedicine*, 2(2 SUPPL.), S881–S883.

Tiwari, S. & Beriha, S. S. (2015). Primary Bacteremia Caused by Rhizobium Radiobacter in Neonate: A Rare Case Report. *Journal of Clinical and Diagnostic Research*, 9(10), DD01–DD02.

Tokpohozin, S. E., Fischer, S., Sacher, B. & Becker, T. (2016). β-d-Glucosidase as "Key Enzyme" for Sorghum Cyanogenic Glucoside (Dhurrin) Removal and Beer Bioflavouring. *Food and Chemical Toxicology*, 97, 217–223.

Tommiska, V., Tuominen, R. & Fellman, V. (2003). Economic Costs of Care in Extremely Low Birthweight Infants during the First 2 Years of Life. *Pediatric Critical Care Medicine*, 4(2), 157–163.

Trufelli, H., Palma, P., Famiglini, G. & Cappiello, A. (2011). An Overview of Matrix Effects in Liquid Chromatography–Mass Spectrometry. *Mass Spectrometry Reviews*, 30(3), 491–509.

Uchide, N., Ohyama, K., Bessho, T., Takeichi, M. & Toyoda, H. (2012). Possible Roles of Proinflammatory and Chemoattractive Cytokines Produced by Human Fetal Membrane Cells in the Pathology of Adverse Pregnancy Outcomes Associated with Influenza Virus Infection. *Mediators of Inflammation*, 2012(1), 32.

Usman, F., Manzoor, Saeqah, Maqsood, S., Arif, A., Hijazi, A. & Manzoor, Sumaira. (2021). Hyperuricemia as a Risk Factor for Preterm Delivery in Women with Pre-Eclampsia. *Pakistan Journal of Medical and Health Sciences*, 15(11), 3157–3158.

Usui, R., Ohkuchi, A., Matsubara, S., Izumi, A., Watanabe, T., Suzuki, M. & Minakami, H. (2002). Vaginal Lactobacilli and Preterm Birth. *Journal of Perinatal Medicine*, 30(6), 458–466.

Vadillo-Ortega, F., Hernandez, A., Gonzalez-Avila, G., Bermejo, L., Iwata, K. & Strauss, J. F. (1996). Increased Matrix Metalloproteinase Activity and Reduced Tissue Inhibitor of Metalloproteinases-1 Levels in Amniotic Fluids from Pregnancies Complicated by Premature Rupture of Membranes. *American Journal of Obstetrics and Gynecology*, 174(4), 1371–1376.

Vanderhoeven, J. P., Bierle, C. J., Kapur, R. P., McAdams, R. M., Beyer, R. P., Bammler, T. K., Farin, F. M., Bansal, A., Spencer, M., Deng, M., Gravett, M. G., Rubens, C. E., Rajagopal, L., Adams Waldorf, K. M., Waldorf, K. M. A. & Sullam, P. M. (2014). Group B Streptococcal Infection of the Choriodecidua Induces Dysfunction of the Cytokeratin Network in Amniotic Epithelium: A Pathway to Membrane Weakening. *PLoS Pathogens*, 10(3), e1003920.

Vandooren, J. (2011). Gelatin Degradation Assay Reveals MMP-9 Inhibitors and Function of O-Glycosylated Domain. *World Journal of Biological Chemistry*, 2(1), 14.

Vannuccini, S., Bocchi, C., Severi, F. M., Challis, J. R. & Petraglia, F. (2016). Endocrinology of Human Parturition. *Annales d'Endocrinologie*, 77(2), 105–113.

Velázquez, E., Peix, A., Zurdo-Piñeiro, J. L., Palomo, J. L., Mateos, P. F., Rivas, R., Muñoz-Adelantado, E., Toro, N., García-Benavides, P. & Martínez-Molina, E. (2005). The Coexistence of Symbiosis and Pathogenicity-Determining Genes in Rhizobium Rhizogenes Strains Enables Them to Induce Nodules and Tumors or Hairy Roots in Plants. *Molecular Plant-Microbe Interactions*, 18(12), 1325–1332.

Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Delanghe, J., Van Simaey, L., De Ganck, C., Temmerman, M. & Vaneechoutte, M. (2004). Cloning of 16S RRNA Genes Amplified from Normal and Disturbed Vaginal Microflora Suggests a Strong Association between Atopobium Vaginae, Gardnerella Vaginalis and Bacterial Vaginosis. *BMC Microbiology*, 4(1), 1–11.

Verma, V., Oza, H., Thaker, R. & Kumar, S. (2019). The Role of 8-Hydroxy-2'-Deoxyguanosine and Total Antioxidant Capacity in Women with Preterm Birth: A Preliminary Study. *International Journal of Health Sciences & Research (www.ijhsr.org)*, 9(7), 12.

Verstraelen, H., Verhelst, R., Claeys, G., De Backer, E., Temmerman, M. & Vaneechoutte, M. (2009). Longitudinal Analysis of the Vaginal Microflora in Pregnancy Suggests That L. Crispatus Promotes the Stability of the Normal Vaginal Microflora and That L. Gasseri and/or L. Iners Are More Conducive to the Occurrence of Abnormal Vaginal Microflora. *BMC Microbiology*, 9.

Vo, N. T., Kusagawa, E., Nakano, K., Moriwaki, C., Miyake, Y., Haruyama, S., Fukuhara, S., Nguyen, N. T., Dang, P. H., Nguyen, M. T. T. & Kataoka, T. (2021). Biological Evaluation of Alkyl Triphenylphosphonium Ostruthin Derivatives as Potential Anti-Inflammatory Agents Targeting the Nuclear Factor KB Signaling Pathway in Human Lung Adenocarcinoma A549 Cells. *BioChem*, 1(2), 107–121.

Vollmer, P., Walev, I., Rose-John, S. & Bhakdi, S. (1996). Novel Pathogenic Mechanism of Microbial Metalloproteinases: Liberation of Membrane-Anchored Molecules in Biologically Active Form Exemplified by Studies with the Human Interleukin-6 Receptor. *Infection and Immunity*, 64(9), 3646–3651.

Voltolini, C., Torricelli, M., Conti, N., Vellucci, F. L., Severi, F. M. & Petraglia, F. (2013). Understanding Spontaneous Preterm Birth: From Underlying Mechanisms to Predictive and Preventive Interventions. *Reproductive Sciences*, 20(11), 1274–1292.

Wagner, B. D., Grunwald, G. K., Zerbe, G. O., Mikulich-Gilbertson, S. K., Robertson, C. E., Zemanick, E. T. & Harris, J. K. (2018). On the Use of Diversity Measures in Longitudinal Sequencing Studies of Microbial Communities. *Frontiers in Microbiology*, 9(MAY), 1037.

Wagner, M., Walter, P., Salla, S., Johnen, S., Plange, N., Rütten, S., Goecke, T. W. & Fuest, M. (2018). Cryopreservation of Amniotic Membrane with and without Glycerol Additive. *Graefe's Archive for Clinical and Experimental Ophthalmology*, 256(6), 1117–1126.

Wahab, M. F., Dasgupta, P. K., Kadjo, A. F. & Armstrong, D. W. (2016). Sampling Frequency, Response Times and Embedded Signal Filtration in Fast, High Efficiency Liquid Chromatography: A Tutorial. *Analytica Chimica Acta*, 907(5), 31–44.

Waitzman, N. J., Jalali, A. & Grosse, S. D. (2021). Preterm Birth Lifetime Costs in the United States in 2016: An Update. *Seminars in Perinatology*, 45(3), 151390.

Walker, J. J. & Morley, L. (2018). BMJBest Practices. *British Medical Journal*. Retrieved January 15, 2019, from https://bestpractice.bmj.com/topics/es-es/326

Walsh, E. M., Li, S. X., Black, L. K. & Kuzniewicz, M. (2019). Incremental Cost of Prematurity by Week of Gestational Age. *AJP Reports*, 9(1), e76–e83.

Walsh, S. W., Nugent, W. H., Alam, S. M. K., Washington, S. L., Teves, M., Jefferson, K. K. & Strauss, J. F. (2020). Protease Amplification of the Inflammatory Response Induced by Commensal Bacteria: Implications for Racial Disparity in Term and Preterm Birth. *Reproductive Sciences*, 27(1), 246–259.

Wang, L., Qu, L., Yang, L., Wang, Y. & Zhu, H. (2020). NanoReviser: An Error-Correction Tool for Nanopore Sequencing Based on a Deep Learning Algorithm. *Frontiers in Genetics*, 11, 900.

Wang, R. & Guo, S. (2021). Phytic Acid and Its Interactions: Contributions to Protein Functionality, Food Processing, and Safety. *Comprehensive Reviews in Food Science and Food Safety*, 20(2), 2081–2105.

Wang, Y. P. & Lei, Q. Y. (2018). Metabolite Sensing and Signaling in Cell Metabolism. *Signal Transduction and Targeted Therapy*, 3(1), 30.

Ward, K. & Theiler, R. N. (2008). Once-Daily Dosing of Gentamicin in Obstetrics and Gynecology. *Clinical Obstetrics and Gynecology*, 51(3), 498–506.

Watari, M., Watari, H., DiSanto, M. E., Chacko, S., Shi, G. P. & Strauss, J. F. (1999). Pro-Inflammatory Cytokines Induce Expression of Matrix-Metabolizing Enzymes in Human Cervical Smooth Muscle Cells. *American Journal of Pathology*, 154(6), 1755–1762.

Waters, T. P. & Mercer, B. (2011). Preterm PROM: Prediction, Prevention, Principles. *Clinical Obstetrics and*

Gynecology, 54(2), 307–312.
Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M. J., Kuipers, O. P., Van Sinderen, D. & Kok, J. (2007). Complete Genome Sequence of the Prototype Lactic Acid Bacterium Lactococcus Lactis Subsp. Cremoris MG1363. *Journal of Bacteriology*, 189(8), 3256–3270.
Weinstein, M. J., Luedemann, G. M., Oden, E. M., Wagman, G. H., Rosselet, J. P., Marquez, J. A., Coniglio, C. T., Charney, W., Herzog, H. L. & Black, J. (1963). Gentamicin, a New Antibiotic Complex from Micromonospora. *Journal of Medicinal Chemistry*, 6(4), 463–464.
Weiss, A., Goldman, S. & Shalev, E. (2007). The Matrix Metalloproteinases (MMPS) in the Decidua and Fetal Membranes. *Frontiers in Bioscience*, 12(1), 649.
Whittaker, R. H. (1965). Dominance and Diversity in Land Plant Communities. *Science*, 147(3655), 250–260.
Wiberg-Itzel, E., Cnattingius, S. & Nordström, L. (2005). Lactate Determination in Vaginal Fluids: A New Method in the Diagnosis of Prelabour Rupture of Membranes. *BJOG: An International Journal of Obstetrics and Gynaecology*, 112(6), 754–758.
Wilks, M., Wiggins, R., Whiley, A., Hennessy, E., Warwick, S., Porter, H., Corfield, A. & Millar, M. (2004). Identification and H(2)O(2) Production of Vaginal Lactobacilli from Pregnant Women at High Risk of Preterm Birth and Relation with Outcome. *Journal of clinical microbiology*, 42(2), 713–7.
Willis, A. D. (2019). Rarefaction, Alpha Diversity, and Statistics. *Frontiers in Microbiology*, 10(OCT), 2407.
Wilm, M. (2011). Principles of Electrospray Ionization. *Molecular and Cellular Proteomics*, 10(7).
Witkin, S. S., Mendes-Soares, H., Linhares, I. M., Jayaram, A., Ledger, W. J. & Forney, L. J. (2013). Influence of Vaginal Bacteria and D- and L-Lactic Acid Isomers on Vaginal Extracellular Matrix Metalloproteinase Inducer: Implications for Protection against Upper Genital Tract Infections. *mBio*, 4(4).
Woessner, J. F. (1991). Matrix Metalloproteinases and Their Inhibitors in Connective Tissue Remodeling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 5(8), 2145–2154.
Xu, H., Zhang, X., Yao, W., Sun, Y. & Zhang, Y. (2019). Characterization of the Vaginal Microbiome during Cytolytic Vaginosis Using High-Throughput Sequencing. *Journal of Clinical Laboratory Analysis*, 33(1), e22653.
Yamanaka, H., Taniguchi, A., Tsuboi, H., Kano, H. & Asami, Y. (2019). Hypouricaemic Effects of Yoghurt Containing Lactobacillus Gasseri PA-3 in Patients with Hyperuricaemia and/or Gout: A Randomised, Double-Blind, Placebo-Controlled Study. *Modern Rheumatology*, 29(1), 146–150.
Yao, C., Chou, J., Wang, T., Zhao, H. & Zhang, B. (2018). Pantothenic Acid, Vitamin C, and Biotin Play Important Roles in the Growth of Lactobacillus Helveticus. *Frontiers in Microbiology*, 9(JUN), 1194.
Yasuda, S., Tanaka, M., Kyozuka, H., Suzuki, S., Yamaguchi, A., Nomura, Y. & Fujimori, K. (2020). Association of Amniotic Fluid Sludge with Preterm Labor and Histologic Chorioamnionitis in Pregnant Japanese Women with Intact Membranes: A Retrospective Study. *Journal of Obstetrics and Gynaecology Research*, 46(1), 87–92.
Yeoman, C. J., Thomas, S. M., Miller, M. E. B., Ulanov, A. V., Torralba, M., Lucas, S., Gillis, M., Cregger, M., Gomez, A., Ho, M., Leigh, S. R., Stumpf, R., Creedon, D. J., Smith, M. A., Weisbaum, J. S., Nelson, K. E., Wilson, B. A. & White, B. A. (2013). A Multi-Omic Systems-Based Approach Reveals Metabolic Markers of Bacterial Vaginosis and Insight into the Disease. *PLoS ONE*, 8(2), e56111.
Yetter, J. (1998). Examination of the Placenta. *American family physician*, 57(5), 242–260.
Yoneyama, Y., Suzuki, S., Sawa, R., Takeuchi, T., Kobayashi, H., Takei, R., Kiyokawa, Y., Otsubo, Y., Hayashi, Z. & Araki, T. (2000). Changes in Plasma Adenosine Concentrations during Normal Pregnancy. *Gynecologic and Obstetric Investigation*, 50(3), 145–148.
Yoon, B. H., Romero, R., Park, J. Y., Oh, K. J., Lee, J. H., Conde-Agudelo, A. & Hong, J. S. (2019). Antibiotic Administration Can Eradicate Intra-Amniotic Infection or Intra-Amniotic Inflammation in a Subset of Patients with Preterm Labor and Intact Membranes. *American Journal of Obstetrics and Gynecology*, 221(2), 142.e1-142.e22.
York, T. P., Strauss, J. F., Neale, M. C. & Eaves, L. J. (2010). Racial Differences in Genetic and Environmental Risk to Preterm Birth. *PLoS ONE*, 5(8), 12391.
Z. Mahmoudabadi, A., Heidarinia, S. & Rezaei-Matehkolaei, A. (2015). Extracellular Phytase in Candida Albicans from Different Sources. *Current Enzyme Inhibition*, 12(2), 134–136.
Zaga, V., Estrada-Gutierrez, G., Beltran-Montoya, J., Maida-Claros, R., Lopez-Vancell, R. & Vadillo-Ortega, F. (2004). Secretions of Interleukin-1β and Tumor Necrosis Factor α by Whole Fetal Membranes Depend on Initial Interactions of Amnion or Choriodecidua with Lipopolysaccharides or Group B Streptococci1. *Biology of Reproduction*, 71(4), 1296–1302.
Zhang, Q., Zhang, L., Wang, Y., Zhao, M., Chen, R., Tao, Z., Lyu, T., Huang, Z. & Liao, Q. (2019). An Optimized 16S

RRNA Sequencing Protocol for Vaginal Microbiome to Avoid Biased Abundance Estimation. *bioRxiv*, 2008(2007), 28–30.

Zhang, Y. Z., Ran, L. Y., Li, C. Y. & Chen, X. L. (2015). Diversity, Structures, and Collagen-Degrading Mechanisms of Bacterial Collagenolytic Proteases. *Applied and Environmental Microbiology*, 81(18), 6098–6107.

7 Appendix A – Optimisation of Fluorescent Gelatin model

7.1 Methods

In a preliminary experiment, 3 conditions were used: bacteria resuspended in PBS, MRS and conditioned cell free media for *L. gasseri*, *L. jensenii* and *L. crispatus* at 1:1, 1:10, 1:100, 1:1000 dilutions. PBS and MRS were used as negative controls, MRS was also diluted in PBS 1:10, 1:100, 1:1000 to control for the brown colour of this media which may obscure fluorescence. Collagenase A from *Clostridium histolyticum* (Roche, COLLA-RO) was used as a positive control at 0.5 and 0.05 U/mL. Fluorescent measurements were taken every 15 minutes for 21hr, all conditions were run in duplicate.

7.2 Results

7.2.1.1 Pilot experiment (20hr)

We used a collagenase/gelatinase assay to investigate enzymes produced by vaginal bacteria that may be able to degrade FM and contribute to PPROM. The assay contains gelatin which when degraded, produces fluorescence that can be measured to quantify degradation.

7.2.1.2 Pilot Experiment – Raw Values

For the pilot experiment (n=1) (Figure 7.1), we tested live *L. gasseri*, *L. jensenii* and *L. crispatus* resuspended in either PBS or fresh MRS and MRS from a 24hr culture that had been centrifuged and filtered to produce conditioned cell-free media, also referred to as "*L. crispatus* media" etc. Live bacteria and conditioned media were normalised to 1:1 (10^7 CFU/mL) before dilution to 1:10 (10^6 CFU/mL), 1:100 (10^5 CFU/mL), 1:1000 (10^4 CFU/mL).

Fluorescence intensity (arbitrary units) ranged from 12.4 - 2332.03 (Figure 7.1a). Collagenase A (0.5 and 0.05 U/mL) was used as a positive control and produced considerably higher fluorescence compared to conditions without collagenase. Conditions using PBS (e.g., *L. crispatus* in PBS), had a higher fluorescence intensity compared to those containing MRS (e.g., *L. crispatus* in MRS), average fluorescence intensity was 66.03 for PBS vs 21.63 for MRS conditions (Figure 7.1b). For this reason, in subsequent analysis, fluorescence intensity was normalised to the value at timepoint one for each condition to show the change in fluorescence rather than raw fluorescence intensity values (arbitrary units).

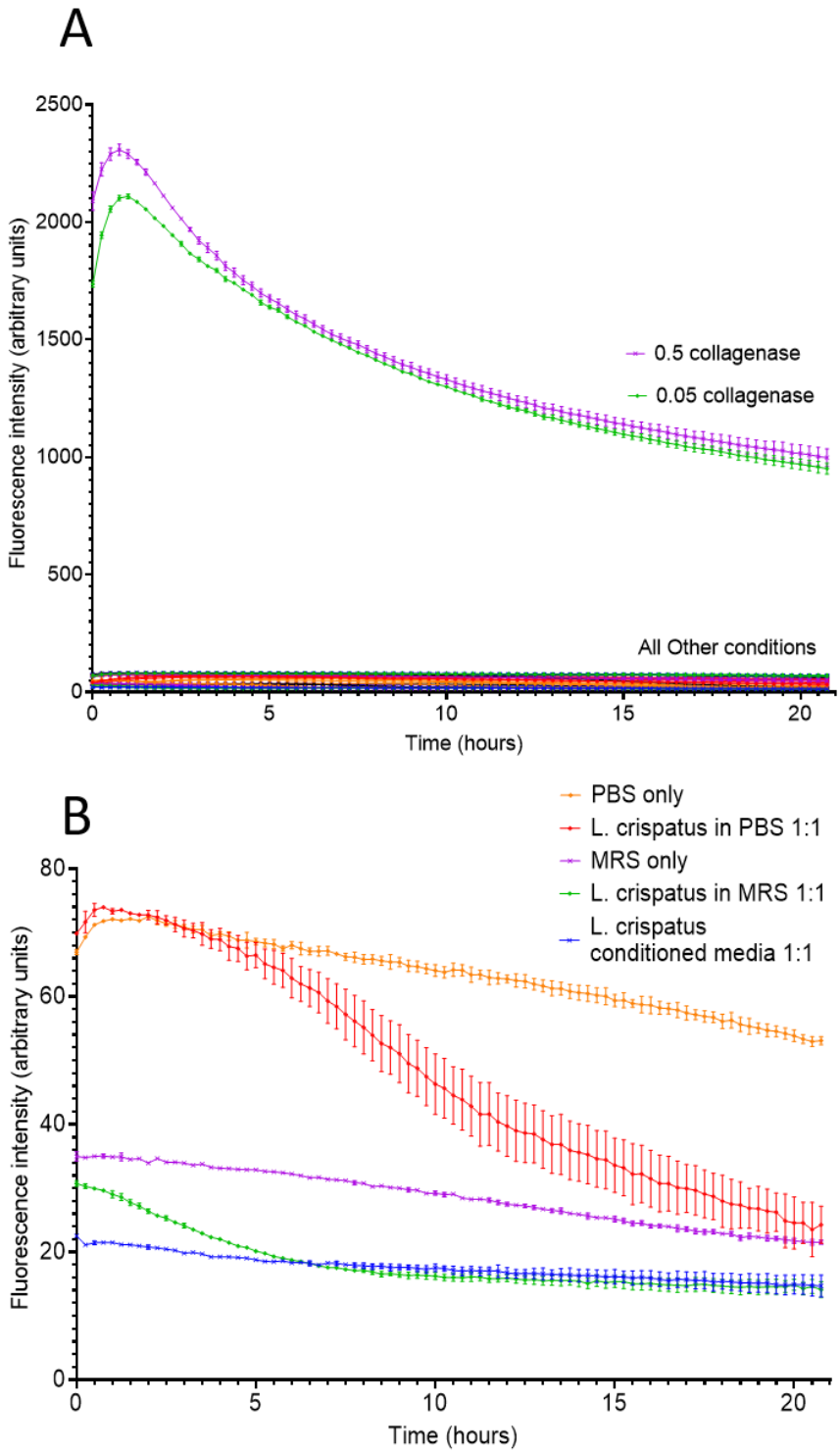


Figure 7.1 Fluorescence intensity (arbitrary units) of **(A)** 0.5 and 0.05 U/mL collagenase (positive control), MRS only, PBS only (negative controls), *L. crispatus* conditioned cell free media, *L. crispatus* suspended in PBS, *L. crispatus* suspended in fresh MRS, 1:1 dilution =10^7 CFU/mL. **(B)** Graph A with positive controls (0.5 and 0.05 U/mL collagenase) removed for better visualisation. (n=1, 2 technical repeats per condition. Points are the mean of technical repeats bars represent SEM). Analysis performed on GraphPad Prism.

7.2.1.3 Pilot Experiment Normalised – Live Bacteria

By normalising fluorescence values to timepoint one we can better visualise differences between conditions. Conditions containing live bacteria, suspended in either PBS (Figure 7.2a) or MRS (Figure 7.2b), generally decreased over time except for *L. gasseri* and *L. jensenii* in Figure 7.2a where fluorescence increased slightly before decreasing.

The rate of fluorescence decrease appears to be dose dependent as demonstrated by *L. jensenii* (Figure 7.2c). The 1:1 dilution decreased immediately, the 1:10 dilution after 30 min and the 1:100 dilution after 1 hr 45 min.

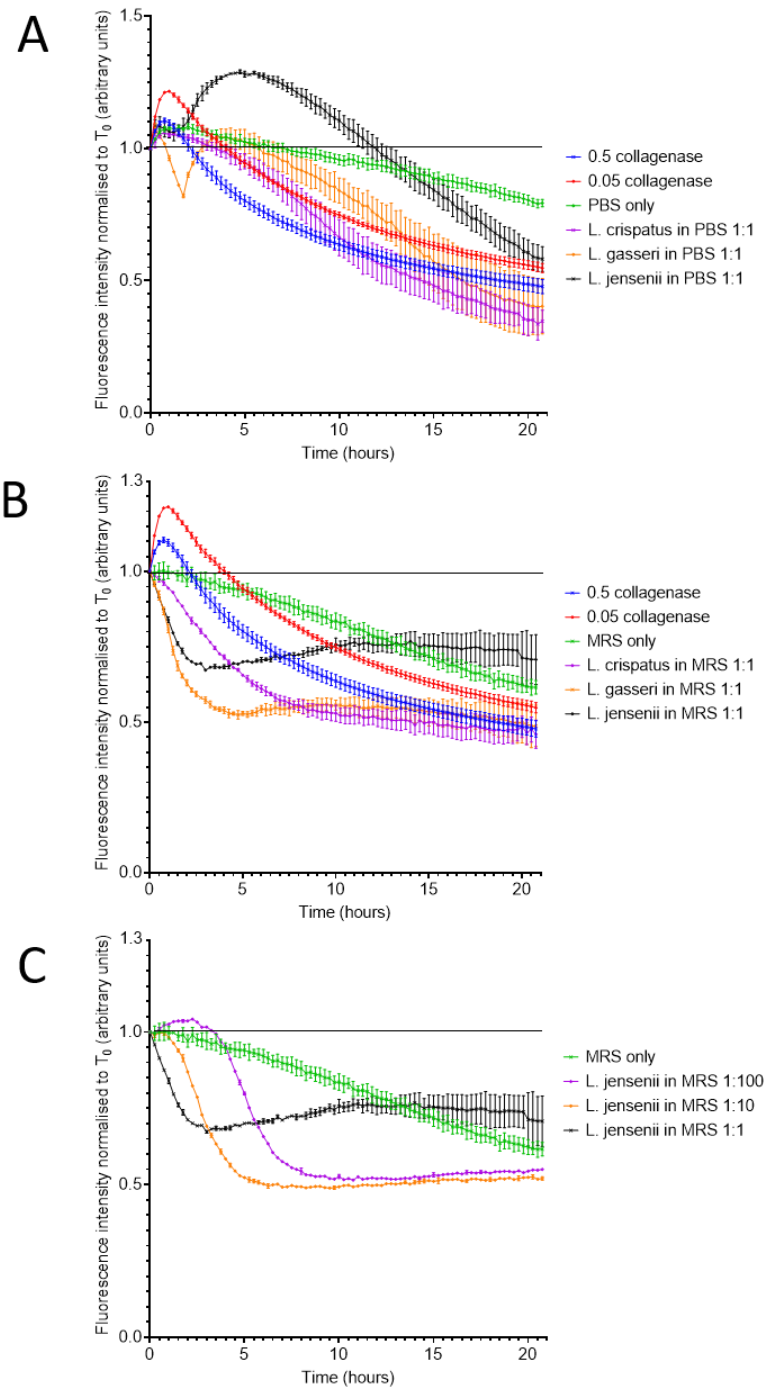


Figure 7.2 Fluorescence intensity (arbitrary units) normalised to the first time point for each condition. **A)** *L. gasseri*, *L. jensenii* and *L. crispatus* 1:1 suspended in **PBS**, 0.5 and 0.05 U/mL collagenase A. **B)** *L. gasseri*, *L. jensenii* and *L. crispatus* 1:1 suspended in fresh **MRS**, 0.5 and 0.05 U/mL collagenase A. **C)** *L. jensenii* suspended in fresh MRS at 1:1, 1:10, 1:100 dilutions. 0.5 and 0.05 U/mL collagenase A. 1:1=10^7 CFU/mL, 1:10=10^6 CFU/mL, 1:100=10^5 CFU/mL. (n=1, 2 technical repeats per condition. Points are the mean of technical repeats bars represent SEM). Analysis performed on GraphPad Prism.

7.2.1.4 Pilot Experiment Normalised – Conditioned Media

Focussing on the conditions containing cell free conditioned media, a considerable increase in fluorescence can be seen in the 1:10 dilution (Figure 7.3). Interestingly, the other dilutions including the non-diluted 1:1 condition did not have the same increase. This trend was observed for *L. gasseri* Figure 7.3a, *L. crispatus* Figure 7.3b, *L. jensenii* Figure 7.3c.

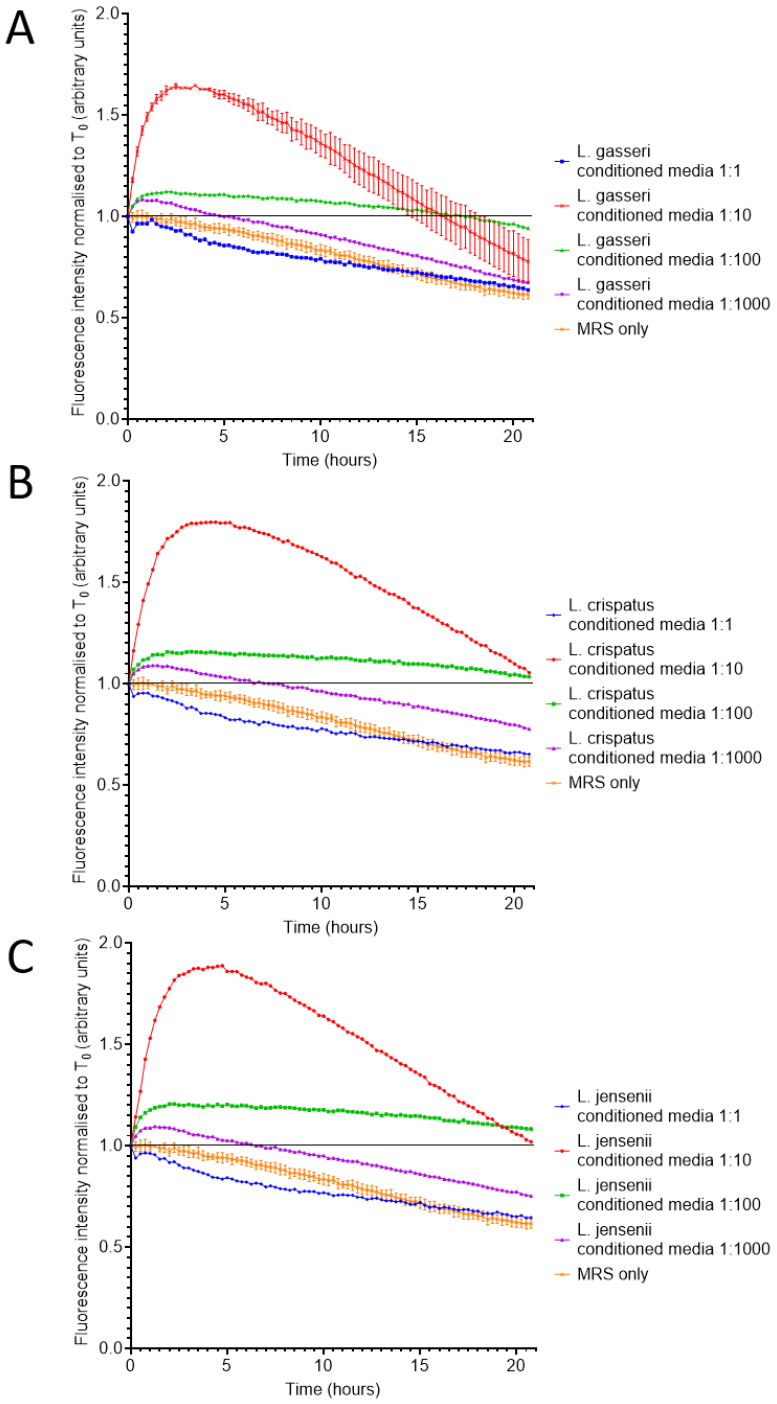


Figure 7.3 Fluorescence intensity (arbitrary units) normalised to the first time point for each condition. 1:1, 1:10, 1:100, 1:100 dilution of conditioned cell free media for **A)** *L. gasseri* **B)** *L. crispatus* **C)** *L. jensenii.* 1:1=10^7 CFU/mL, 1:10=10^6 CFU/mL, 1:100=10^5 CFU/mL, 1:1000=10^4 CFU/mL. (n=1, 2 technical repeats per condition. Points are the mean of technical repeats bars represent SEM). Analysis performed on GraphPad Prism.

Comparison of only the 1:10 conditions showed varying rates of fluorescence increase for the different *Lactobacillus* species over both 20 hr (Figure 7.4a) and 5 hr (Figure 7.4b).

Based on these results, we performed subsequent experiments for 5 hours as this captures the fluorescence increase and subsequent plateau in all conditions (Figure 7.4b).

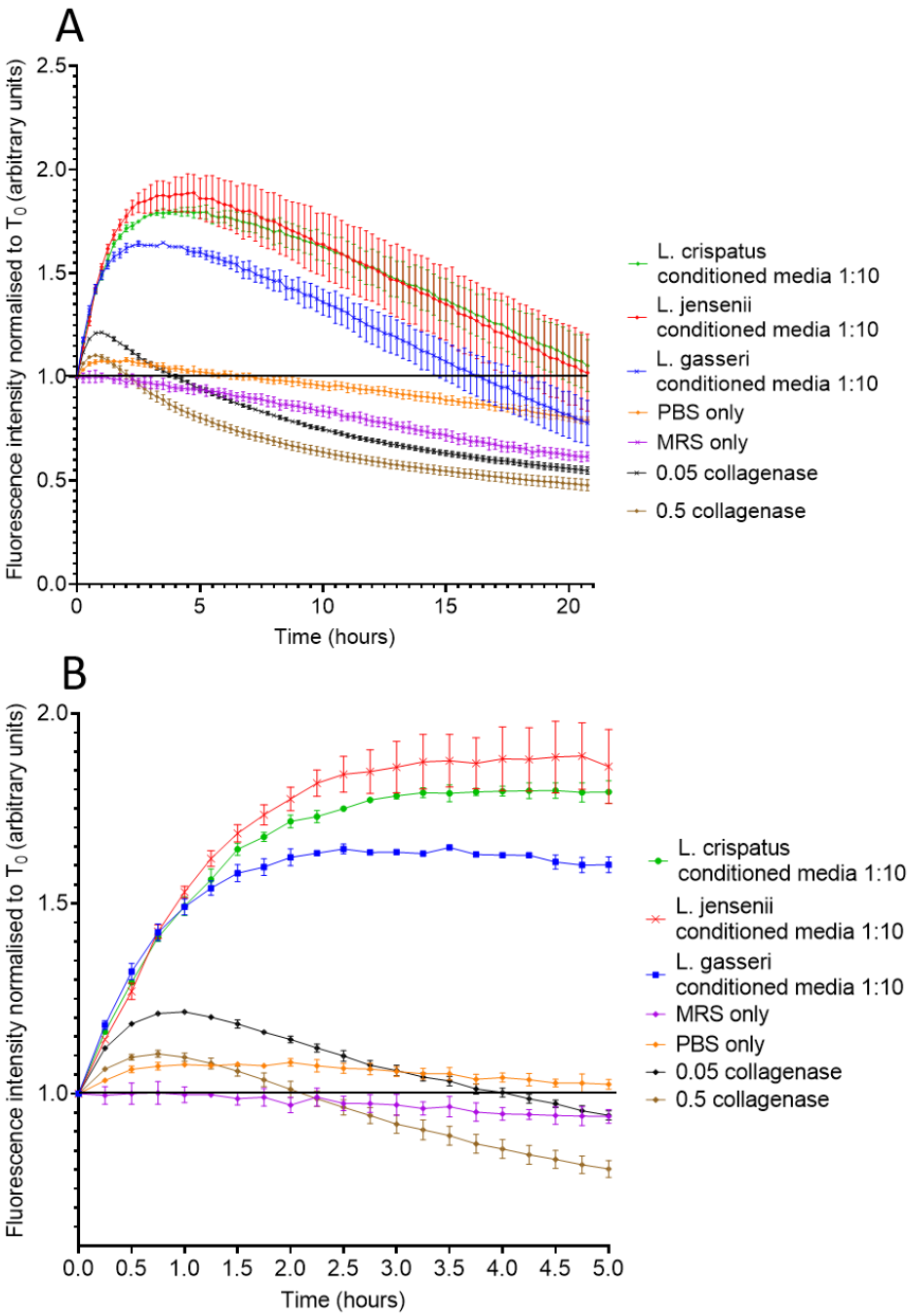


Figure 7.4 Fluorescence intensity (arbitrary units) normalised to the first time point for each condition: 0.5 and 0.05 U/mL collagenase A, *L. gasseri*, *L. jensenii* and *L. crispatus* conditioned cell free media 1:10 dilution 1:10=10^6 CFU/m for **A)** 20 hours **B)** Cropped to 5 hours for better visualisation. Analysis performed on GraphPad Prism.

7.3

8 Appendix B - Optimisation of Primary Human Amnion Epithelial Cell Isolation

8.1 Methods

8.1.1 Optimisation of Human Amnion Epithelial Cells (hAEC) Isolation

For details on patient recruitment and fetal membrane processing please see section 4.3.1.

Firstly, we reviewed the literature on hAEC isolation from fresh amnion. A recent review of 7 studies recommended methods and key points for cell isolation (Motedayyen et al., 2017).

For all methods, large pieces of membrane were removed from the placenta within 6 hours post-delivery. The membranes were washed twice in PBS to remove blood and the chorion which is opaque and pink in colour was removed from the amnion by scraping with blunt instruments. The amnion was then cut into 2 cm x 2 cm pieces for further enzymatic processing.

8.1.1.1 Method 1

As recommended by (Motedayyen et al., 2017), three digestion steps were carried out. 1st digestion - amnion pieces were placed into 20 ml of 0.05% trypsin/EDTA, warmed to 37°C, with shaking for 10 minutes and the supernatant was discarded (to remove blood clots and cellular debris). 2nd digestion -The tissue was then transferred to 20 ml of 0.05% trypsin/EDTA, warmed to 37°C, with shaking (64 rpm) for 30 minutes to detach epithelial cells from the membrane. 3rd digestion -the tissue was again transferred to 20 ml of 0.05% trypsin/EDTA, warmed to 37°C, with shaking (64 rpm) for an additional 30 minutes.

Immediately after tissue has been removed from the digestion (for both 2nd and 3rd digestion steps) 20 ml of ice cold F12 media was added to the cell suspension to neutralise the trypsin and prevent degradation of the cells. The cell suspension from 2nd and 3rd digestion was pooled and filtered through a 70 µm cell strainer. The strained cell suspension was then centrifuged at 500 x g for 10 min before the supernatant was discarded and the pellet resuspended in 5 ml of ice cold DMEM/F12 media for counting.

No details regarding trypsin were given in (Motedayyen et al., 2017), so we tested three types of trypsin using method 1.

1. Trypsin-EDTA with phenol red 0.25%, (ThermoFisher 25200072) usually used for cell culture, containing 2.5 g porcine trypsin and 0.2 g EDTA per litre of Hanks' Balanced Salt Solution.
2. Trypsin from bovine pancreas (Sigma Aldrich, T1426-50MG) essentially salt-free, lyophilized powder, ⩾10,000 BAEE units/mg protein dissolved in Hanks' Balanced Salt Solution.
3. Trypsin-EDTA (0.5%), no phenol red (ThermoFisher,15400054) from porcine pancreas.

After unsuccessfully testing the accepted protocol from the literature with various enzymes we then tested several other methods using alternative enzymes.

8.1.1.2 Method 2

Amnion pieces were constantly rotated overnight at 4°C in a solution containing 1.5% Pronase (Sigma, 10165921001) before straining as described in method 1.

8.1.1.3 Method 3

As previously done by (Janzen et al., 2017) the amnion was finely minced with scissors and was digested in 1 mg/ml collagenase A (Sigma Aldrich, 10103578001) + 0.025% Trypsin-EDTA (ThermoFisher, 15400054) before straining as described in method 1.

8.1.1.4 Method 4

Finally, for method 4, amnion pieces were placed into trypsin 0.25% (From porcine pancreas SIGMA T4799) + EDTA 0.05% (SIGMA E6758) dissolved in DMEM F12 (Serum free), pre-warmed to 37°C. The tissue was incubated at 37°C with shaking (100 rpm) in 50 ml falcon secured horizontally to allow more movement of the tissue in digestion media. After incubation, warmed FBS was added to neutralise the enzymes and the falcon tube was shaken to further dislodge cells from the tissue. The digestion mixture was then filtered using a 70-micron cell strainer. Tissue pieces caught in the cell strainer were placed into a new tube containing DMEM F12, were shaken again to dislodge further cells and strained again with new cell strainer (Majority of cells were in this second strain).

Strained cell suspensions were centrifuged at 500 x g for 10 minutes and the supernatant was removed as in method 1, however, the cell pellet was resuspended in prewarmed DMEM F12 + EGF in preparation of cell culture.

8.1.1.5 Cell counting

Number of viable cells were counted using a haemocytometer. 50 µl of freshly isolated cell suspension was mixed with the same volume of trypan blue and left at room temperature for 1 min before loading 10 µl of cell suspension into the chamber. Total number of viable cells (intact cells not stained blue) in 4 large squares were counted and the equation below was used to calculate total number of viable cells in the cell suspension, accounting for dilution.

$$Cells/mL = \frac{Total\ N^o viable\ counted}{4}\ x10{,}000\ x2$$

8.1.2 hAEC Culture

After counting, freshly isolated hAEC were placed into either a T25 cell culture flask or a 24 well plate containing DMEM/F-12 GlutaMAX (ThermoFisher) with additional: 10% FBS (FisherScientific) and Recombinant Human epithelial growth factor (Biolegend UK, 585505) and incubated in 5% CO_2 at 37 ˚C. Monolayers were passaged when 80% confluency was attained. Media was removed and cells were washed with 5 mL Phosphate-buffered saline (PBS) to remove any trace of serum. 2 mL of trypsin was added, and cells were incubated at 37˚C for 2 minutes or until all cells had detached from the flask. In order to inactivate the detachment enzyme (trypsin), 4 mL of media (×2 the volume of trypsin) was added to the flask and pipetted over the surface of the flask several times to ensure all cells were suspended in solution. The solution was then centrifuged at 500 x g for 5 minutes after which the supernatant was discarded, and pellet resuspended in 10 mL of media. Cells were counted as described above and placed into a flask or plate with additional media for 200,000 cells per well.

8.1.3 hAEC validation

To visualise epithelial and mesenchymal cell markers, hAEC and MRC-5 cells (used as a positive vimentin control) were washed with PBS, fixed using 4% PFA, incubated for 30 min at room temperature and washed x3 in PBS. To ensure a receptor specific binding, blocking was

undertaken using 1% BSA for 1 hr at room temperature. The epithelial marker CD324 Monoclonal Antibody, DECMA-1, (ThermoFisher, 202504) (E-Cadherin) plus the mesenchymal marker Recombinant Anti-Vimentin antibody (abcam, EPR3776) (vimentin) were added to the cells at 1:100, 1:250, 1:500, incubated overnight at 4 ˚C and washed with PBS. DAPI was then added 1:1000, incubated for 30 minutes at room temperature, removed and washed x3 with PBS. As with the live/dead staining, coverslips were removed and placed cell-side down onto glass slides containing a drop of ProLong™ Gold Antifade Mountant (Fisher Scientific). The slides were allowed to cure in the dark for 1-2 hrs at RT before imaging on fluorescent Olympus IX73 microscope.

8.2 Results

8.2.1 Human Amnion Epithelial Cells

8.2.1.1 hAEC Isolation

Four methods of hAEC isolation were tested and method number 4 was found to be the most successful (see section 8.1.1.4). This method produced the largest cell pellet indicating cells were liberated from the tissue but were not digested during the isolation process (Figure 8.1). This method also produced the largest number of viable cells during culture after isolation (**Figure 8.2**). After isolation, we successfully confirmed that the cells had an epithelial morphology using a common epithelial marker E-Cadherin and a common mesenchymal marker Vimentin (Figure 8.3) when compared to mesenchymal MRC-5 cells (Figure 8.4).

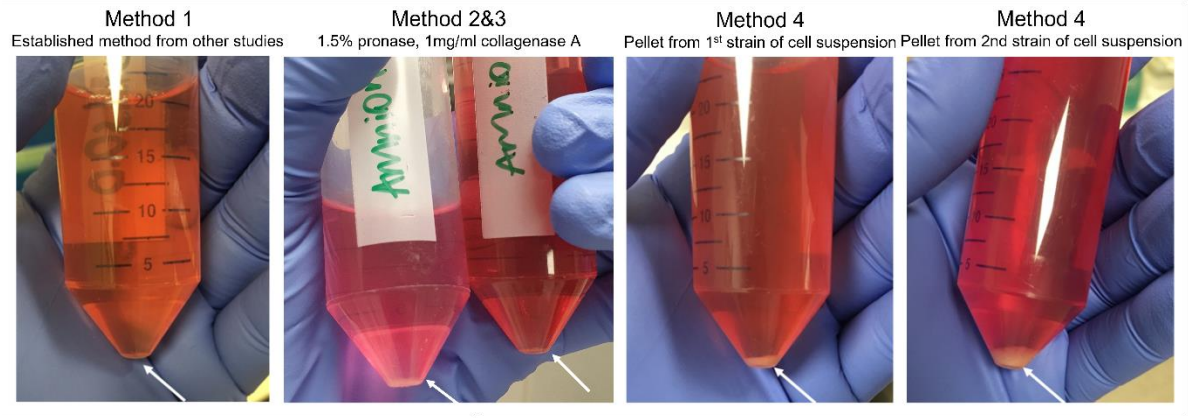


Figure 8.1 Cell pellets from Human amnion epithelial cell isolation using 4 different methods 1) three digestion steps were carried out all with 0.05% trypsin/EDTA. 2) constantly rotated overnight at 4°C in 1.5% Pronase (Sigma, 10165921001). 3) finely minced and digested in 1 mg/ml collagenase A (Sigma Aldrich, 10103578001) + 0.025% Trypsin-EDTA (ThermoFisher, 15400054). 4) trypsin 0.25% (From porcine pancreas SIGMA T4799) + EDTA 0.05% (SIGMA E6758) dissolved in DMEM F12, 37°C with shaking. Suspension filtered and tissue shaken again in DMEM F12 then strained again (final picture).

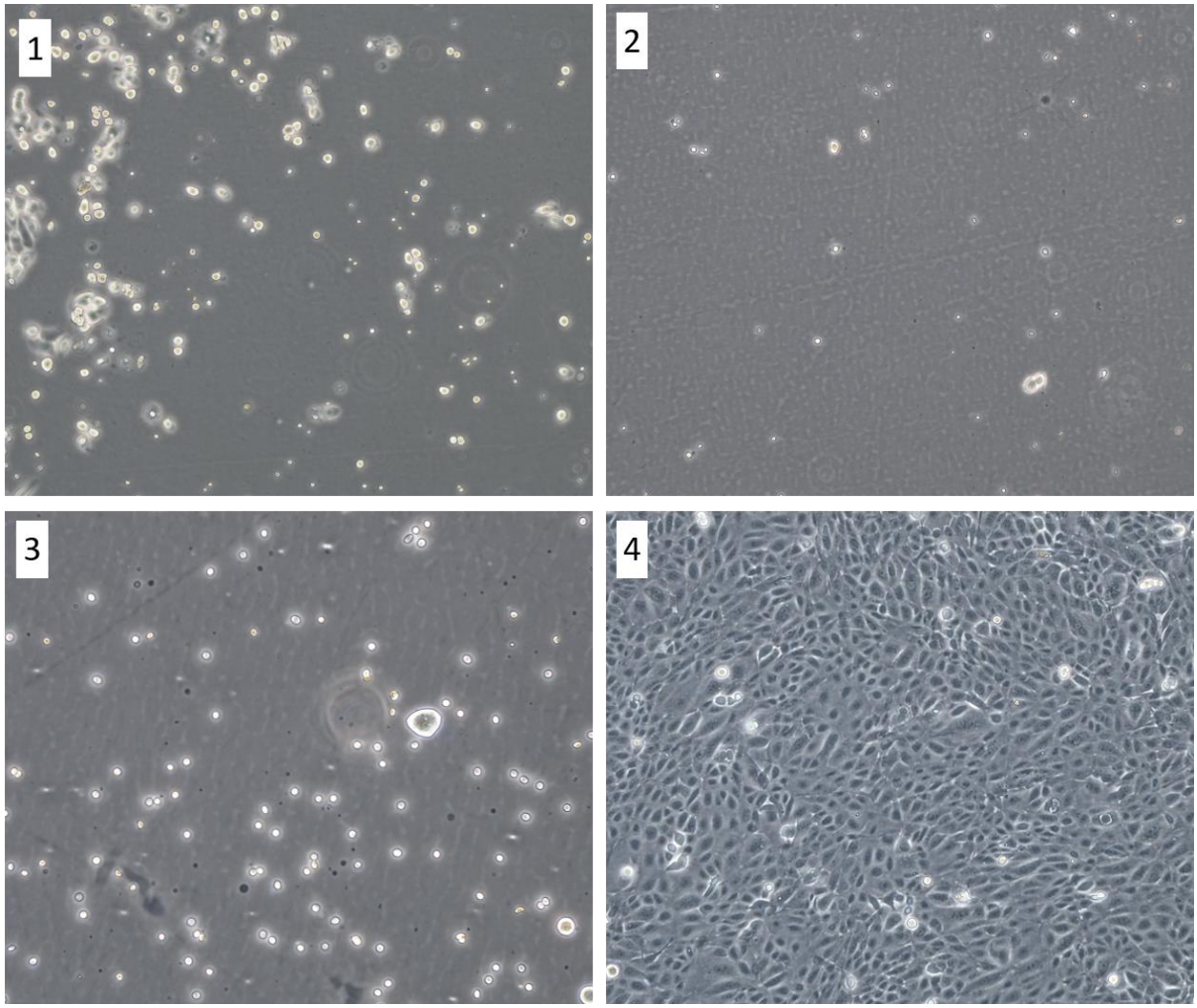


Figure 8.2 Human amnion epithelial cells 24 hours after isolation using 4 different methods. 1) three digestion steps were carried out all with 0.05% trypsin/EDTA. 2) constantly rotated overnight at 4°C in 1.5% Pronase (Sigma, 10165921001). 3) finely minced and digested in 1 mg/ml collagenase A (Sigma Aldrich, 10103578001) + 0.025% Trypsin-EDTA (ThermoFisher, 15400054). 4) trypsin 0.25% (From porcine pancreas SIGMA T4799) + EDTA 0.05% (SIGMA E6758) dissolved in DMEM F12, 37°C with shaking. All images taken with x10 magnification

8.2.1.2 hAEC Validation

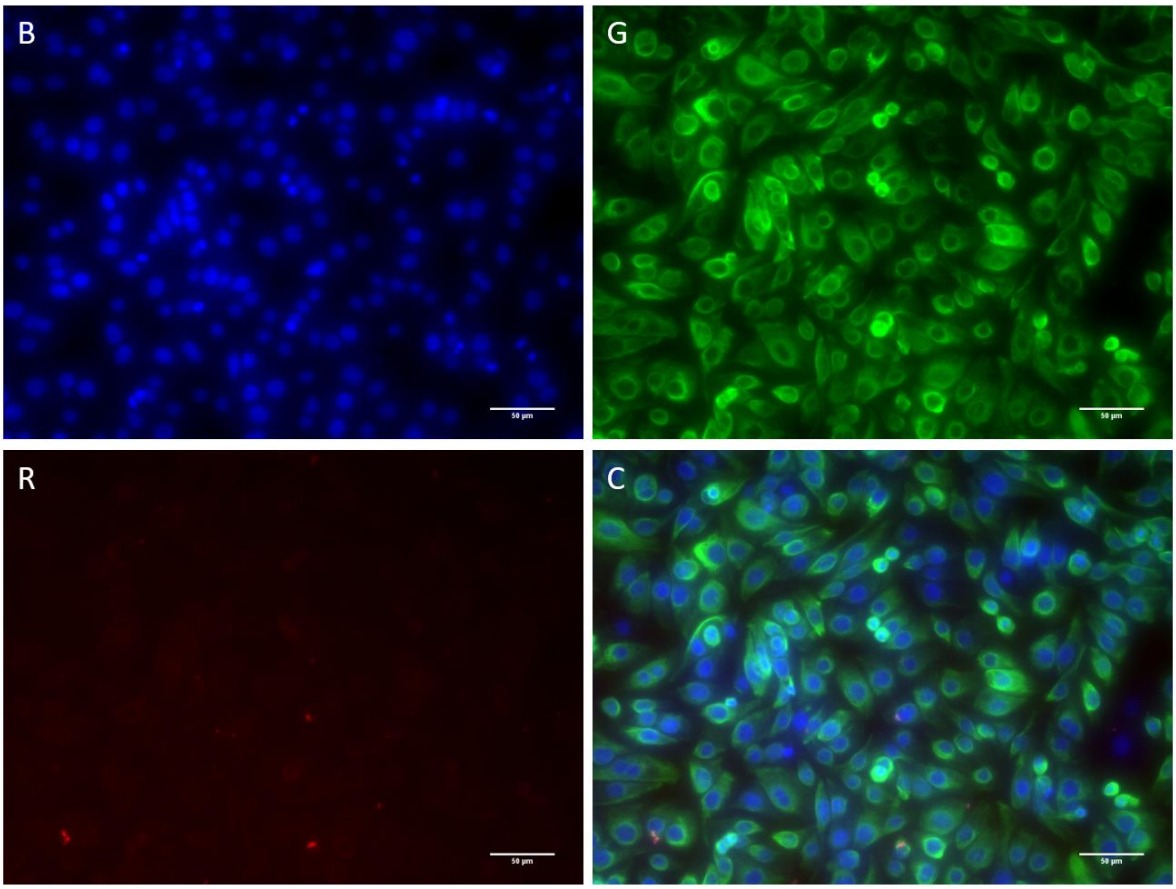


Figure 8.3 hAEC cells 24 hours after isolation. Glass coverslip was mounted onto a glass slide before imaging at x20/0.30 magnification using Olympus IX73 microscope. Images were normalised and overlaid using ImageJ software. **B)** DAPI staining nucleus of all cells **G)** E-Cadherin (CD324 Monoclonal Antibody, DECMA-1, ThermoFisher, 202504) **R)** Vimentin (Recombinant Anti-Vimentin antibody (abcam, EPR3776) **C)** Composite image of RGB channels

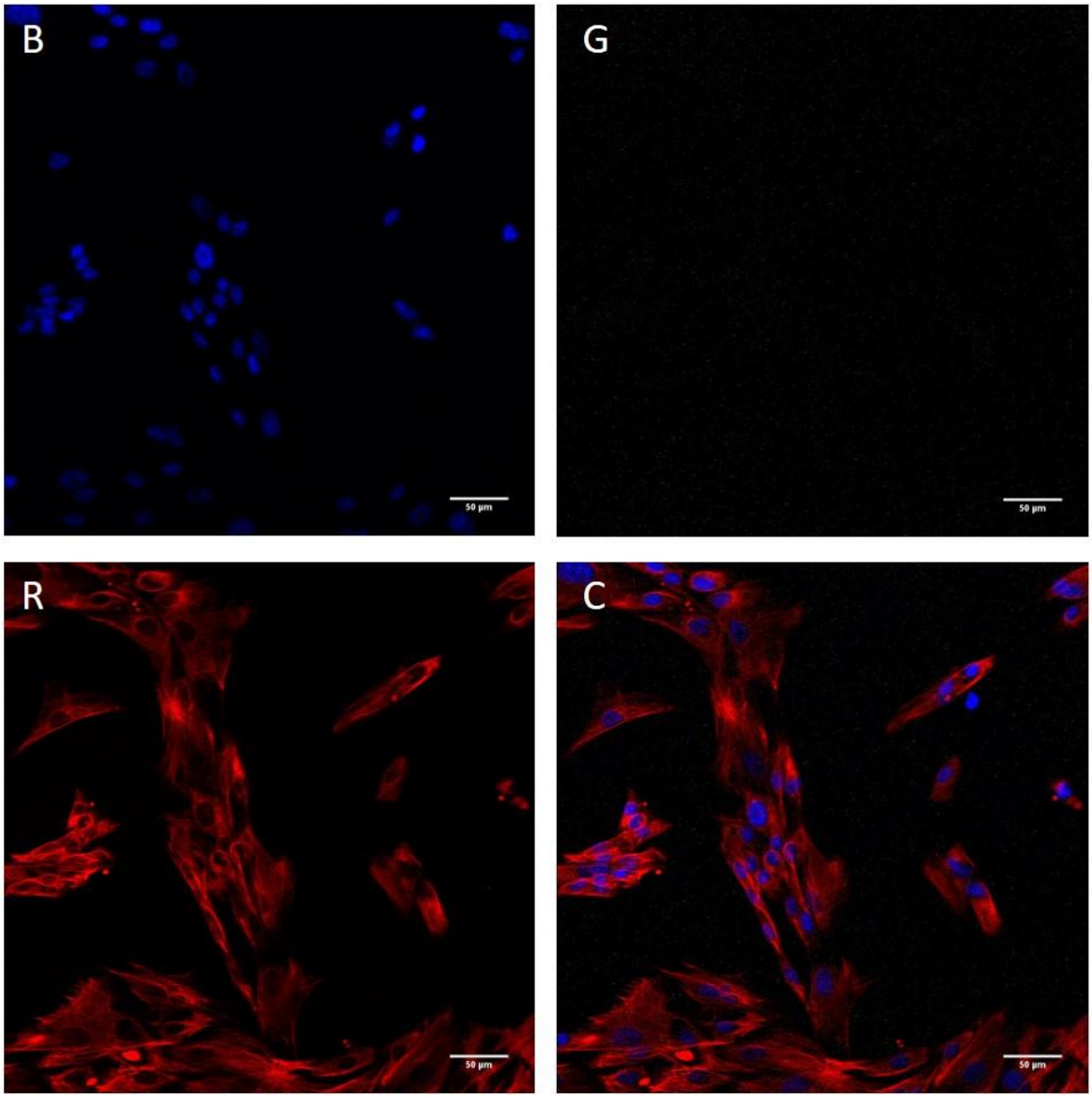


Figure 8.4 MRC-5 cells. Glass coverslip was mounted onto a glass slide before imaging at x20/0.30 magnification using Olympus IX73 microscope. Images were normalised and overlaid using ImageJ software. **B)** DAPI staining nucleus of all cells **G)** E-Cadherin (CD324 Monoclonal Antibody, DECMA-1, ThermoFisher, 202504) **R)** Vimentin (Recombinant Anti-Vimentin antibody (abcam, EPR3776) **C)** Composite image of RGB channels

8.3 Comments

We successfully optimised hAEC isolation after testing 4 methods and 4 types of trypsin (Figure 8.1). Our protocol used higher concentrations of trypsin for a shorter time with only one degradation step which reduced overall time by ~2hours compared to the current standard method (Gottipamula & Sridhar, 2018; Motedayyen et al., 2017; Tabatabaei et al., 2014) but did not decrease cell viability.

We discovered that the type of trypsin used for degradation was essential to cell isolation and viability. Trypsin-EDTA with phenol red 0.25%, (ThermoFisher 25200072) (usually used for cell passaging) and Trypsin from bovine pancreas (Sigma Aldrich, T1426-50MG) were not able to isolate sufficient cells from the membrane using any method. Trypsin-EDTA 0.5% from porcine pancreas, no phenol red (ThermoFisher, 15400054) isolated significantly fewer cells compared to trypsin 0.25% from porcine pancreas (SIGMA T4799) + EDTA 0.05% (SIGMA E6758) which we found to isolate sufficient numbers of viable epithelial cells using method 1 and 4. We also tested alternative enzymes such as pronase and collagenase A which significantly decreased the number of viable cells in culture due to degradation of isolated cells.

The variation in trypsin is supported by Tabatabaei et al, (2014) who specifically investigated the effect of 4 commercially available trypsins on hAEC isolation. They used: trypsin powder from porcine pancreas (Gibco 27250018, Sigma T4799, Fluka 93613) and Trypsin liquid (2.5%), no phenol red from porcine pancreas (Gibco 15090046) and found varying immunophenotypes and growth depending on isolation conditions.

After optimising isolation, we successfully validated the morphology of hAEC via the epithelial cell marker E-cadherin and the mesenchymal marker vimentin, using MRC-5 cells as a mesenchymal control. It is crucial to validate the extraction method to ensure that mesenchymal cells in the basement membrane are not also isolated as these cells require the same extraction protocol but with additional incubation time (Bilic et al., 2008).

Additionally, this staining method should be performed at all stages of an infection experiment to assess the cells adaptation to culture. Janzen et al., (2017) observed increased vimentin after TNF-α stimulation, however, vimentin was also observed to increase in the

untreated condition after 8 days of culture. (Alcaraz et al., 2013) found that E-cadherin expression was lost from hAEC after 3 passages.

Preliminary experiments and optimisations detailed in this appendix were all completed before March 2020. Lab work was continued when SARS-CoV-2 university restrictions permitted in July 2020. However, due to new safety guidelines such as reduced lab capacity, permission to work only half days, required lab booking, clinical restrictions regarding access to labour ward, and new clinical protocols on placenta disposal resulting in tissue collection challenges, continuation of hAEC infection experiments was not feasible. Instead, infection models utilising whole tissue from human fetal membranes was prioritised (see chapter 2). Despite these limitations, the preliminary results and extensive protocol optimisation in this chapter will form the base of future studies.

We successfully optimised and validated isolation of hAEC, our data highlights the necessity of consistent reagents when replicating published protocols as the same product from different manufacturers can produce unwanted differences.

In addition to amnion epithelial cells, decidual cells can be isolated from fetal membranes. Models utilising hAEC demonstrate response to more severe intraamniotic infection whereas models utilising decidual cells can better demonstrate response from vaginal organisms without infection as these are the first cellular layer of membrane above the cervix.