Safety, Tolerability and Colonisation Dynamics of a Vaginally Administered *Lactobacillus crispatus* Live Biotherapeutic Product in Pregnancy

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Statement of originality

I hereby state that all work presented in this thesis was performed by myself unless otherwise stated. Dr Katie Mountain contributed to the collection of metadata presented in Chapter 3 (Group A). Lauren Roberts provided and prepared DNA from 24 *L. crispatus* isolates as described in Chapter 4. Lauren Roberts also extracted DNA from lyophilised *L. crispatus* CTV-05.

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Abstract

The female reproductive tract is colonised by high levels of bacteria and other microbes collectively referred to as the microbiota. Vaginal microbiota dominated by *L. crispatus* associates with reduced preterm birth (PTB) risk, whilst *L. iners* dominance and *Lactobacillus* deplete, high diversity communities associate with increased risk. This thesis investigated whether a live vaginal biotherapeutic, *L. crispatus* CTV-05 (LACTIN-V) in pregnancy could modulate the microbiota towards a protective state. It was hypothesised that LACTIN-V would be safe, well-tolerated and acceptable, leading to successful colonisation of *L. crispatus* CTV-05 and reduced local inflammation.

LACTIN-V was administered to 62 women at high-risk of PTB once daily for five days from 14^{+0} weeks' gestation and then once weekly for six weeks. Women were assessed at enrolment and at 15^{+0}, 18^{+0}, 20^{+0}, 28^{+0} and 36^{+0} weeks gestation, and at delivery. Adverse events and compliance were assessed throughout, and a questionnaire determined acceptability. Vaginal microbiota and *L. crispatus* CTV-05 colonisation was assessed by metataxonomics, quantitative polymerase chain reaction (qPCR) and culture. Cervicovaginal fluid cytokine concentrations were measured by immunoassays.

LACTIN-V was found to be safe, well tolerated and acceptable during pregnancy (Chapter 3). A persistent shift towards *L. crispatus* dominance of the vaginal microbiota with *L. crispatus* CTV-05 colonisation was demonstrated in 65% of women at 28^{+0} weeks, eight weeks after administration (Chapter 4), alongside reduced cervicovaginal concentrations of IL-1ß, IL-8 and IL-6, particularly in women who transitioned from a high-risk microbiota at baseline.

In conclusion, LACTIN-V use in pregnancy is safe, well tolerated and leads to effective colonisation of *L. crispatus* in most women, displacing less favourable bacteria and reducing cervicovaginal inflammation. These results support the concept that vaginal administration of a live biotherapeutic containing *L. crispatus* can modulate the microbiota and immune milieu to a protective state, and so could reduce PTB.

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Articles in preparation

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Mountain KE, Yatham SS, Adan M, Arulkumaran S, **Bayar E,** Brown RG, Chandiramani M, Cook J, Chilvers J, Groom KM, Jones B, Kindinger L, Lewis HV, Merrick A, Ng S, Zarasvand Z, Mayer E, Glampson B, Terzidou V, Teoh TG, Bennett PR and Sykes L. Pregnancy outcomes in women attending preterm birth prevention services in the UK: a cohort study. *Submitted to Plos Medicine.*

Peer-reviewed articles

Bayar E, MacIntyre DA, Sykes L, Mountain K, Parks TP, Lee PP and Bennett PR, 2023. Safety, tolerability, and acceptability of *Lactobacillus crispatus* CTV-05 (LACTIN-V) in pregnant women at high-risk of preterm birth. *Beneficial Microbes*, *14*(1), pp.45-55.

Bayar E, Bennett PR, Chan D, Sykes L and MacIntyre DA, 2020. The pregnancy microbiome and preterm birth. *Seminars in immunopathology* (Vol. 42, pp. 487-499).

Zarasvand S, **Bayar E,** Adan M, Mountain K, Lewis H, Joash K, Teoh TG, Bennett PR, Das S and Sykes L, 2020. Rapid quality improvement in a preterm birth clinic care pathway during the COVID-19 pandemic. *BMJ Open Quality*, *9*(4), p.e001049.

Published conference abstracts

Bayar E, MacIntyre D, Sykes L, Lee Y, Parks T, Lee P, Bennett PR, 2022. Safety, tolerability and acceptability of Lactobacillus crispatus CTV-05 (LACTIN-V) in pregnancy. *BJOG* (Vol. 129, pp. 53-53).

Bayar E, Brown R, Chan D, Merrick A, Mountain K, Lewis H, Sykes L, Teoh TG, Bennett PR, 2022. Do emergency cervical cerclages save babies lives? *BJOG* (Vol. 129, pp. 48-49).

Gimeno-Molina B, **Bayar E,** Lee Y, Muller I, Botto M, MacIntyre DA, Bennett PR, Kropf P, Sykes L, 2022. Cervicovaginal inflammation and neutrophil infiltration/activation in women at high-risk of prematurity. *BJOG* (Vol. 129, pp. 54-54).

Bayar E, Zarasvand S, Adan M, Sykes L, Lee Y, Parks T, Lee P, MacIntyre DA, Bennett P, 2021. Acceptability of live vaginal biotherapeutics in a cohort of pregnant women. *BJOG* (Vol. 128, No. 8, pp. E32-E33).

Book chapter

Khoo C and **Bayar E** (2023). 'Chapter 16: Self-Assessment' in Bennett P, Williamson C, Sykes L, MacIntyre DA, Dixon PH (5th Edition) *Basic Science in Obstetrics & Gynaecology, a textbook for MRCOG Part 1.* Elsevier, pp. 278-304.

Conference Oral Presentations

Bayar E, Love RL, Lee YS, Ng S, Gimeno-Molina B, Parks T, Lee P, Bennett PR, Sykes L, MacIntyre DA. Live biotherapeutics for preterm birth prevention: vaginal administration of *Lactobacillus crispatus* CTV-05 in pregnancy leads to persistent colonisation and reduces inflammation. Society for Reproductive Investigation, Canada (2024).

Bayar E, Love RL, Lee YS, Ng S, Gimeno-Molina B, Parks T, Lee P, Bennett PR, Sykes L, MacIntyre DA. Live biotherapeutics for preterm birth prevention: vaginal administration of Lactobacillus crispatus CTV-05 in pregnancy leads to persistent colonisation and reduces inflammation. UK Annual Preterm Birth Conference, Liverpool (2024). (Oral Poster Pitch).

Bayar E, Love RL, Lee YS, Ng S, Gimeno-Molina B, Parks T, Lee P, Bennett PR, Sykes L, MacIntyre DA. Live biotherapeutics for preterm birth prevention. Annual Academic Meeting Royal College of Obstetricians and Gynaecologists, London (2024).

Gimeno-Molina B, **Bayar E,** Mountain KE, Love RL, Merrick AV, Riani-El-Achhab S, Lee Y, Muller I, David AL, Terzidou V, Bennett PR, MacIntyre DM, Botto M, Kropf P, Sykes L. Cervical shortening and spontaneous preterm birth are associated with cervical neutrophil migration and complement activation. UK Annual Preterm Birth Conference, Liverpool (2024).

Capuccini K, Manolia E, Correia G, **Bayar E,** Gimeno-Molina B, Sykes L, Takats Z, Bennett PR, MacIntyre DA. Direct on-swab metabolic profiling by DESI-MS for monitoring and stratification of live biotherapeutic intervention during pregnancy. Metabolomics in Life Science Conference, Sweden (2024).

Gimeno-Molina B, **Bayar E,** Love RL, Merrick AV, Riani-ElAchhab, S, Lee YS, Muller I, David AL, Terzidou V, Bennett PR, MacIntyre DA, Botto M, Kropf P, Sykes L. Cervical shortening is associated with cervical neutrophil migration and complement activation. Society for Reproductive Investigation, Australia (2023).

Mountain KE, Yatham SS, **Bayar E,** Lewis HV, Kindinger L, Terzidou V, Teoh TG, Bennett PR, Sykes L. Pregnancy outcomes in women attending preterm birth prevention services in the UK: a cohort study. Royal Society of Medicine, Obstetrics & Gynaecology Meeting (2023).

Gimeno-Molina B, **Bayar E,** Mountain K, Love R, Lee Y, Muller I, Dell A, Haslam S, Grassi P, Wu G, MacIntyre D, Bennett PR, Kropf P, Sykes L. The role of cervical

neutrophils in cervicovaginal inflammation in women at high-risk of delivering preterm. The International Society for Immunology of Reproduction (2023).

Gimeno-Molina B, Love R, Mountain KE, **Bayar E,** Lee YS, David AL, Terzidou V, Kropf P, Bennett PR, MacIntyre DA, Botto M, Sykes L. The complement system in cervical shortening and spontaneous preterm birth. The American Society for Immunology of Reproduction (2023).

Gimeno-Molina B, Love RL, **Bayar E,** Merrick AV, Riani-El-Achhab S, Lee YS, Muller I, David AL, Terzidou V, Bennett PR, MacIntyre DA, Botto M, Kropf P, Sykes L. Cervical shortening is associated with cervical neutrophil migration and complement Activation. Royal Society of Medicine, Obstetrics & Gynaecology Meeting (2023).

Bayar E, Lee YS, Ng S, Gimeno-Molina B, Parks T, Lee P, Bennett PR, Sykes L, MacIntyre DA. Live vaginal biotherapeutics as a prevention strategy for preterm birth. Invited to present at the Royal Society of Medicine, Luke Zander Research Bursary Prize winner (2022).

Gimeno-Molina, B, **Bayar E,** Love RL, Merrick AV, Riani-ElAchhab, S, Lee YS, Muller I, David AL, Terzidou V, Bennett PR, MacIntyre DA, Botto M, Kropf P, Sykes L. Cervical shortening is associated with cervical neutrophil migration and complement Activation. Victor Bonney Academic Meeting (2022).

Bayar E, Zarasvand S, Adan M, Sykes L, Lee YS, Parks T, Lee PP, MacIntyre DM, Bennett PR. Acceptability of live vaginal biotherapeutics in a cohort of pregnant women. Annual Academic Meeting Royal College of Obstetricians and Gynaecologists (2021). (Oral Poster Pitch).

Bayar E, Teoh TG, Bennett PR, Sykes L. The role of fetal fibronectin in guiding the management of threatened preterm labour. Annual Academic Meeting Royal College of Obstetricians and Gynaecologists (2021). (Oral Poster Pitch).

Bayar E, Zarasvand S, Sykes L, Lee YS, Parks TP, Lee PP, MacIntyre DA, Bennett PR. Tolerability and compliance of live vaginal biotherapeutics in a cohort of pregnant

women at high risk of preterm birth. 7th International Congress on Probiotics, Prebiotics in Paediatrics (Virtual, 2021).

Mountain KE, Yatham SY, **Bayar E**, Adan M, Zarasvand S, Terzidou V, Teoh TG, Bennett PR, Sykes L. Pregnancy outcomes in preterm birth prevention services: approaching individualised patient risk profiles. UK Annual Preterm Birth Conference (Virtual, 2021).

Bayar E, Teoh TG, Bennett PR, Sykes L. Fetal fibronectin in guiding the management of threatened preterm labour. Royal College of Obstetrics & Gynaecologists World Congress, Oman 2020. *Congress cancelled due to COVID-19 pandemic.*

Conference Poster Presentations

Gimeno-Molina B, Lee YS, Chan D, Love RL, Mountain KE, **Bayar E,** Botto M, David AL, Terzidou V, Bennett PR, MacIntyre DA, Kropf P, Collado MC, Sykes L. Opsonisation of vaginal microbiota in women at high-risk of delivering preterm. UK Annual Preterm Birth Conference, Liverpool (2024).

Capuccini K, Manoli E, Correia G, **Bayar E**, Gimeno-Molina B, Sykes L, Takats Z, Bennett PR and MacIntyre DA. Direct on-swab metabolic profiling by DESI-MS for monitoring and stratification of live biotherapeutic intervention during pregnancy. UK Annual Preterm Birth Conference, Liverpool (2024).

Gimeno-Molina B, **Bayar E,** Love RL, Merrick AV, Riani-ElAcha, S, Lee YS, Muller I, David AL, Terzidou V, Bennett PR, MacIntyre DA, Botto M, Kropf P, Sykes L. Cervical Shortening is Associated with cervical neutrophil migration and complement activation. Annual Academic Meeting Royal College of Obstetricians and Gynaecologists (2023).

Mountain KE, Yatham SY, **Bayar E,** Adan M, Zarasvand S, Brown R, Lewis HV, Cook J, Chandiramani M, Jones B, Kindinger L, Terzidou V, Teoh TG, Bennett PR, Sykes L. Pregnancy outcomes in women attending preterm birth prevention services in the

UK: a cohort study. 4th European Spontaneous Preterm Birth Congress, The Netherlands (2023).

Gimeno-Molina B, Lee YS, Chan D, Mountain KE, **Bayar E,** Botto M, David AL, Terzidou V, Bennett PR, MacIntyre DA, Kropf P, Collado MC, Sykes L. Opsonisation of vaginal microbiota in women at high-risk of delivering preterm. 4th European Spontaneous Preterm Birth Congress, The Netherlands (2023).

Gimeno-Molina B, Lee YS, Chan D, Mountain KE, **Bayar E,** Botto M, David AL, Terzidou V, Bennett PR, MacIntyre DA, Kropf P, Collado MC, Sykes L. Opsonisation of vaginal microbiota in women at high-risk of delivering preterm. The American Society for Immunology of Reproduction (2023).

Gimeno-Molina B, **Bayar E,** Love RL, Merrick AV, Riani-El-Achhab S, Lee YS, Muller I, David AL, Terzidou V, Bennet PR, MacIntyre DA, Botto M, Kropf P, Sykes L. Cervical Shortening and Spontaneous Preterm Birth are Associated With Cervical Neutrophil Migration and Complement Activation. 4th European Spontaneous Preterm Birth Congress, The Netherlands (2023).

Gimeno-Molina B, **Bayar** E, Lee YS, Muller I, Botto M, MacIntyre DA, Bennett PR, Kropf P, Sykes L. Cervicovaginal inflammation and neutrophil infiltration/activation in women at high-risk of prematurity. Royal College of Obstetrics & Gynaecologists World Congress, London (2022).

Gimeno-Molina B, **Bayar E,** Lee YS, Muller I, Botto M, Dell A, Haslam S, Grassi P, Wu G, MacIntyre DA, Bennett PR, Kropf P, Sykes L. Cervicovaginal inflammation is associated with cervical neutrophil infiltration and activation in women at high-risk of delivering preterm. Society for Reproductive Investigation, USA (2022).

Budwig L, Brown RG, Lee YS, Mountain K, Gimeno-Molina B, Adan M, **Bayar E,** MacIntyre DA, Bennett PR, Sykes L. The use of peripheral blood neutrophil counts in the prediction of funisitis following preterm prelabour rupture of membranes. Society for Reproductive Investigation, USA (2021).

Mountain KE, Yatham SY, **Bayar E,** Adan M, Zarasvand S, Terzidou V, Teoh TG, Bennett PR, Sykes L. Pregnancy outcomes in preterm birth prevention services: approaching individualised patient risk profiles. Society for Reproductive Investigation, USA (2021).

Zarasvand S, **Bayar E,** Adan M, Mountain M, Lewis H, Joash K, Teoh TG, Bennett PR, Das S, Sykes L. Rapid quality improvement in a preterm birth clinic during the COVID-19 pandemic. UK Annual Preterm Birth Conference (Virtual 2021).

Bayar E, Quinlan R, Akers R, Adan M, Smith-Junkere K, Sykes L, MacIntyre D, Bennett PR. Acceptability of vaginal probiotics in a cohort of pregnant women. RCOG congress 2020. Royal College of Obstetrics & Gynaecologists World Congress, Oman (2020). *Congress cancelled due to COVID-19 pandemic.*

Prizes

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Letter of Appreciation: Global COVID-19 Pandemic. Imperial College NHS Trust (Professor Tim Orchard, Chief Executive), 2020.

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Abbreviations

PTB	Preterm birth
PVL	Peri-ventricular leukomalacia
IVH	Intra-ventricular haemorrhage
IQ	Intelligence quotient
BPD	Bronchopulmonary dysplasia
NEC	Necrotising enterocolitis
EOS	Early onset neonatal sepsis
LOS	Late onset neonatal sepsis
TLR	Toll-like receptor
IL	Interleukin
iNOS	Induced nitric oxide synthase
HA	Hyaluronan
GAC	Glycosaminoglycans
TNF-α	Tumour necrosis factor-α
NF-κB	Nuclear factor kappa-B
COX	Cyclooxygenase
PGE2	Prostaglandin E2
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitor matrix metalloproteinase
CRH	Corticotrophin releasing hormone
PIP_2	Phosphatidylinositol-4, 5-bisphosphate
IP_3	Inositol-1, 4, 5-triphosphate
PR	Progesterone receptor
CRHBP	Corticotrophin releasing hormone binding protein
cAMP	Cyclic adenosine monophosphate
sPTB	Spontaneous preterm birth
DHEA	Dehydroepiandrosterone
PAPP-A	Pregnancy associated plasma protein-A
DNA	Deoxyribonucleic acid
PPROM	Preterm premature rupture of membranes
STI	Sexually transmitted infection
UTI	Urinary tract infection
BV	Bacterial vaginosis
HPV	Human papilloma virus
PCR	Polymerase chain reaction
CMV	Cytomegalovirus
IUD	Intra-uterine death
NND	Neonatal death
MTL	Mid-trimester loss
LLETZ	Large loop excision of the transformation zone
FDCS	Fully dilated caesarean-section
BMI	Body mass index
TVUS	Transvaginal ultrasound scanning
NICE	National Institute for Health and Clinical Excellence

fFN	Fetal fibronectin
IGFBP-1	Insulin-like growth factor binding protein-1
PAMG-1	Placental alpha-microblobulin-1
CVF	Cervicovaginal fluid
NPV	Negative predictive value
QUiPP App	Quantitative Innovation in Predicting Preterm Birth App
BAPM	British Association of Perinatal Medicine
phIGFBP-1	Phosphorylated form of insulin-like growth factor binding protein-1
PPV	Positive predictive value
RCT	Randomised control trial
IM	Intra-muscular
GBS	Group B streptococcus
GA	Gestational age
NICU	Neonatal intensive care unit
RNA	Ribonucleic acid
16S rRNA	16S ribosomal RNA
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
CST	Community state type
DC	Dendritic cell
PPR	Pattern recognition receptors
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular pattern
NK cells	Natural killer cells
Treg	Regulatory T cells
Th cells	T-helper cells
CRP	C-reactive protein
MBL	Mannose-binding lectin
MAC	Membrane attack complex
APC	Antigen presenting cell
TCR	T cell receptor
MHC	Major histocompatibility
IFN-γ	Interferon-γ
TGF	Transforming growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leucocyte antigens
IL-1ra	Interleukin-1 receptor antagonist
ICAM-1	Intercellular adhesion molecule-1
PBMC	Peripheral blood mononuclear cell
MIP	Macrophage inflammatory protein
Ig	Immunoglobulin
H_2O_2	Hydrogen peroxide
MRS	De-Man-Rogosa-Sharpe
OTU	Operational taxonomic unit
LACTIN-V	*Lactobacillus crispatus* CTV-05
PBS	Phosphate-buffered saline
NaOAC	Sodium acetate
HCl	Hydrochloride
EDTA	Ethylenediaminetetraacetic acid
DEPC	Diethyl pyrocarbonate

PI	Protease inhibitor
dNTP	Deoxyribonucleotide phosphate
TBE	Tris/Borate/EDTA
RT-PCR	Real time-PCR
NaOH	Sodium hydroxide
bp	Base pair
rpm	Revolutions per minute
CFU	Colony forming units
FLIP-1 trial	Feasibility of LACTIN-V in Pregnancy-1 trial
PPI	Patient and Public Involvement
VMET2	Vaginal Microbiome and Metabolome in Pregnancy
HRA	Health Research Authority
QCCH	Queen Charlotte's and Chelsea Hospital
qPCR	Quantitative PCR
T	Timepoint
ELCS	Elective caesarean-section
UV	Ultraviolet
BASHH	The British Association for Sexual Health and HIV
STRB	ChromID Strepto B
MALDI-TOF	Matrix-assisted laser desorption/ionisation coupled to time-of-flight mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
IQR	Interquartile range
PDSA	Plan, Do, Study, Act
ID	Identification
DCDA	Dichorionic diamniotic
CIN	Cervical intraepithelial neoplasia
NIH	National Institute of Health
HBLC	High bacterial load colonisation
iPTB	Iatrogenic preterm birth
NC	Negative control
PC	Positive control
ASV	Amplicon sequence variant
RT-qPCR	Real time-quantitative PCR
DESI-MS	Direct on-swab desorption electrospray ionization mass spectrometry
EMCS	Emergency caesarean-section
JAK/STAT	Janus kinase-signal transducer
OOR	Out of range
sPT	Spontaneously preterm
PT	Preterm
iPT	Iatrogenic preterm
VMT	Vaginal microbiota transplantation

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1 Introduction

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1.1 Preterm birth

1.1.1 Scope of the problem

Preterm birth (PTB) is defined as delivery before 37^{+0} weeks($^{+days}$) gestation and is the leading cause of death among children aged under 5. Over the past two decades, the worldwide under-5 mortality has halved, from 9.65 million in 2000 to 5.05 million in 2019. However, despite advancements in maternal and neonatal care the proportion of deaths due to PTB have increased from 14.5% to 17.7% (1). The global prevalence of PTB varies according to region. Although 90% of PTBs occur in low or middle-income countries, high-income countries are not exempt (Figure 1.1) (2).

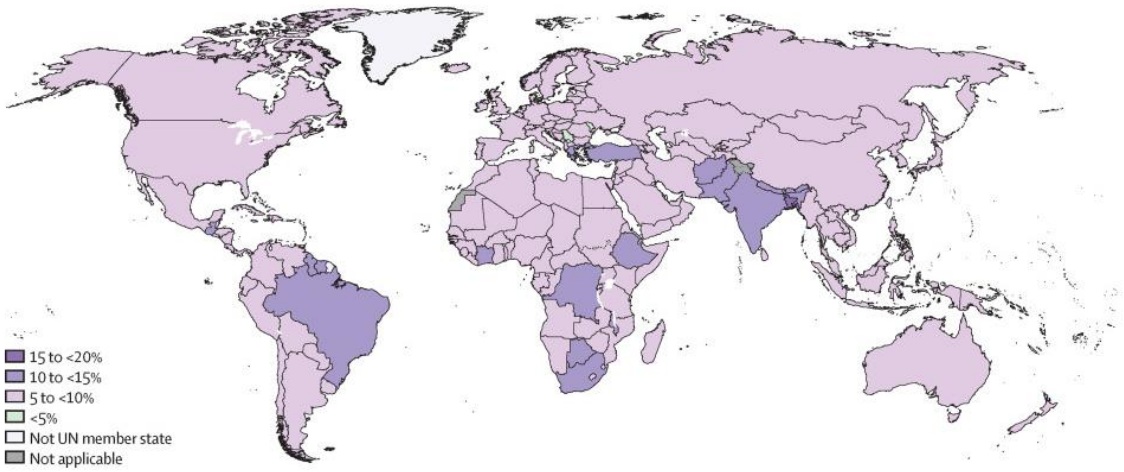


Figure 1.1 – Global PTB rates in 2020, reproduced from Ohuma et al 2023 (3).

The estimated global prevalence of PTB in 2020 was 9.9%, equating to 13.4 million preterm livebirths. Bangladesh had the highest rate of PTB in 2020 (16.2%). High rates of PTB were also observed in high-income countries including Greece (10%) and the USA (10%).

When compared to other European countries, PTB rates in the UK are high (4, 5). Data from the Office for National Statistics shows that the PTB rate in England and Wales is consistently 7%, of which, 11% are born between 28^{+0} - 32^{+0} weeks' gestation and 5% at $<28^{+0}$ weeks' gestation (6). A recent review of PTB global prevalence showed no changes since 2010 (3). Between 1995 to 2006 rates of neonatal morbidity

as a result of prematurity remained unchanged (7). Comparisons of neonatal morbidity between 2007 to 2016 has also showed no improvement (8).

1.1.2 Neonatal survival

The World Health Organisation defines prematurity as extremely preterm (<28^{+0} weeks), very preterm (28^{+0} to 31^{+6} weeks) and late preterm (32^{+0} to 36^{+6} weeks) (9). Gestational age at birth is inversely related to neonatal mortality and morbidity (10). The use of antenatal corticosteroids, assisted ventilation and advances in neonatal intensive care facilities, have led to improved neonatal survival since 1995, with the greatest improvement seen in those born between 22^{+0} and 23^{+6} weeks' gestation (11). Survival rates require cautious consideration as they may include survival that is disproportionately associated with disability. The EPIPAGE-2 study showed that survival without adverse outcomes was significantly higher in those born between 25^{+0} - 31^{+0} weeks' gestation in 2011 when compared to 1997 (12). Peri-viability represents the stage of fetal development that lies at the cusp of extra-uterine survival and includes 22^{+0} - 26^{+0} weeks' gestational age (13). Studies of survival rates in infants born in the peri-viable phase have been conflicting. The EPIPAGE-2 study demonstrated that improved survival without morbidity did not occur in those born before 24^{+0} weeks' gestation between 1997 and 2011 (12). However, analysis of >4000 peri-viable infants born at 22^{+0} - 24^{+0} weeks' gestation showed survival without morbidity to improve from 16% to 20% from 2000 to 2011 (14). Different definitions of neonatal morbidity at differing childhood stages may contribute to contradicting results. The management of peri-viable neonates comes with clinical and ethical challenges requiring a balance of survival with long-term quality of life.

1.1.3 Neonatal morbidity

During the last 15 weeks of gestation the fetal brain undergoes profound maturation (15). Prematurity predisposes neonates to brain injury including peri-ventricular leukomalacia (PVL) and intra-ventricular haemorrhage (IVH) and can result in lifelong disability (Figure 1.2) (15). The fragile microvasculature, immature vessel walls, fluctuations in cerebral blood flow and lack of autoregulation are important factors

predisposing preterm neonates to IVH (16). The most severe cases of IVH occur in those born at 24^{+0} weeks' gestation and impacts up to 22% (12). Cerebral palsy is defined as a group of disorders affecting an individual's ability to move and balance and is the primary motor disorder following PTB (17).

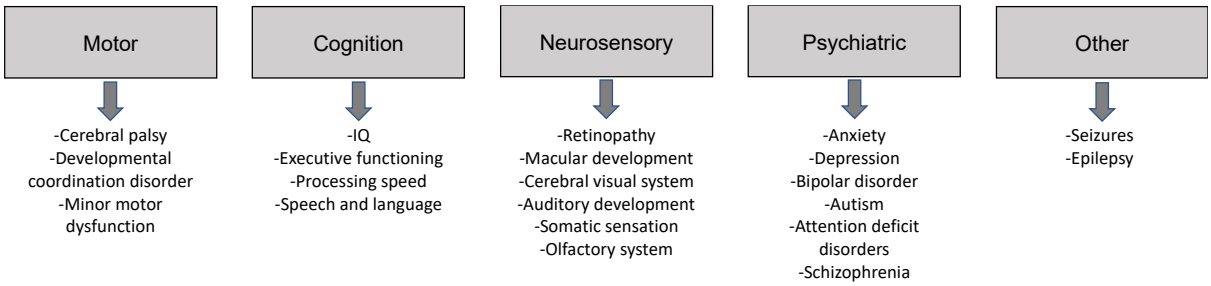


Figure 1.2 - Neurological sequelae of PTB. A range of neurological consequences can occur in infants born preterm and can be divided into neurosensory, cognitive, motor and psychiatric categories. Such consequences can impact life-long health (18). IQ = intelligence quotient.

Neurological impact can persist from childhood through to adolescence and adulthood. In the absence of cerebral palsy, children born $\leq 32^{+0}$ weeks' display impaired motor performance from infancy through to the age of 15 when compared to term children (19).

Preterm infants are also at risk of developing bronchopulmonary dysplasia (BPD), which affects 45% of infants born before 29^{+0} weeks' gestation (20). BPD remains a life-long systemic condition, predisposing infants to recurrent respiratory infections and asthma. Necrotising enterocolitis (NEC) is an inflammatory condition of the gastrointestinal tract impacting preterm infants, causing feeding difficulties, bowel necrosis and perforation (21). Preterm neonates are at risk of sepsis, with the incidence being inversely proportional to gestational age at birth (22). This can be divided into early onset neonatal sepsis (EOS), which develops within 72 hours of life and late onset neonatal sepsis (LOS), which develops >72 hours of life. *Streptococcus agalactiae* and *Escherichia coli* account for 70% of EOS (23).

1.1.4 Maternal impact

Prematurity impacts on the psychosocial and emotional wellbeing of women and their families. Parental relationships with preterm infants can be disrupted due to the environment of neonatal intensive care units (24, 25). Depression and anxiety are more common in mothers of preterm infants when compared to mothers of term infants (26). In the first year of life after PTB, higher levels of psychological distress, traumatic and depressive symptoms are seen, when compared to term mothers (27). These responses can persist with post-traumatic stress symptoms reported in up to 41% of mothers 18 months after a PTB (28). The infant's physical appearance has been identified as one of the greatest sources of stress for parents, exacerbated by the lights and sounds of equipment in neonatal intensive care units (29). Preterm delivery has even been identified as a risk factor for early mortality in women. Analysis of outcomes of over 2 million women delivering between 1973 and 2015 identified that the length of pregnancy was inversely associated with women's mortality (30).

1.1.5 Financial impact

In England and Wales, PTB is estimated to cost £3.4 billion per year (31). Although most costs relate to the inpatient hospitalisation period, the financial impact persists. Healthcare costs per preterm child surviving to the age of 18 are higher when compared to term children (32). Unsurprisingly, long-term morbidity associated with PTB, such as BPD and NEC contribute to long-term healthcare costs (33).

PTB also places financial strain on other sectors of the economy, families and wider society. Such categories are less widely studied but remain important. Non-healthcare costs to families include travel costs and loss of employment (34). Mothers experiencing PTB take longer to return to employment, have a lower weekly income and have higher spending when compared to mothers delivering at full term (35).

1.2 Physiology of term parturition

Parturition, the process of giving birth, involves carefully orchestrated changes that occur in the myometrium, decidua and cervix. Transition from a quiescent state of pregnancy to labour involves regulation of anatomical, immunological, biochemical and endocrine mechanisms. Three key events precede labour: cervical remodelling, membrane activation and myometrial activation. Understanding the physiology of normal parturition has facilitated the development of pharmacological agents used in the induction of labour and as tocolytics. Four phases of parturition have been described (36). These include:

1. Quiescence: Maintenance of myometrial quiescence and cervical mechanical integrity.
2. Activation: Gradual change from quiescence to active labour whereby the myometrium is responsive to signalling and cervical tissue integrity changes to allow cervical remodelling.
3. Stimulation: This includes active labour, whereby uterine contractions, cervical effacement and dilatation occur allowing for delivery of the fetus and placenta.
4. Involution: Uterine involution, lactation and recovery mechanisms begin.

1.2.1 Cervical remodelling

During pregnancy, the cervix functions as a protective barrier from micro-organisms and provides structural rigidity and support for a growing fetus and uterus. Endocervical epithelial cells confer protection from microorganisms by secreting defensins and thick mucus, and increasing expression of Toll-like receptors (TLRs) which bind to bacterial toxins (37). Structural support is accomplished by maintaining strength at the internal os to prevent protrusion of fetal membranes through the cervical canal. In early pregnancy, 85% of the cervix consists of type I and type III collagen fibres, providing strength (37). The remainder of the cervical tissue consists of elastin, fibroblasts, smooth muscle cells and white blood cells (37).

Transvaginal ultrasound assessments show that most women maintain a cervical length of 30 to 40mm throughout pregnancy (38). In normal pregnancy, the cervical length gradually shortens by 3mm during the second trimester and by 5mm at term (37, 39). A dichotomy exists whereby the cervix must remain long and closed throughout pregnancy, whilst having capability to soften and dilate during labour. Cervical remodelling describes cervical transitioning from a rigid to an elastic state, permissive of delivery. The stages of cervical change during pregnancy include softening, ripening, dilation and repair (Figure 1.3) (37).

Cervical softening can be seen as early as the first trimester and occurs due to increased vascularity and oedema of the cervix, hypertrophy of cervical stroma and hyperplasia of cervical glands (37). In early pregnancy, increased collagen synthesis and solubility as well as cervical growth maintains tensile strength and integrity (40). Prior to pregnancy, endocervical epithelial cells are sparse. During pregnancy, endocervical epithelial cells proliferate, occupying 50% of the entire cervical mass (37). Sonographic assessments have shown cervical gland area to increase with pregnancy leading to cervical softening (41). Additionally, endocervical epithelial cells produce enzymes which contribute to prostaglandin synthesis, release protease inhibitors and chemokines including interleukin (IL)-8 (42, 43).

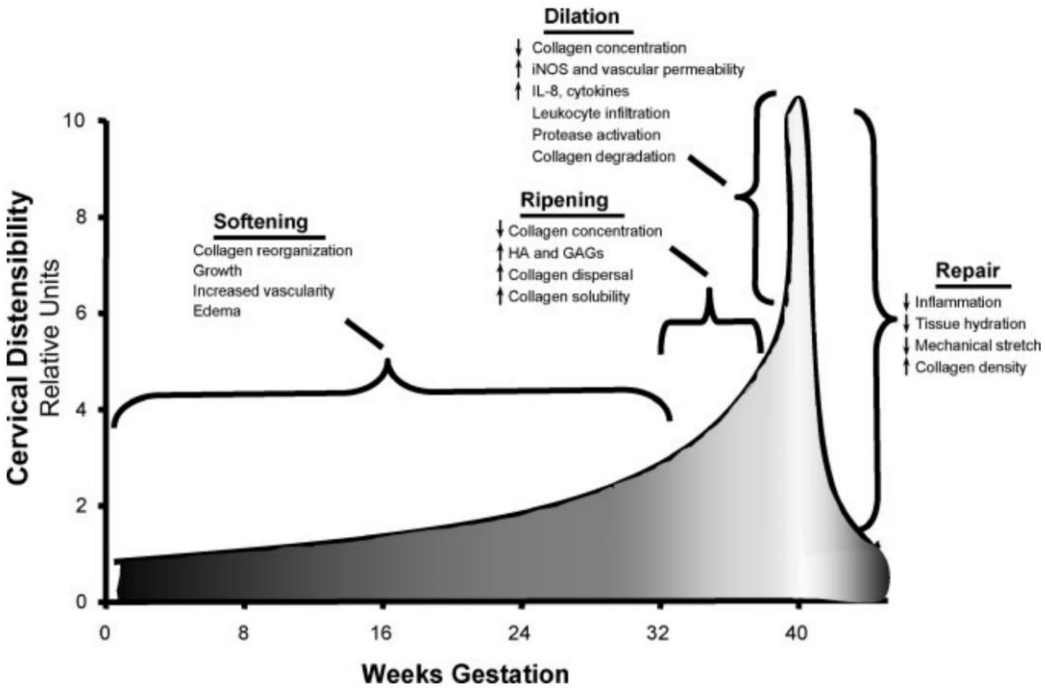


Figure 1.3 – Cervical remodelling in pregnancy, reproduced from Word et al (2007) (37). The stages of cervical change throughout pregnancy include softening, ripening, dilation and repair. The phases occur overlappingly and are each controlled by individual cellular pathways. Abbreviations: iNOS (induced nitric oxide synthase), IL-8 (interleukin-8), HA (hyaluronan), GACs (glycosaminoglycans)

Cervical ripening occurs in the last few weeks of pregnancy and is characterised by transition to a thin, soft and stretchable state. Regulation of cervical remodelling is not fully understood, however corresponds with an increase in cervical cytokines such as IL-6, IL-8, IL-1β, tumour necrosis factor-α (TNF-α) and cervical influx of activated macrophages, T-lymphocytes, mast cells, neutrophils and eosinophils (44, 45). Culture and cytokine assays using cervical tissue have shown its capacity to produce large amounts of IL-8, through which neutrophil-mediated cervical ripening occurs (46). During cervical ripening, collagen concentrations reduce and collagen fibrils become more widely dispersed, due to the synthesis of hydrophilic glycosaminoglycans (GACs) causing a reduction in tensile strength and cervical thinning (47). Increases in hyaluronan (HA) causes collagen dispersal and can stimulate macrophages to initiate inflammatory cascades (48, 49). Cytokines stimulate angiogenesis, vasodilatation and vessel permeability, allowing for further influx of

leucocytes and release of degradative enzymes permitting additional collagen breakdown and cervical ripening (50).

During cervical dilatation infiltrating leucocytes produce collagenase, leading to collagen degradation and matrix remodelling (51). This is largely caused by the release of proteolytic granules from infiltrating inflammatory cells, including neutrophils and monocytes. Cytokine and chemokine release (IL-8, IL-1 and TNF-α) from neutrophils activate the nuclear factor kappa-B (NF-κB) signalling pathway, which stimulates expression of labour associated genes such as cyclooxygenase (COX-2) and prostaglandin E2 (PGE2) (52). Following delivery there is a rapid repair response whereby relief of mechanical distension terminates the described signalling pathways.

1.2.2 Rupture of fetal membranes

The fetal membrane is a bilayer structure surrounding and protecting the developing fetus and is composed of the amnion and chorion (53). The amnion represents the innermost layer which is in contact with the amniotic fluid, is avascular and functions to provide structural strength, mechanical protection of the fetus and produce prostaglandins (54). The chorion is highly vascular and serves as an immunological barrier for fetal protection (55).

Rupture of the fetal membranes is a sequential process. Firstly, there is distension and loss of elasticity, followed by separation of the chorion and amnion, disruption of the chorion, herniation of the amnion and finally amnion rupture (56, 57). Loss of elasticity and thinning occurs because of similar biochemical triggers of cervical remodelling, due to local production of pro-inflammatory cytokines, prostaglandins and matrix metalloproteinases (MMPs) (57-59). Disruption of the chorion occurs due to collagen reduction. Collagen turnover within the fetal membranes is regulated by a balance of MMPs which breakdown collagen and TIMPs (tissue inhibitor matrix metalloproteinases) which inhibit MMPs (60). As pregnancy advances, MMP activity dominates over TIMP activity, leading to a loss of collagen and thinning of the fetal membranes (61). This effect is most marked from 38^{+0} weeks of pregnancy and at the portion of the fetal membranes overlying the internal os (61). Prostaglandin release

from the cervix, decidua, chorion and amnion also contribute to increases in MMPs (62). Following term rupture of fetal membranes, 70% and 95% of women enter labour within 24 and 72 hours respectively (63).

1.2.3 Uterine contractions

The uterus consists of three layers and includes the innermost endometrium, myometrium and serosal layers. The myometrium consists mostly of smooth muscle cells known as myocytes that express high levels of contractile proteins, actin and myosin (64, 65). Sliding between actin thin filaments and myosin thick filaments leads to myometrial contraction and is stimulated by myosin light chain kinase and inhibited by myosin light chain phosphatase. Activation of myosin light chain kinase and phosphatase are regulated by many intracellular signalling pathways (66, 67).

1.2.4 Hormonal influence

Hormones such as oxytocin, progesterone and corticotrophin releasing hormone (CRH) also play a role in the initiation of parturition.

Oxytocin is an uterotonic produced by the hypothalamus and released from the posterior pituitary gland. On binding to its G-protein coupled receptor, oxytocin activates phospholipase C, which subsequently hydrolyses phosphatidylinositol-4, 5-bisphosphate (PIP_2) to inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol, leading to calcium mobilisation (68, 69). This increase in calcium release allows for the formation of the calcium-calmodulin complex, which triggers myosin light chain kinase activity (70). Myosin light chain kinase phosphorylates the myosin light chains, allowing acto-myosin cycling and myometrial contractions (70, 71). Oxytocin also stimulates secretion of CRH and prostaglandin F2α from trophoblast cells (72, 73).

Progesterone is produced from the corpus luteum and plays a vital role in pregnancy maintenance. The name ‘progesterone’ stems from its function as a pro-gestational steroid hormone (74). From about 7^{+0} to 9^{+0} weeks gestation, the placenta becomes the main source of progesterone production. Progesterone binds to progesterone

receptors (PRs), which are ligand-activated nuclear transcription regulators, expressed in gestational tissues (75). The two PR receptors include PR-A and PR-B. In response to progesterone, the two receptors become transcriptionally active, affecting the expression of different genes (76). PR-A and PR-B have opposite effects on pro-inflammatory gene expression. When the PR-A to PR-B ratio favours PR-B, progesterone causes a decrease in pro-inflammatory gene expression (76). In contrast, a ratio that favours PR-A, leads to increased pro-inflammatory gene expression. For the most of human pregnancy, PR-B dominate and promote myometrial quiescence through PR-B mediated anti-inflammatory actions (76). During labour, a rise in PR-A expression occurs, which promotes parturition by inhibiting the anti-inflammatory actions of PR-B and triggering pro-inflammatory gene expression (76).

Placental CRH release increases throughout pregnancy and plays a role in initiating myometrial contractions. CRH binding protein (CRHBP) limits concentrations of free CRH. At term, CRHBP levels fall, resulting in an increase in CRH. On binding to its receptor, CRH increases COX-2 within the amnion (77). This stimulates MMP production, which in turn increases cyclic adenosine monophosphate (cAMP) production, thus inducing myometrial contractions (78).

1.2.5 Regulation of term parturition

The mechanisms that synchronise timing of human parturition involve tightly regulated endocrine, paracrine, mechanical and immune factors. Various types of ‘gestational clocks’ have been described, according to tissue compartments and molecular pathways (Figure 1.4). The gestational period aims to support development of the fetus to a point whereby it can maintain homeostasis self-sufficiently *ex-utero*. It is therefore unsurprising that fetal maturation plays a role in regulating parturition. Spontaneous PTB (sPTB) can occur when there is a breakdown in these biological clocks.

The placental clock has been described as a key determinant for the length of gestation. A rapid increase in circulating levels of bioavailable CRH at term triggers

parturition (79, 80). CRH measurements at 16^{+0} to 20^{+0} weeks of pregnancy have shown ability to identify women destined to deliver early or post-dates (79). Women delivering preterm show higher and earlier CRH rises (81). Peaks in CRH concentrations precede IL-6 surges, suggesting that CRH release also influences cytokine release (82). Uteroplacental transfer of CRH to the fetus occurs, acting on the fetal pituitary and adrenal glands, leading to production of corticotrophin, cortisol and dehydroepiandrosterone (DHEA) (83). Fetal surfactant production occurs as a response to cortisol, accelerating fetal lung maturation.

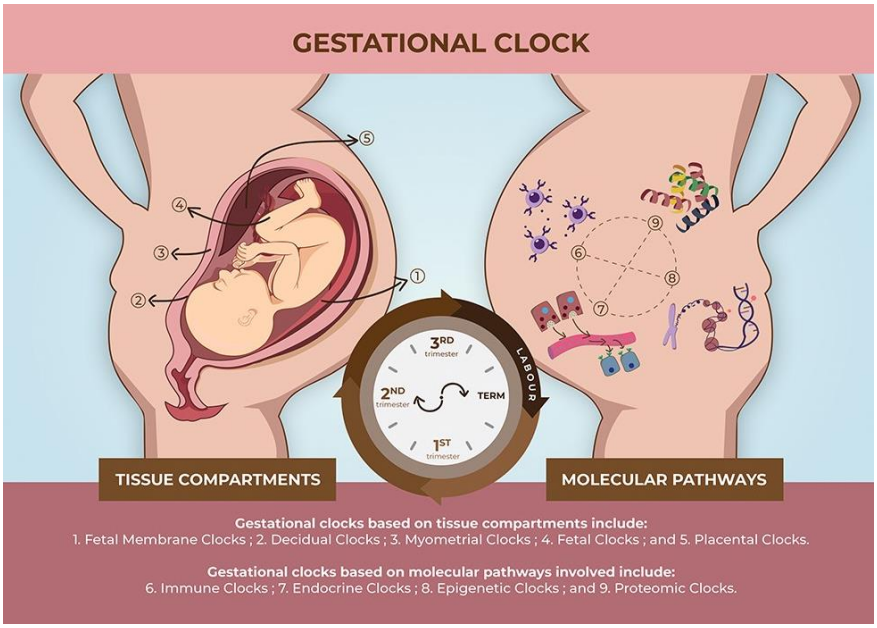


Figure 1.4 – Gestational clocks, reproduced from Wijaya et al (2020) (84). Various gestational clocks have been described to regulate the onset of term parturition. Gestational clocks at tissue compartments include the fetal membrane, decidua, myometrium, fetus and placenta. Gestational clocks according to molecular pathways include immune, endocrine, epigenetic and proteomic clocks.

The endocrine clock represents the interaction between maternal, placental and fetal hormones and the onset of labour (85, 86). This includes the impact of progesterone withdrawal, oxytocin and prostaglandins. Animal studies show a drop in circulating progesterone levels with parturition (87). In contrast, humans do not demonstrate a substantial drop in progesterone with the onset of labour (88, 89). Instead, inactivation of progesterone at the target tissue, changes in the affinity or distribution of PRs and production of endogenous anti-progestin inhibiting the function of progesterone

occurs, leading to a functional progesterone withdrawal (89, 90). The immune clock refers to an alteration in immune cells prior to the onset of labour (discussed in section 1.8.6) (91). The proteomic clock refers to changes in plasma proteomes that occur throughout pregnancy and reflect underlying changes of the host immune response to pregnancy. Circulating plasma proteomes including interleukins, pregnancy associated plasma protein-A (PAPP-A) and placental growth factor are altered in physiological and pathological pregnancies, reflecting underlying changes in immunoregulation and inflammation associating with sPTB (92). The epigenetic clock describes how deoxyribonucleic acid (DNA) methylation associates with the onset of labour (93, 94). Alterations in DNA methylation in placental tissue, myometrium, cervical samples and cord blood have relate to sPTB (93, 94).

Mechano-physical factors also contribute to parturition. Membrane over-distension leads to increased levels of PGE2, following increased expression of COX-2 from the amnion (95). Acute over-distension induces IL-8 upregulation in amniotic epithelial cells (96). Through this mechanism multiple pregnancies, fibroids and polyhydramnios associate with sPTB (97-99). Congenital uterine anomalies, such as an unicornuate, bicornuate or didelphys uterus that occur due to interrupted Müllerian duct development, associate with a three-fold increase risk of sPTB reflecting an impaired ability of the uterine cavity to distend with advancing gestation (100).

Overall, the regulation of labour is complex and involves multiple molecular pathways at different tissue compartments. Synchronised signalling between the mother, placenta and fetus controls the physiological timing of parturition.

1.3 Pathophysiology of preterm birth

PTB can occur in the following three contexts; spontaneous preterm labour with intact membranes (40-45%), preterm premature rupture of membranes (PPROM) (25-30%) and iatrogenic (30-35%), whereby delivery by caesarean-section or by induction is initiated due to maternal or fetal reasons such as pre-eclampsia or intra-uterine growth restriction (101, 102). Spontaneous PTB refers to both spontaneous onset of labour with intact membranes and PPROM. PPROM occurs in 3% of pregnancies and is

defined by spontaneous rupture of membranes prior to 37^{+0} weeks' gestation for at least one hour before the onset of contractions (102). When PPROM occurs between 24^{+0} – 28^{+0} weeks' gestation, the median time to delivery is nine days (103). Latency length reduces after 29^{+0} weeks' gestation and a small proportion of women remain undelivered for weeks or months (103). Subclinical infection plays a causative role in PPROM (104). At the same time, PPROM introduces the risk of ascending infection. The pathogenesis of sPTB is not fully understood and in view of its multiple contributing mechanisms, sPTB can be considered as a 'preterm parturition syndrome' (105).

1.3.1 Infection

Infection causes at least one-third of sPTB cases, with the most likely source being ascending vaginal infection (106, 107). The term 'infection' and 'inflammation' are often erroneously used synonymously. As infection causes an intense inflammatory response, assessment of elevated inflammatory markers is frequently used as a surrogate marker for investigating infection. However, inflammation can be caused by non-infectious means (e.g. bleeding). Despite this, much evidence exists to support the role of pathogenic microbes as triggers of early parturition. Animal models involving inoculation with bacteria and endotoxins show progression to sPTB (108-110). In humans, systemic infections such as pneumonia, pyelonephritis and malaria predispose to sPTB (111-113). Local infections including sexually transmitted infections (STIs) and urinary tract infections (UTIs) have also been implicated (111, 114, 115). Expectedly, antibiotic elimination of active infection reduces sPTB risk (116).

Timing of infection

The association between infection and sPTB is more pronounced in earlier gestations. Infection is associated with 15%, 80-85% and 90% of deliveries at 34^{+0} - 36^{+0}, 28^{+0} and 24^{+0} weeks' of gestation respectively (117). Amniotic fluid sampling taken as early as 5^{+0} to 20^{+0} weeks' gestation, where the pathogens *Ureasplasma urealyticum* and *Mycoplasma hominis* have been isolated, relate to spontaneous delivery at 24 weeks'

gestation (118, 119). Early intra-uterine infection may silently coincide with significant inflammation, triggering sPTB.

Specific organisms

Specific organisms have been associated with sPTB. STIs, including *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoea* elevate sPTB risk (115). *Chlamydia trachomatis* infection during the first 24 weeks of pregnancy correlates with a three-fold increase in sPTB risk (120). *Chlamydia trachomatis* has been associated with a short cervix, suggesting that infection may directly cause cervical remodelling (120). Alternatively, a shortened cervix may develop secondary to *Chlamydia trachomatis*, acting as a further gateway for ascending infection. A recent systematic review and meta-analysis identified *Neisseria gonorrhoea* to increase the chance of PPROM, sPTB and perinatal mortality (121). *Mycoplasma* and *Ureaplasma* spp. are well-studied pathogens in their association with sPTB and are commonly found in cord blood cultures or fetal tissues in sPTB (122-124). Bacterial vaginosis (BV) is discussed in section 1.7.3.

Viruses have also been implicated in sPTB pathogenesis, including human papilloma virus (HPV), herpes viruses and influenza (125). In heel-prick blood samples taken from day three to five of neonatal life, polymerase chain reaction (PCR) detection of cytomegalovirus (CMV) associated with sPTB, suggesting that fetal CMV exposure predisposes to sPTB (126). The mechanisms by which viral infection drives PTB remain unclear. Murine models show that HPV exposed embryos display altered trophoblast adhesion, suggesting that viral infection of extra-villous trophoblast cells interferes with placental development, causing placental dysfunction and subsequent sPTB (127). Cervical dysplasia, secondary to HPV exposure is a common risk factor for sPTB even in the absence of cervical excision (128).

Maternal periodontal disease has also been associated with sPTB through haematogenous spread of bacteria to the placenta, chorion and fetus. Analysis of amniotic fluid using PCR detected *F. nucleatum*, a common oral pathogen, is significantly associated with miscarriage, intra-uterine death (IUD), neonatal death

(NND), sPTB and PPROM (129). The association between periodontal disease and PTB has been disputed; treatment of periodontal disease does not reduce sPTB (130).

Intra-uterine infection

The amniotic cavity is regarded as a sterile environment and detection of microorganisms within the amniotic cavity is considered pathological (131). Microbial invasion of the amniotic cavity associates with cervical insufficiency. Positive amniotic fluid cultures are seen in 9% of women with cervical shortening (132, 133). Pathogens most found in the amniotic cavity include *Mycoplasma* spp., *Ureaplasma urealyticum*, *Streptococcus agalacticae*, *Escherichia coli*, *Fusobacterium* spp. and *Gardnerella vaginalis* (134, 135). The detection of these bacteria has mainly relied on culture-based techniques, which are limited to those bacteria readily culturable under laboratory conditions. It is therefore likely that culture-based approaches underestimate the true prevalence of intra-uterine infection on sPTB. Consistent with this, the use of sensitive, molecular-based methods have recently described positive PCR results for *Ureaplasma urealyticum* in sPTB cases with negative amniotic fluid cultures (136).

The mechanism by which infection activates pathways initiating early parturition are detailed in sections 1.7.3 and 1.8.7.

1.3.2 Uterine overdistension

Throughout pregnancy, physiological uterine distension occurs as gestational age advances. Intra-amniotic pressure is kept consistent, despite fetal and uterine growth due to the effects of progesterone and nitric oxide on myometrial relaxation (137, 138). Uterine overdistension can, however, increase myometrial contractility, prostaglandin release, myometrial oxytocin receptors and alter expression of gap junction proteins, triggering uterine activity (139-141). *In-vitro* stretching of the fetal membranes increases collagenase, pre-B-cell colony-enhancing factor, IL-8 and PGE2 (142-144). Uterine overdistension therefore predisposes to sPTB, by activating membrane rupture, cervical remodelling and stimulating uterine contractions. Uterine

overdistension as seen in multiple pregnancies, fibroids and polyhydramnios, is associated with sPTB (97-99).

1.3.3 Cervical insufficiency

Cervical insufficiency describes the inability of the uterine cervix to retain a pregnancy in the absence of contractions or labour and is considered to be a cause of mid-trimester loss (MTL) and sPTB (145). As cervical remodelling occurs before signs or symptoms of labour, cervical insufficiency typically presents with increased vaginal pressure, caused by protruding fetal membranes followed by membrane rupture. Cervical insufficiency is painless, but can present with mild lower back discomfort and increased vaginal discharge without vaginal bleeding (146). Labour is typically short. There is no diagnostic test for cervical insufficiency. Although diagnostic techniques involving assessment of the cervix at hysterosalpingogram, insertion of cervical dilators without resistance or an inflated Foley catheter through the internal os have been described, these techniques do not reliably diagnose cervical insufficiency (147). Determining the true incidence of cervical insufficiency is challenging as the diagnosis is typically made following recurrent MTL or recurrent sPTB.

The causes of cervical insufficiency are multi-faceted (148). The loss of connective tissue following cervical surgery, as seen following a cone biopsy or large loop excision of the transformation zone (LLETZ) is one cause (149). Intra-uterine infection also contributes to cervical insufficiency; microbial invasion of the amniotic cavity is a common finding (132). Other types of cervical trauma causing damage to cervical connective tissue include repeated surgical termination of pregnancies and advanced stage caesarean-sections (150-152). Fully dilated caesarean-sections (FDCS) pose the highest risk for future cervical insufficiency (153, 154). In fact, cervical dilatation at the time of caesarean-section is proportionally related to the risk of subsequent PTB; caesarean-sections performed at 6cm, 7cm, 8cm, 9cm and 10cm dilatation correspond to a 2.8%, 4.1%, 6.0%, 8.7% and 13% increased risk of sPTB respectively (155). A contributing factor is inadvertent cervical trauma occurring as the incision involves cervical rather than uterine tissue. Other causes of cervical trauma include cervical tissue involvement in the uterine closure and cervical extensions that may

occur at fetal delivery (155-157). Cervical tears from vaginal or instrumental deliveries also predispose to future cervical insufficiency. Other less common causes of cervical insufficiency include *in-utero* exposure to diethylstilbestrol, connective tissue disorders including Ehlers-Danlos syndrome and congenital uterine anomalies (146, 158-161).

1.3.4 Placental causes

Uteroplacental ischaemia can predispose to sPTB. Placental vascular lesions, causing uteroplacental ischaemia, occur in 35% women with PPROM versus 11.8% of term deliveries (162). First trimester uteroplacental dysfunction relates to both iatrogenic and sPTB. The same factors used to predict pre-eclampsia, including Black ethnicity, chronic hypertension, diabetes mellitus, low PAPP-A and uterine artery pulsatility index may also predict sPTB $<33^{+0}$ weeks (163).

The renin-angiotensin system has been implicated in sPTB. In the presence of uteroplacental ischaemia, renin production increases, increasing angiotensin II which triggers myometrial contractility and prostaglandin production (164-166). In cases of extensive uteroplacental ischaemia, necrosis and haemorrhage can occur. Thrombin can further stimulate myometrial contractility and MMP production (167-169). Intra-uterine haematomas in the first trimester increase the risk of sPTB (170, 171).

1.3.5 Genetics

Genetic association studies suggest that a genetic predisposition to sPTB exists. Within families, sisters and daughters of those who delivered preterm are also at risk (172, 173). Differences between ethnic groups exist, reflecting potential genetic predispositions. The risk of sPTB for Black women is double that of White women when socio-economic factors are considered (174). Single-nucleotide polymorphisms that link genes involved in inflammation and cell signalling have been identified as risk factors for sPTB among African-American women (175). The relationship between ethnicity and sPTB represents a complex interaction between genetics, vaginal microbiota and sociodemographic factors (discussed in section 1.7.3) (176). Genetic factors also predispose White women to sPTB. A genome wide study of over 40,000

European women revealed variants of genes involved in the binding capability of oestrogen to its receptor to impact sPTB risk (177).

1.3.6 Maternal stress

Many different forms of stress including physical, nutritional, psychological, occupational and psychosocial, are associated with sPTB. Physically demanding work, long working hours and night shifts elevate the risk of sPTB (178-180). Mothers with anxiety disorders, post-traumatic stress, depression or schizophrenia also display a greater sPTB risk (181, 182). Aberrant autonomic, neuroendocrine and immune pathways are thought to contribute (183). Interestingly, cortisol release secondary to maternal stress can induce vaginal dysbiosis, through inhibition of oestrogen-related vaginal epithelial cell maturation and glycogen accumulation (184). Domestic violence also predisposes women to sPTB, antepartum haemorrhage and stillbirth (185).

1.3.7 Risk factors for preterm birth

Based on the pathophysiology of PTB (discussed in section 1.3), several antenatal risk factors exist (Figure 1.5).

Socio-demographics

The risk of sPTB in Black women is double that of White women (174). Although socio-demographic factors such as maternal education contribute, a complex interaction between genetics and the microbiome is also likely to occur (discussed in section 1.7.3).

Obstetric history

Previous sPTB predisposes women to a 2 to 2.5-fold increased risk of subsequent sPTB, with earlier sPTB more predictive of future PTB (186, 187). Previous MTL, PPROM and a history of Ehlers-Danlos syndrome can cause cervical insufficiency influencing sPTB risk (discussed in section 1.3.3). Short inter-pregnancy interval has

been associated with sPTB. An inter-pregnancy interval of <6 months significantly associates with sPTB <36^{+0} weeks and NND (188).

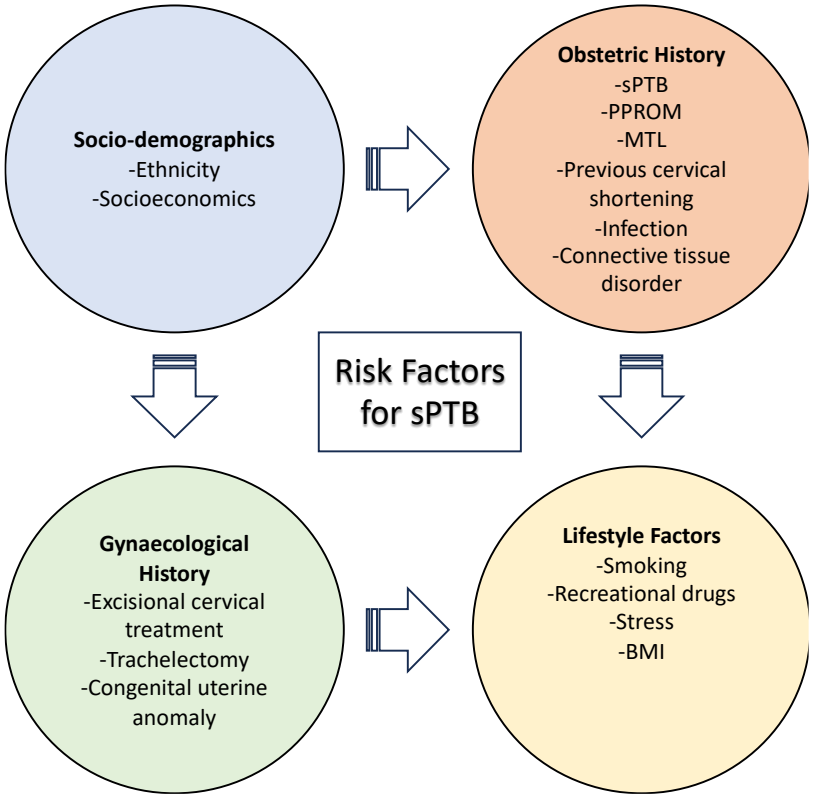


Figure 1.5 – Risk factors for sPTB. Risk factors include socio-demographics factors, lifestyle factors, obstetric history and gynaecological history. BMI = body mass index.

Gynaecological history

A woman's gynaecological history can contribute to sPTB risk. Congenital uterine anomalies, cervical dysplasia, cone biopsies and LLETZ procedures can lead to cervical insufficiency (discussed in section 1.3.3). A trachelectomy describes the surgical removal of the cervix, typically as part of cancer treatment. Ironically, as the uterus is spared from surgery, trachelectomies are considered fertility sparing. However, the major cervical disruption causes loss of mechanical support and compromises protection from ascending infection, predisposing women to MTL, sPTB and PPROM (189).

Lifestyle factors

Extremes of body mass index (BMI) have also been linked to sPTB (190). Obese women are also at a higher risk of gestational diabetes and pre-eclampsia, which can subsequently predispose to sPTB. A low pre-pregnancy BMI may represent nutrient deficiency, associating with sPTB (191). Deficiencies in iron and zinc may compromise immune functioning and increase susceptibility to infection (192). Maternal smoking predisposes to sPTB; even one cigarette a day increases the risk (193). Recently, electronic cigarettes are marketed as less harmful alternatives but still pose a risk of sPTB in view of their nicotine content (194). Recreational drug use also relates to sPTB risk. States within the USA that have introduced marijuana legalisation have seen a rise in sPTB (195). Environmental exposure to pollution and particulate matter also increases sPTB (185).

Overall, the recognition of antenatal risk factors is crucial for patient education, lifestyle modification and referral to PTB prevention clinics for specialist input.

1.4 Prediction of preterm birth

The three key aspects in PTB care include prediction, prevention and preparation. Previous sPTB is the single most important predictor of subsequent sPTB. A history of sPTB birth increases subsequent sPTB risk by 32% (196). Despite this, women with a history of sPTB only account for 10% of sPTB <34^{+0} weeks' leaving a need for further predictive tools (197, 198). In addition to identifying risk factors, current prediction strategies include transvaginal ultrasound scanning (TVUS) of the cervical length and the use of biomarkers.

1.4.1 Cervical length

Mid-trimester cervical length assessment by TVUS has been shown to be more superior than maternal history for sPTB prediction (199). A short mid-trimester cervical length, defined as ≤25mm, predicts sPTB and associates with a relative risk of 3.3 for sPTB (200). A population-based prospective multi-centre study involving 39,284

women showed that the detection rate of sPTB <32^{+0} weeks' gestation was 38%, 55% and 69% for maternal risk factors, cervical length measurement and a combination of the two, respectively. A short cervical length has been consistently shown in large multi-centre trials to associate with sPTB risk. In one study, only 1.7% of participants had a short cervical length <15mm, but accounted for 86% and 58% of sPTB before 28^{+0} and 32^{+0} weeks' gestation, highlighting its value (201).

The optimum time to measure cervical length is between 16^{+0} and 24^{+0} weeks. As the lower segment of the uterus is poorly developed in early pregnancy, cervical length assessment is challenging prior to 16^{+0} weeks and has limited value in sPTB prediction (202, 203). Most studies assessing outcomes of interventions use 24^{+0} weeks as the gestational cut-off for intervention, therefore measurements after 24^{+0} weeks are not recommended (204). Nonetheless, the National Institute for Health and Clinical Excellence (NICE) guidelines recommend cervical length assessment in women who are ≥30^{+0} and symptomatic of threatened preterm labour. A cervical length of >15mm is reassuring, whilst a measurement of ≤15mm with symptoms is considered diagnostic of preterm labour.

Advantages of TVUS assessment of the cervix include its safety, acceptability and ability to yield relevant clinical information that cannot be detected through speculum or digital examination (205-207). When compared with transabdominal cervical length measurements, TVUS is advantageous in that it is unaffected by raised BMI, cervical position or shadows produced from fetal parts (208, 209). The challenges associated with TVUS include lack of certification for cervical length measurement, with over a quarter of images not meeting the standards for cervical length assessment when performed by sonographers without cervical length specialist training (210). Training is required to maintain intra and interobserver reproducibility (211, 212). With appropriate training, a low inter-observer variation rate of 5-10% exists (213, 214). Although multiple pregnancies are associated with a higher risk of sPTB, TVUS cervical length assessment in asymptomatic multiple pregnancies with no risk factors for sPTB is a poor predictor (215, 216).

1.4.2 Biomarkers

The most extensively studied biomarkers that are integrated into clinical practice include fetal fibronectin (fFN), insulin-like growth factor binding protein-1 (IGFBP-1) and placental alpha-microblobulin-1 (PAMG-1) (31, 217).

Fetal fibronectin

fFN is an extracellular glycoprotein, produced by the decidua and chorion (218). In early pregnancy, fusion of the decidua and fetal membranes is incomplete and therefore fFN can be detected in the cervicovaginal fluid (CVF) up to 21^{+0} weeks' gestation (219, 220), therefore limiting its capacity to detect PTB early in pregnancy. Between 35^{+0} - 37^{+0} weeks fFN can again be detected, as a physiological phenomenon (221). fFN is released when disruption at the choriodecidual interface occurs, either by mechanical or inflammatory mediated injury, therefore fFN presence beyond 22^{+0} weeks associates with sPTB (222).

Lockwood et al (1991) were the first to show that fFN associated with sPTB (223). Following this, a multi-centre study involving symptomatic women demonstrated the ability of fFN to predict delivery within seven days (224). The negative predictive value (NPV) for sPTB prediction within seven days was 99% (224). The capability of fFN to predict sPTB in symptomatic women has been reproduced by others (225-230). Owing to its impressive NPV, integration of fFN into clinical practice has seen fewer hospital admissions, leading to cost savings (231, 232).

The role of fFN in women asymptomatic of sPTB has also been explored. Fortnightly measurements in asymptomatic women from 22^{+0} to 30^{+0} weeks' gestation showed an ability to predict 60% of very early sPTBs, but authors concluded testing would not reduce sPTB due to limitations in available interventions (233). However, an analysis of fFN testing in both symptomatic and asymptomatic women has shown improvements in clinical management, including hospitalisation, increased clinical surveillance, advice to travel abroad, decision to administer steroids, tocolytics and place cervical cerclages (101, 234).

A threshold of 50 ng/mL has been validated for sPTB prediction. Levels below and above 50 ng/mL are deemed as negative and positive respectively (223, 235). Application of a single threshold runs the risk of increasing false positive or false negative results, hence quantitative fFN has been explored (236). fFN concentration and sPTB risk have a directly proportionate relationship and quantitative fFN testing may be of more value (237, 238).

A significant improvement is achieved when combining fFN with cervical length (239). This has led to the development of the Quantitative Innovation in Predicting Preterm Birth (QUiPP) App, a free, validated predictive model which combines clinical history, gestational age, quantitative fFN and cervical length measurement to quantify and individualise a patient's sPTB risk (240-243). Using a threshold of 10% has a positive likelihood ratio of 1.3 and negative likelihood ratio of 0.8 for predicting sPTB $<34^{+0}$ weeks (244). Use of the QUiPP app is recommended by NHS England and the British Association of Perinatal Medicine (BAPM) (245, 246).

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

Actim® Partus is simple, in that it does not require specialist training or equipment, is a rapid and cost-effective bedside test using monoclonal antibodies that bind to phIGFBP-1, a protein produced by decidualised endometrium. Generation of a binary result allows for simple interpretation by performing healthcare providers. Disruption of cells at the choriodecidual interface causes leakage of phIGFBP-1 into the CVF (247). Similar tests such as the Actim® Prom test, detect insulin-like growth factor binding protein-1 (IGFBP-1). High quantities of IGFBP-1 exist in amniotic fluid, aiding identification of those with ruptured membranes (248).

The sensitivity, specificity, positive predictive value (PPV) and NPV of phIGFBP-1 testing in symptomatic women is 81.1%, 97.1%, 95.2% and 87.7% respectively (249). Validity of phIGFBP-1 testing has been challenged; half of women with a positive Actim® Partus result deliver at term (250). The ideal biomarker for sPTB prediction should identify women who will deliver within 48 hours to seven days, to prompt administration of corticosteroids and magnesium sulphate (251). A meta-analysis concluded that whilst phIGFBP-1 identifies symptomatic women who will not deliver

within 48 hours, prediction of delivery within seven to 14 days in both symptomatic and asymptomatic women was low (252). Prediction of sPTB using Actim® Prom and Actim® Partus can guide clinicians to hospitalise women and prepare for delivery. However, as testing is prompted by clinical presentation with threatened preterm labour or PPROM, at the point of a positive result the opportunity to intervene with preventative measures has usually passed.

Placental alpha-microblobulin-1 (PAMG-1)

Another studied biomarker is PAMG-1. First isolated from amniotic fluid in 1975, PAMG-1 testing is used in clinical practice and marketed as AmniSure® or PartoSure®. This point-of-care test detects PAMG-1, which is released from the decidua into the amniotic cavity (253). Concentrations of PAMG-1 are high in amniotic fluid and low in CVF (254). Single-centre studies show that detection of CVF PAMG-1 fluid correlates with imminent delivery (255-257). The positive and negative predictive values for PAMG-1 have been reported to be 12.5% and 97% respectively (256).

1.5 Prevention of preterm birth

1.5.1 Modifiable risk factors

Optimisation of risk factors such as smoking cessation and support with recreational drug use are important in sPTB prevention (258, 259). The saving babies' lives care bundle (version 3) highlights the importance of smoking cessation to reduce PTB risk, as well as the commencement of low dose aspirin in women at risk of hypertensive disorders and testing for asymptomatic bacteriuria at clinical visits (246, 258).

1.5.2 Cervical cerclage

Cervical cerclage, a suture placed around the cervix, can prevent sPTB and MTL in high-risk women (260). Cerclages preserve the structural strength of the cervix,

creating both mechanical and immunological integrity and protecting against ascending infection (261, 262).

The efficacy cervical cerclage in sPTB prevention is controversial, with conflicting results reported in the literature (263, 264). Randomised trials have not consistently shown significant sPTB prevention (265, 266). Such trials have been criticised for reaching conclusions in low-risk populations that cannot be extrapolated to high-risk women. The pathophysiology of cervical shortening in low-risk women is likely to differ to that seen in high-risk women (267). The true controversy lies in the correct identification of women who would benefit from a cerclage.

Several factors influence outcomes of cervical cerclage insertion, including suture material and placement technique. Two commonly used suture materials include a monofilament or braided suture (268). A monofilament suture does not alter the vaginal microbiome whereas a shift towards dysbiosis and reduced *Lactobacillus* spp. is seen with braided suture use (269). Additionally, braided sutures associate with increased sPTB risk when compared to monofilament sutures (269). C-STICH1 randomised women with singleton pregnancies requiring a history or ultrasound indicated cerclage to receive either monofilament or braided sutures (270). C-STICH1 showed no difference in pregnancy loss (defined as miscarriage, stillbirth or NND within seven days of life) or gestational age at delivery between the two groups. However, maternal sepsis and chorioamnionitis occurred in a significantly higher proportion of women receiving braided sutures when compared to monofilament. Further, confounders such as placement technique and antibiotic use were not accounted for.

There are two main described techniques for cerclage: McDonald and Shirodkar. The McDonald technique involves applying a cerclage around the cervix in a purse-string manner, with an anteriorly tied knot (Figure 1.6) (271). In contrast, the Shirodkar technique involves a transverse anterior colpotomy to allow for dissection of the bladder towards the internal cervical os and placement of the suture superficially, with the cerclage secured posteriorly beneath the vaginal epithelium (272). Whilst more technically challenging, the Shirodkar permits higher cerclage placement closer to the internal os, allowing for a longer post-cerclage cervical length (273). The Shirodkar

technique correlates with reduced rates of sPTB when compared to McDonald placement (274).

Women with a history of sPTB, MTL, PPROM, cervical trauma (FDCS, LLETZ, cone biopsy) and a short cervix (≤25mm) can be offered a cerclage (31). PTB prevention clinics identify appropriate cervical cerclage candidates within high-risk populations.

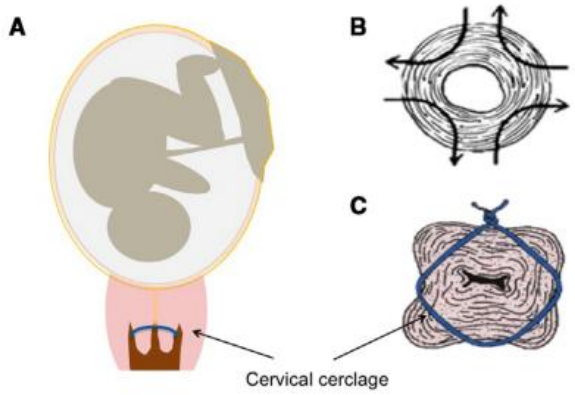


Figure 1.6 – Cervical cerclage placement using the McDonald technique, reproduced from Kindinger & David (2019) (275). Cervical cerclage placement is shown in panel A. The technique for suture placement is shown (panel B) and a purse-string approach for knot tying is performed (panel C).

Emergency cervical cerclage is offered to women with evidence of cervical shortening/dilatation in the absence of established labour and chorioamnionitis. However, the take home baby rate following emergency cervical cerclage is only 50% (276). Studies assessing the role of emergency cervical cerclage placements have been limited to observational studies (277). In one trial, women were randomised to receive an emergency cerclage with indomethacin or bedrest. A longer latency period was seen with emergency cerclage placement, however this trial was limited by its small sample size (n=23), inability to distinguish effects of tocolysis and the inclusion of twin pregnancies (278). NICE guidelines suggest consideration of an emergency cervical cerclage between 16^{+0} and 27^{+6} weeks' gestation, when the cervix is dilated, exposed and unruptured but acknowledge the need for further randomised control trials (RCTs) (31). The C-STICH2 trial is randomising women to receive an emergency

cerclage versus conservative management and will provide insightful results into the benefits of emergency cerclage (279).

In women with a history of failed cervical cerclage, a transabdominal cerclage can be inserted pre-pregnancy or in early pregnancy (280). Transabdominal cerclages can be performed laparoscopically or at laparotomy. Observational studies show abdominal cerclages to be safe and successful. The MAVRIC RCT randomised women to receive a transabdominal cerclage, high vaginal cerclage or low vaginal cerclage and demonstrated transabdominal cerclage to be superior for women with a history of failed cerclage in the reduction of early sPTB and MTL (281-283).

1.5.3 Progesterone

Progesterone was first reported to reduce sPTB in 1975 (284). Two preparations of progesterone for use in PTB prevention exist; vaginal progesterone and intramuscular (IM) 17-hydroxyprogesterone acetate, the latter historically used but no longer recommended in the USA (285). Progesterone has anti-inflammatory properties and suppresses pro-labour pathways that lead to cervical remodelling and uterine activation (286). Progesterone maintains uterine quiescence by a number of mechanisms, including increased expression of calcium channels, which diminish electrical activity of myometrial cells, downregulation of receptors involved in myometrial contraction and dampening down of proteins involved in the cross-linking of actin and myosin filaments (287). Progesterone also inhibits myosin phosphorylation, which is required for myometrial contractility (288).

Some authors show progesterone to be as effective as cerclage placement for PTB prevention (289). Current guidance recommends offering progesterone to women with a history of sPTB $<34^{+0}$ weeks of pregnancy or MTL ($>16^{+0}$) and a short cervix measuring ≤25mm between 16^{+0} and 24^{+0} weeks of pregnancy, whilst considering progesterone for women with either a history of sPTB/MTL or a shortened cervix (31, 290, 291). However, evidence behind progesterone efficacy remains inconclusive. The OPTIMUM trial randomised women at high-risk women of sPTB to receive vaginal progesterone or placebo and found no significant sPTB reduction (292). The

PROLONG study randomised women with a history of sPTB to receive IM progesterone and similarly did not report a reduction in sPTB (293). In cases of established PPROM, progesterone has not shown to prolong pregnancy (294). Most studies showing a benefit have been conducted in women a short cervix (295, 296). Progesterone use in women with a cervical length of 10 - 20mm had a significant reduction in sPTB (296). Conflicting results regarding progesterone use are likely to reflect heterogeneity of studied populations and differing causes of sPTB (297).

1.5.4 Cervical devices

The Arabin pessary is the most studied pessary for sPTB prevention. It is a silicone device that aims to keep the cervix closed whilst alleviating the pregnancy weight from the internal os (Figure 1.7). A cervical pessary is less invasive than a cervical cerclage, does not require anaesthesia and can be inserted with ease in the outpatient setting (298). A meta-analysis involving three RCTs did not find cervical pessaries to prevent PTB or improve perinatal outcomes in singleton pregnancies with a short cervix (299).

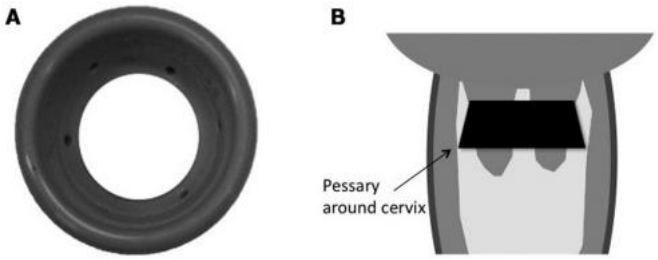


Figure 1.7 – The Arabin cervical pessary, reproduced from Kindinger and David (2019) (275). The Arabin cervical pessary (panel A) and its positioning around the cervix (panel B) is shown.

1.5.5 Antibiotics

The association between infection and sPTB has led to interest regarding antibiotics in mitigating sPTB risk. Unsurprisingly, antibiotic treatment of asymptomatic bacteriuria, pyelonephritis and pneumonia have demonstrated reductions in sPTB (300-302). However, untargeted use of antibiotics as a general approach to reduce sPTB is not an effective strategy, could lead to harm as seen in the ORACLE study,

and could have a devastating impact on antibiotic resistance in the general population. Antibiotic treatment of BV in sPTB prevention is discussed in section 1.7.3.

1.5.6 Preterm birth prevention clinics

The saving babies' lives care bundle (version 3) states that every UK maternity care provider should offer PTB prevention clinics to facilitate the provision of specialised care to women with risk factors (246). In the past decade, the number of PTB prevention clinics across the UK have increased. Between 2012 and 2017, the provision of PTB prevention clinics increased by 44% in UK hospitals, reflecting a response to national guidance (303). Further improvements have been described, including a 160% increase in PTB prevention clinics between 2017 and 2021 (304). Combining obstetric history TVUS cervical length assessment provides an effective approach for PTB prevention within specialised prevention clinics (305). Additionally, serial cervical length measurements are more informative than isolated measurements and PTB prevention clinics provide opportunity to monitor cervical length over time (200). Women report that being regarded as 'high-risk' generates negative feelings and anxiety, but describe feeling reassured by frequent clinic appointments (306). Continuity of care within PTB prevention clinics has been shown to reduce PTB rates and ameliorates maternal stress (307). PTB prevention clinics also provide an opportunity to address modifiable risk factors, such as smoking cessation (188, 308, 309).

1.6 Preparation for preterm birth

When PTB is imminent, efforts to cease preterm labour are ineffective and risk prolonging fetal exposure to infection. Management of preterm labour therefore focuses on optimising the conditions for the arrival of a preterm neonate.

1.6.1 Intra-uterine transfer

Place of birth is a crucial consideration. The saving babies' lives care bundle (version 3) highlights the importance of optimising place of birth and arranging transfer of women to units with appropriate neonatal facilities according to gestational age (246).

1.6.2 Tocolysis

Tocolysis involves the use of medication to delay labour by inhibiting uterine contractions. Tocolytics include beta-adrenergic agonist receptors, calcium channel blockers, COX inhibitors and magnesium sulphate. However, tocolysis will not permanently cease ensuing labour nor will it significantly lengthen the gestational period (310). Tocolysis can be beneficial in circumstances where delaying delivery will allow for administration of antenatal corticosteroids, magnesium sulphate and intra-uterine transfer (311).

1.6.3 Antenatal corticosteroids

Corticosteroids should be offered when preterm labour is suspected, established, or planned for 24^{+0} and 33^{+6} weeks of pregnancy (31). Respiratory morbidity is a serious complication of prematurity and antenatal corticosteroids provide protection by accelerating fetal lung maturation. Antenatal corticosteroids have been one of the most effective treatments in perinatal medicine and can reduce the risk of NND, respiratory distress syndrome, NEC and IVH (312). The maximum benefit is seen when corticosteroids are given between 1 and 7 days before birth (313, 314). As prediction of sPTB is not straightforward, implementation of this ideal timescale can be challenging. The timing of corticosteroid administration is sub-optimal in up to 60% of cases (315). Animal studies show that repeated courses of antenatal corticosteroids impact myelination in the developing brain (316). Human studies show that repeated corticosteroids courses associate with neurodevelopmental abnormalities and increased rates of aggression and hyperactivity in children (317, 318). Beyond 34^{+0} weeks' neonatal morbidity is less common, the benefit of corticosteroids declines and a risk on childhood neurodevelopment may emerge (319). Antenatal corticosteroids are not recommended during the late preterm period.

1.6.4 Magnesium sulphate

Magnesium sulphate should be offered to women between 24^{+0} and 29^{+6} weeks of pregnancy and can be considered up to 33^{+6} weeks of pregnancy in women who are expected to deliver within 24 hours (31). Magnesium sulphate offers fetal neuroprotection and substantially decreases the risk of cerebral palsy (320).

1.6.5 Antibiotics

The role of antibiotics in the management of PTB and PPROM is controversial. Throughout Europe antibiotic use varies, reflecting a lack of robust evidence regarding antibiotic use in the context of PTB and PPROM (321).

In the ORACLE I trial, 4,826 women with PPROM were randomised to receive erythromycin, co-amoxiclav or placebo. Women on erythromycin were significantly less likely to deliver within 48 hours and although cases of NND, chronic lung disease and major cranial abnormalities were reduced with erythromycin, statistical significance was not reached. Significantly fewer women on co-amoxiclav delivered within 48 hours and 7 days, however co-amoxiclav associated with significantly increased rates of NEC (322).

In the ORACLE II trial, 6,295 women in spontaneous preterm labour with intact membranes and without evidence of infection were assigned to receive erythromycin, co-amoxiclav or placebo (323). Results showed no difference in the rates of NND, chronic lung disease or ultrasound evidence of cranial abnormalities between the three groups. A seven-year follow up of children included in the ORACLE II trial showed that children born in the context of intact membranes treated with erythromycin, had increased functional impairment at aged seven (324). A Cochrane review including 14 studies demonstrated no benefit when antibiotics were used in women in preterm labour with intact membranes (325). Although maternal infection may be reduced, antibiotics did not reduce perinatal deaths or admission to neonatal intensive care and long-term studies show that antibiotic use in fact associates with childhood functional impairment and cerebral palsy (325). A Cochrane review looking at antibiotic use in women with PPROM involving 22 studies showed that antibiotics significantly reduced chorioamnionitis, delivery within 48 hours, neonatal morbidity,

neonatal infection, oxygen therapy and cerebral ultrasound abnormalities (326). Reassuringly, a seven-year follow up of children born from mothers receiving antibiotics for PPROM in the ORACLE I trial showed no association between erythromycin or co-amoxiclav with behavioural difficulties or educational attainment (327).

As a result of these studies, clinical guidance recommends avoiding antibiotics in suspected preterm labour with intact membranes, yet recommends a 10-day course of oral erythromycin in cases of PPROM (31). Once labour is established, intravenous benzylpenicillin is recommended in all cases of PPROM as well as in women with confirmed group B streptococcus (GBS) colonisation with intact membranes (328). If chorioamnionitis is suspected, treatment with benzylpenicillin, gentamicin and metronidazole are recommended to protect the mother from Gram-positive or negative aerobic/anaerobic bacteria, whilst concomitantly preventing neonatal group B streptococcal infection (31).

1.6.6 Peri-viability

The peri-viable phase poses a challenge to obstetricians, neonatologists and parents as considerable uncertainty regarding mode of delivery, resuscitation and life-sustaining care exists. Recent case reports describing the survival of extremely preterm infants weighing <300g born between 22^{+0} and 23^{+0} weeks of pregnancy exist (329, 330). In scenarios involving the peri-viable fetus, a multi-disciplinary discussion including the neonatologist, obstetrician and parents regarding neonatal resuscitation should take place with consideration of prognostic features (331) (Figure 1.8).

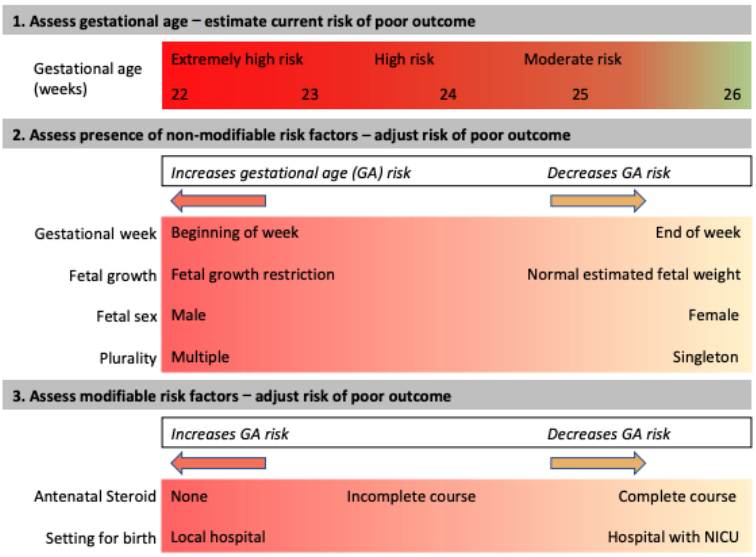


Figure 1.8 – Risk stratification for the perinatal management of extreme PTB before 27^{+0} weeks of gestation, reproduced from the BAPM (331). For the management of PTB involving a peri-viable fetus, several prognostic features including gestational age, fetal growth, fetal sex, plurality and birth setting should be considered. Abbreviations: GA = gestational age, NICU = neonatal intensive care unit.

1.6.7 Postnatal support

Following preterm delivery, delayed cord clamping, immediate skin-to-skin (known as kangaroo care) and breastfeeding are effective and inexpensive techniques that can benefit the preterm infant (332). These approaches can be implemented with ease in low-, middle- and high-income countries.

1.7 The vaginal microbiota

1.7.1 Profiling the vaginal microbiota

The study of microorganisms was historically limited to isolation and culture outside of the human body. More recently, the use of culture-independent techniques utilising molecular-based approaches have enabled a more comprehensive understanding of polymicrobial communities including bacteria, viruses, fungi and archaea that co-exist within the human body (333). The term ‘microbiota’ describes the presence of micro-organisms in a defined environment whereas the term ‘microbiome’ refers to the entire

habitat, including microorganisms, their genomes and surrounding conditions (334). The human microbiome is dynamic and influenced by several factors (Figure 1.9). Initial studies of the human microbiome have focused on the gastrointestinal tract, which harbours the largest collection of microorganisms. However, it is now widely recognised that other body niches including the vaginal microbiome host communities of microbes that play a critical role in shaping health and disease states.

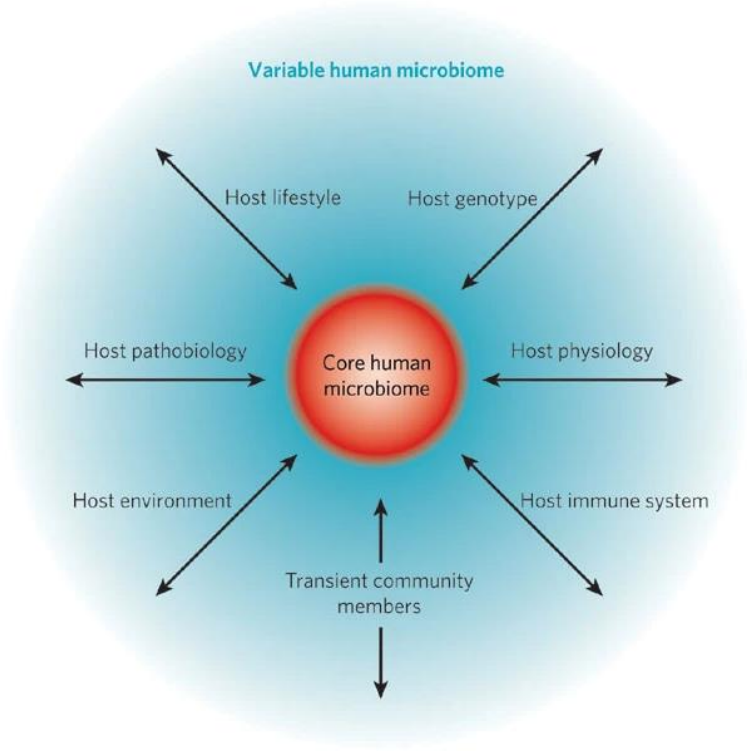


Figure 1.9 – Influencing factors of the human microbiome, reproduced from Turnbaugh et al (2007) (333). Factors influencing the human microbiome include the host genotype, physiological mechanisms, environmental factors, pathogens and transient community members. These factors also influence the composition of the vaginal microbiome.

There exist two major sequencing-based methodologies used to study the microbiota: i) metagenomics and ii) metataxonomics. Metagenomics involves the study of genomes and genes from within a microbial community and is obtained through shotgun sequencing of all DNA in a sample (334). Metataxonomics refers to amplification and sequencing of specific short-length regions of microbial taxonomic marker genes. For the study of bacteria, the 16S ribosomal RNA (ribonucleic acid) gene (16S rRNA) is targeted. The bacterial 16S rRNA gene consists of highly

conserved regions of nine hyper-variable regions, V1-V9. PCR primers designed to bind to these highly conserved regions amplify these variable regions, allowing identification of bacterial species (335). In some cases, mapping sequences to existing 16S rRNA gene databases allow for bacteria classification to bacterial strain level (335).

1.7.2 The vaginal microbiota in reproductive health

The vaginal microbiota plays an important role in reproductive health (336). Throughout a woman's life, the vaginal microbiota composition alters under the influence of endogenous and exogenous factors. Prior to menarche, circulating oestrogen levels are low. This associates with a high-diversity microbial milieu, involving a combination of aerobic, anaerobic and enteric bacteria (337). With the initiation of menarche, circulating oestrogens increase and impact the biochemical and microbial structure of the reproductive tract. Circulating oestrogen stimulates vaginal epithelial cell proliferation. Glycogen deposition and breakdown products are used by *Lactobacillus* spp. as a carbon source. Ultimately, this environment favours the dominance of *Lactobacillus* spp., resulting in a lowered vaginal pH (338). This acidic environment results in a microenvironment that is hostile to other microbial species, conferring protection against BV, candidiasis, STIs, human immunodeficiency virus (HIV), herpes simplex virus (HSV) and HPV (339-344).

Using next generation sequencing and hierarchical clustering of relative abundance data obtained from the metataxonomic analysis of vaginal samples, Ravel and colleagues originally describe five vaginal community state types (CST) that were characterised on the basis of relative abundances of major bacterial species (Table 1.1) (339). Since then, the five CSTs originally described by Ravel et al (2011) have been subcategorised into further sub-groups (345). This was achieved using the VALENCIA algorithm, which classifies samples according to a reference set of 13,160 taxonomic profiles collected from 1,975 women of reproductive age and has been validated in multiple ethnic populations (345). The VALENCIA algorithm works by initially identifying seven major CSTs according to the species with highest relative abundance. These seven CSTs can be further broken into 13 sub-CSTs. CST I and III

are broken into A and B groups, with A referring to samples with a higher abundance of the focal species. CST-IV refers to samples without a high relative abundance of *Lactobacillus* and is sub-classified to CST IV-A, IV-B and IV-C. CST IV-A describes a high relative abundance of *Candidatus Lachnocurva vaginae,* moderate *G. vaginalis* abundance and moderate relative abundance of *Atopobium vaginae*. CST IV-B describes a high relative abundance of *G. vaginalis*, low relative abundance of *Candidatus Lachnocurva vaginae* and moderate relative abundance of *Atopobium vaginae.* CST IV-C describes a low relative abundance of *Lactobacillus* spp., *G vainalis*, *A. vaginae* and *Candidatus Lachnocurva vaginae.* CST IV-C instead describes the abundance of diverse anaerobic bacteria and is further divided into five sub-CSTs; CST IV-C0, CST IV-C1, CST IV-C2, CST IV-C3 and CST IV-C4 and respectively represent moderate *Prevotella*, dominance with *Streptococcus*, *Enterococcus*, *Bifidobacterium* and *Staphylococcus*.

Table 1.1 – Bacterial composition within vaginal CSTs according to characterised by the VALENCIA algorithm.

CST	Dominating species
I I-A I-B	*L. crispatus*
II	*L. gasseri*
III III-A III-B	*L. iners*
IV	*Lactobacillus* deplete
IV-A	*Candidatus Lachnocurva vaginae*
IV-B	*G. vaginalis*
IV-C	Diverse anaerobic bacteria
V	*L. jensenii*

Longitudinal profiling of the vaginal microbiota shows some women maintain compositional stability throughout the menstrual cycle (346). For others, falling oestrogen and progesterone levels during the menstrual cycle, along with endometrial shedding and menstrual bleeding, can reduce *Lactobacillus* spp. abundance, which later recovers in the follicular phase of the menstrual cycle (346, 347). Unsurprisingly, menses is a risk factor for BV.

During pregnancy, endocrine stability exists, whereby an increase in circulating oestrogen occurs and menstruation ceases, favouring *Lactobacillus* spp. dominance (348). Healthy pregnant women maintain a vaginal microbiota dominated by *Lactobacillus* spp. throughout pregnancy. Post-partum, oestrogen levels fall and alongside the passage of lochia, the vaginal microbiota shifts towards a *Lactobacillus* spp. deplete state (348). This state of *Lactobacillus* spp. depletion exists for up to one year post-partum (349).

1.7.3 The vaginal microbiome and preterm birth

Intra-uterine infection secondary to ascending pathogens from the vagina is a well-established contributor to sPTB (102). In sPTB, the similarity of bacterial species found in the amnion and chorion with those in the vagina, support this theory (350, 351). Additionally, histological evidence of chorioamnionitis seen at the site of membrane rupture in the lower uterine segment and close to the cervix again supports ascending infection as a trigger for sPTB (134). Furthermore, in twin pregnancies, histological chorioamnionitis and microbial invasion of the amniotic cavity are usually seen in the presenting twin (351).

The role of ascending vaginal bacteria has led to an interest in characterisation of the vaginal microbiota. PTB risk is associated with *L. iners* dominance, whereas *L. crispatus* abundance confers protection from PTB (352). Within a UK population, *L. iners* dominance as early as 16^{+0} weeks significantly associated with cervical shortening and sPTB before 34^{+0} weeks (353). Meanwhile, *L. crispatus* dominance was predictive of term birth (353).

BV, a displacement of *Lactobacillus* spp. with anaerobic bacteria such as *Gardnerella vaginalis*, *Bacteroides* spp., *Mobiluncus* spp., and *Mycoplasma hominis,* is also a risk factor for sPTB and PPROM. The first diagnostic criteria developed for BV diagnosis involved Gram staining of vaginal smears, known as the Spiegel criteria. A more specific criteria was published by Nugent in 1991 (Figure 1.10) (354). The Amsel criteria uses four parameters to diagnose BV including: thin, white/yellow vaginal discharge, clue cells on wet microscopy, a vaginal pH >4.5 and a fishy odour with 10% potassium hydroxide (355). If at least three of these criteria are met, a diagnosis of BV is made. The British Association of Sexual Health and HIV (BASHH) recommend using the Hay/Ison criteria, a validated simplified grading system involving Gram staining, for the clinical diagnosis of BV in busy clinical settings (356, 357). BV is treated with antibiotics including metronidazole, which kill anaerobic bacteria without impacting vaginal lactobacilli (358).

0 = no morphotypes
1 = <1 morphotype
2 = 1- 4 morphotypes
3 = 5-30 morphotypes
4 = >30 morphotypes

Score[b]	Lactobacillus morphotypes	*Gardnerella* and *Bacteroides* spp. morphotypes	Curved gram-variable rods
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

Figure 1.10 – The Nugent Scoring System, reproduced from Nugent, Krohn and Hillier (1991) (354). Vaginal smears are plated on microscopic slides in oil immersion and assessed by microscopy for the presence of three morphotypes: *Lactobacillus, Gardnerella* and curved gram rods. Scores for the presence of each morphotype are summated, the highest score can be ten. A score of 7 or higher is suggestive of BV.

Antibiotic treatment of BV to reduce sPTB risk is conflicting (359, 360). These inconsistencies have partly been due to heterogeneity between study designs, such as differences in BV diagnosis, assessed antibiotics, antibiotic regimens and participant demographics (361, 362). The PREMEVA trial is the largest trial yet, assessing BV treatment for sPTB prevention and showed no difference in risk of late miscarriage (16^{+0} - 21^{+0} weeks) or sPTB (22^{+0} - 32^{+0} weeks) between women receiving

clindamycin, versus placebo (363). The PREMEVA trial has been critiqued for the effectiveness of clindamycin in BV treatment and compliance to clindamycin (361, 364). However, results were in keeping with the most recent Cochrane review which concluded that BV eradication in pregnancy does not reduce sPTB (359).

Variations in the associations between vaginal microbial composition and sPTB risk is seen depending on geographical location and ethnicity of study participants (365). Molecular-based study of the vaginal microbiota has shown that pregnant African American and Hispanic women have a greater abundance of *L. iners*, whereas Caucasian women have a greater abundance of *L. crispatus* and *L. gasseri* in pregnancy in general (366). One of the first studies to use metataxonomic profiling of the vaginal microbiota in pregnancy reported no differences in the relative abundance of bacterial taxa or CST differences in women delivering term or preterm, however, women within this cohort were mostly of African American ethnicity and the sample size was small (367). In contrast, a larger UK-based study published soon after, reported that women with a vaginal microbiome dominated by *L. crispatus* were at reduced risk of sPTB whereas women with a *L. iners* dominated microbiome, had an increased risk of sPTB (353). A Canadian study, consisting of mostly White women, showed through 16S rRNA analysis, that *L. gasseri*, *L. johnsonii*, *L. crispatus*, *L. acidophilus, L. iners, R. solanacearum, B. longum* and *B. breve* associated with a lowered sPTB risk, whilst *Lactobacillus* depletion and the presence of BV-associated bacteria related to an increased sPTB risk (368). Ethnicity as a determinant of the vaginal microbiome and consequently sPTB risk has been suggested by other authors. In a case-control study of pregnant women consisting of mostly Caucasian women, CST IV was inversely correlated to gestational age at delivery and the risk of sPTB was further impacted by increased abundances of *Gardnerella* or *Ureasplasma* (349). In a follow up study, authors performed 16S rRNA sequencing on Caucasian and African American women. Whilst *Lactobacillus* depletion and greater *Gardnerella* abundance were more common in Black women, this only predisposed Caucasian women to PTB (369). Meanwhile, *L. crispatus* was protective against sPTB in both ethnic groups (369). As part of the Human Microbiome Project, the vaginal microbiome in African women was compared between women delivering term and preterm. Metataxonomic analysis showed that those delivering preterm had lower levels of *L. crispatus* and higher levels of *Prevotella* species (370).

Characterisation of the vaginal microbiota in women experiencing PPROM has also been an area of research. PPROM provides entry for ascending pathogenic microbes. Whether infection is a cause or consequence of PPROM is a long-standing conundrum. On one hand, the ascension of pathogenic vaginal microorganisms may trigger a pro-inflammatory cascade, culminating in cervical remodelling and rupture of fetal membranes. On the other hand, rupture of fetal membranes may permit entry of pathogens leading to chorioamnionitis and funisitis. Half of women with *Lactobacillus* spp. dominance prior to the onset of PPROM develop dysbiosis following PPROM, the effect of which is worsened by erythromycin (371). Furthermore, vaginal dysbiosis has been shown to predispose to chorioamnionitis and EOS (371). Subsequent studies have shown that PPROM is associated with a shift towards greater diversity within the second trimester of pregnancy (372).

Outcomes of women experiencing preterm cervical dilatation have been insightful. Women undergoing an emergency cervical cerclage demonstrate a bimodal distribution of delivery, with delivery occurring either before 25^{+0} weeks' or after 34^{+0} weeks gestation (373). This bimodal distribution may reflect the underlying cause of preterm cervical dilatation, with subclinical infection predisposing women to earlier delivery. A prospective analysis of women presenting with cervical dilatation and exposed fetal membranes showed that in 40% of cases, *Lactobacillus* spp. abundance was reduced in comparison to 10% in gestational age-matched controls (374). *Gardnerella vaginalis* was over-represented in women experiencing eventual miscarriage. This study highlights two possible aetiologies; whilst some women may experience a cervical mechanical abnormality and respond well to cerclage placement, a second group of women whereby pathogenic colonisation of the cervicovaginal niche exists, respond less favourably to emergency cerclage placement.

Many studies of the vaginal microbiome in pregnancy have now been undertaken, facilitating recent metanalyses on harmonised datasets. Huang et al (2022) performed an integrated analysis of data published from 12 different cohorts involving pregnant women whose vaginal microbiomes were assessed with 16s rRNA sequencing (375). Although these 12 cohorts had variations in study methodology, sequencing technique, PTB definitions and population demographics, the vaginal microbiome was

shown to have PTB predictive ability. *L. crispatus* abundance negatively correlated with PTB whereas *L. iners* dominance or high diversity (*Prevotella* and *Gardnerella* spp.) associated with a higher sPTB risk (375). Similarly, Golob et al (2023) aggregated published data from nine studies and identified VALENCIA CSTs to have sPTB predictive ability, particularly for early PTB (<32^{+0} weeks') (376).

1.7.4 Placental microbiome

Ascending vaginal bacteria into the 'sterile' uterine environment is a well accepted pathway leading to sPTB. Some argue that the placenta is not a sterile environment and efforts have been made to characterise a potential genuine placental microbiome and its influence on pregancy.

Haematogenous spread of microbes and subsequent placental colonisation have been postulated as an infective pathway leading to sPTB (377). Oral to placental transmission of pathogens has been illustrated in animal models (378). Although common oral pathogens have been identified in the placenta of women with sPTB, treatment of peridontal disease during pregnancy does not prevent sPTB (379, 380).

Metataxonomic and metagonomic analysis of placental samples obtained under sterile conditions identified taxa comparable to the human oral microbiome, including microbiota from the Firmicutes, Tenericutes, Proteobacteria, Bacteriodetes, and Fusobacteria phyla (381). Authors suggest that the placenta constitutes a low-abundance microbiome, consisting of non-pathogenic commensals seen within the human oral microbiome. In view of the low biomass reported, these bacteria are unlikely to play a metabolic role. Moreover, the microbes identified in this study have only been described to exist in environments such as soda lakes, saline habitats and marine environments, and are likley to represent reagent or experimental contamination. Other studies using culture-based, metatoxonomic or metagonomic analysis have failed to demonstrate a placental microbiome (382, 383).

1.7.5 Neonatal gut microbiome

The neonatal gut microbiome plays an important role in early infant health such as development of the immune system, metabolism, gastrointestinal health, allergies and autoimmune disease (384, 385). The mother's microbiota directly influences the neonatal microbiota and mother-to-child vertical transmisison of bacterial strains have been described (386). Amniotic fluid sampling from mothers who deliver preterm show microbial DNA and pro-inflammatory proteins present (387). The fetus swallows large amounts of amniotic fluid during the second and third trimesters of pregnancy and it has been postulated that ingestion of amniotic fluid can influence fetal gut colonisation, triggering a fetal immune response that leads to preterm parturition (385). The impact of the vaginal microbiome and *in-utero* environment on the neonatal gut microbiota is undetermined. Whether a difference in the neonatal gut microbiome occurs as a result of prematurity is unknown.

1.8 Host immune response

1.8.1 Innate immune system

The immune system can be divided into innate and adaptive immune responses. The innate immune response provides non-selective but immediate defence and consists of physical barriers such as mucous membranes, immune cells including neutrophils, dendritic cells (DCs), macrophages and monocytes, and soluble proteins such as cytokines, chemokines and complement. Neutrophils are the dominant immune cell type within the cervix during pregnancy (388). Epithelial cells lining the female reproductive tract act as a first line of defence. They produce mucus consisting of anti-microbial proteins such as defensins, which function to prevent pathogen entry. Mucus consists of complex, high molecular mass glycoproteins, known as mucins, which can trap pathogenic microorganisms (389). Other anti-microbial proteins that are released by epithelial cells include calprotectin, lysozymes, lactoferrin, secretory leucoprotease inhibitor, human neutrophil peptides and human β-defensins (390).

Macrophages and DCs recognise viral or bacterial infection through pattern recognition receptors (PRRs) including TLRs and nod-like receptors (NODs). Invading pathogens express pathogen-associated molecular patterns (PAMPs), which on binding to PRRs, activate the innate immune system (391). The recognition of PAMPs leads to a release of pro-inflammatory cytokines (IL-1β, TNF-α and IL-8) and anti-microbial peptides (392). Such interactions act as a bridge with the adaptive immune response; DCs interact with PRRs to stimulate maturation of naive T cells, which further influence both innate and adaptive responses. Non-immune cells such as trophoblasts and decidual cells also express TLRs, contributing to innate immunity within the feto-maternal interface (393).

1.8.2 Cytokines

Cytokines are soluble signalling proteins that enable intercellular communication and function to regulate cellular functions (394). A summary of the function of major cytokines in pregnancy is presented in Table 1.2. Overall, cytokines play an important role in regulating different immunological phases of pregnancy. Aberrant cytokine signalling and functioning is seen in pathological pregnancy-associated conditions including sPTB, pre-eclampsia and gestational diabetes mellitus (395).

Table 1.2 – Cytokines and their roles in normal pregnancy. Several pro-inflammatory and anti-inflammatory cytokines are involved with physiological processes of pregnancy. Abbreviations: NK cells= natural killer cells, Treg cells = regulatory T cells, Th cells = T-helper cells, CRP = C-reactive protein, DC = dendritic cells, MMP = matrix metalloproteinase.

	Cytokine	Function
Pro-inflammatory	IFN-γ	-Released by uterine NK cells -Contribute to spiral artery remodelling (396) -Involved in differentiation of Th0 cells to Th1 cells
	TNF-α	-Released by maternal peripheral blood mononuclear cells (397) -Multipotent cytokine -Contributes to hormone and steroid synthesis -Influences embryo development -Involved in placental differentiation and parturition (396)
	IL-1β	-Released by innate immune cells, such as monocytes and macrophages (398) -Also released by vaginal epithelial cells (399) -Influences myometrial contractions during labour (400)
	IL-2	-Regulates NK cell activation (401)
	IL-6	-Pro-inflammatory effects -Produced by almost all immune and non-immune cells such as monocytes, macrophages, T cells, neutrophils and endothelial cells (402, 403). -IL-6 migrates to the liver stimulating release of CRP (404). -Involved in T cell proliferation and cell migration

		-Regulates leukocyte and haemopoietic cell function (405)
	IL-8	-Involved in timing of delivery (406) -Recruits neutrophils and macrophages (403, 407)
	IL-12	-Released by maternal peripheral blood mononuclear cells -Maintains balance between Th1 and Th2 cells (408)
	IL-17	-Released by Th17 cells, CD4+ cells, NK cells and monocytes -Functions to recruit neutrophils -Reduces Treg cells (409)
Anti-inflammatory	IL-4	-Involved in the differentiation of T0 cells to Th2 cells -Released by activated T cells, mast cells, basophils, eosinophils and NK cells -Mediates Th2 function (410) -Mediates Treg cells -Inhibits Th1 response
	IL-10	-Released by Th1, Th2 Th17 cells, monocytes, macrophages, DCs, human B cells, granulocytes, eosinophils and mast cells (410) -Also released by non-immune cells such as epithelial cells (410) -Expressed in placental villi -Regulates extravillous cytotrophoblast invasion and inhibits MMP expression (411) -Regulates recruitment, activation and suppression of immune and non-immune cells (410)

1.8.3 Complement system

The complement system involves over 20 proteins, which normally exist in an inactive state. In response to recognition of microorganisms, proteins of the complement system are activated sequentially, triggering an enzyme cascade. The complement system consists of three pathways: the classical, alternative and mannose binding lectin pathways. Following microbial recognition, all three complement pathways converge at the activation of C3 convertase and C5 convertase, leading to the subsequent formation of the membrane attack complex (MAC) (412). Cleavage of C3 into C3a and C3b occurs. C3a is a potent chemoattractant of immune cells including neutrophils. Cleavage of C5 to C5a also occurs, which acts as a further chemoattractant (413). The MAC forms pores in the cell membranes of pathogens, leading to cell lysis.

1.8.4 Adaptive immune system

The adaptive immune system refers to the body's second line of defence, is antigen-specific and therefore takes time to amount a response. Cells of the adaptive immune system include T cells and B cells. Activation by antigen presenting cells (APCs) and cytokines trigger T cells to release cytokines and B cells to release antibodies (414). Through their T cell receptors (TCRs), naive T cells recognise antigen fragments presented by major histocompatibility (MHC) molecules on the surface of APCs. The interaction between an MHC-antigen complex and TCRs contributes to further cytokine release and T cell differentiation into cytotoxic T cells (CD8+ cells) or T-helper (Th) cells (CD4+ cells). Cytotoxic T cells directly destroy infected cells or cancer cells. T-helper (Th) cells can further be categorised into Th1, Th2 and Th17 cells. Under the influence of interferon-γ (IFN-γ) and IL-4, naive T cells develop into Th1 and Th2 cells respectively. Th1 cells produce pro-inflammatory cytokines including IL-2, IFN-γ and TNF-α and mediate phagocytosis against intracellular pathogens, whilst Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (392, 415). IL-4 and IL-10 function as anti-inflammatory cytokines, whereas IL-6 is pro-inflammatory (416, 417). Importantly, IL-6 and IL-10 are not uniquely Th2 cytokines, but can in fact be produced by a variety of cells including Th1 cells, macrophages, B cells and fibroblasts (418, 419).

Hormones including progesterone, oestradiol and prostaglandin D2 also promote a Th2 profile (420-422). The balance between a Th1 and Th2 response during different phases of pregnancy is key to a successful outcome. Rather than functioning as separate entities, Th1 and Th2 cells work in alliance. Th17 cells produce IL-17, a pro-inflammatory cytokine that recruits neutrophils. Paradoxically, Th17 differentiation occurs in the presence of anti-inflammatory transforming growth factor (TGF)-β and the Th2 cytokine IL-6 (423). IL-17 enhances production of Th1 cytokines, including IL-1β and TNF-α.

1.8.5 Immunology of pregnancy

Pregnancy represents a unique immunological phenomenon, where acceptance of the semi-allogenic fetus is balanced against maintaining robust immune protection of the mother. Historically, it was hypothesised that pregnancy represented a state of immune suppression (424). However, a more complex adaptation to pregnancy occurs.

A pro-inflammatory phase is seen during implantation and placentation. Throughout implantation, a high level of Th1 cells and cytokines are seen, including IL-6, IL-8, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine ligand 1 and TNF-α, which are produced by endometrial stromal cells and infiltrating immune cells (425, 426). The uteroplacental unit is densely populated with immune cells, including uterine natural killer (NK) cells (65-70%), APCs such as macrophages (10-20%) and DCs (427). Uterine NK cells release IL-8, IFN-inducible protein-10 chemokines and angiogenic factors that promote vascular development within the decidua (428). Depletion of uterine DCs in mice shows embryo resorption, highlighting the role of uterine DCs in uterine receptivity and successful implantation (429).

Cytotrophoblast cells fail to express human leucocyte antigen (HLA) class I and II molecules and instead express HLA-G and HLA-E. HLA-G has limited polymorphism, meaning that paternally derived HLA-G will present similarly to maternally derived cells, avoiding recognition by maternal T lymphocytes (430).

Following successful implantation, an anti-inflammatory Th2 state is seen during the second trimester, which associates with fetal growth and development. A number of immune cells play a role in establishing the anti-inflammatory environment during the second trimester, such as macrophages, decidual NK cells and regulatory T (Treg) cells (431). Decidual NK cells interact with $CD14^+$ macrophages and trigger Treg production (432). Decidual macrophages release anti-inflammatory cytokines which play a role in tissue renewal and placental growth (433). Their phagocytic function leads to the engulfment of apoptotic trophoblastic cells (434). Apoptotic cell clearance is crucial for maintaining an anti-inflammatory milieu. During pregnancy, Tregs protect fetal cells that express paternal antigens, thus preventing maternal rejection (435). Non-immune cells such as trophoblasts also contribute to a Th2 cytokine milieu (436). Additionally, progesterone potently induces Th2 cytokines, such as IL-4 and IL-5 (437). *In-vitro* work demonstrates the immunomodulatory effects of progesterone, whereby progesterone inhibits Th1 differentiation whilst promoting Th2 differentiation (438). *In-vivo* research has confirmed these findings. Peripheral blood sampling of pregnant women receiving progesterone or placebo, shows an increase in anti-inflammatory IL-10 and decrease in pro-inflammatory IL-1β and IL-8 mRNA levels with progesterone use (439). Alterations in genes that regulate Th2 cytokine release, alter the Th1/Th2 balance in pregnancy, contributing to sPTB pathogenesis (440, 441).

In the third trimester, there is a shift towards a pro-inflammatory state which ultimately leads to the initiation of parturition. The pro-inflammatory NF-κB signalling pathway is involved in initiating parturition (52).

A successful pregnancy depends on the ability to transition from one immunological state to another, as pregnancy progresses. Successful pregnancy associates with significantly higher IL-10 levels whereas miscarriage associates with significantly increased IFN-γ (442). Murine models show that injection of IL-10, anti-IFN-γ and anti-TNF-α agents prevent fetal loss (443). The shift from a Th1 to Th2 state is reflected by the clinical course of autoimmune conditions in pregnancy. Rheumatoid arthritis is largely Th1 mediated and unsurprisingly up to 70% of women with rheumatoid arthritis experience disease remission during pregnancy (444). On the other hand, systemic lupus erythematosus, characterised by a disordered Th2 response, can worsen (445).

The immune changes that occur in pregnancy are far more complex than an altered Th1 and Th2 response. A number of factors influence an individual's host immune response, such as host genetics, age, ethnicity, co-morbidities and host microbiome (446). For example TNF-α, IFN-γ and IL-6 gene polymorphisms may predispose to sPTB (447).

During pregnancy, alterations in the complement system also occur. There is evidence of complement activation in pregnancy with higher concentrations of C3a, C4a and C5a complement proteins within the circulating blood, enhancing host protection (448).

Analysis of immune responses in pregnant and non-pregnant women have shown that during influenza A infection, monocytes and DCs of pregnant women display an exaggerated pro-inflammatory immune response, characterised by increased expression of IFN-γ-induced protein and macrophage inflammatory protein-1β, when compared to non-pregnant women (449). This may explain why influenza A causes greater morbidity in pregnancy.

Changes in maternal circulating immune cells have provided insight into the immune changes of pregnancy. Profound changes have been reported in the peripheral blood of pregnant women including an increase in the total white cell count, neutrophils and monocytes (450). Analysis of peripheral blood lymphocyte subsets in pregnant and non-pregnant women has shown an increase in T lymphocytes, with the most remarkable increase seen in the first trimester (451). In contrast, B cells, cytotoxic $CD8^+$ and helper $CD4^+$ T lymphocytes remain stable throughout pregnancy (451). Assessment of peripheral blood in pregnancy shows a decrease in IL-2 and IFN-γ production, followed by an increase in IL-4 and IL-10 production, all of which are most profound in the third trimester, supporting a Th2 shift (452). Flow cytometric analysis of circulating immune cells shows the normal trajectory in pregnancy to include reduced production of Th1 cytokines (IL-2 and IFN-γ) and greater Th2 cytokine (IL-4) production (453).

The study of serum cytokines in healthy pregnancy has been compared to non-pregnant women. Serum IL-6 concentrations are higher in pregnant women than non-

pregnant women, and peak in the second and third trimesters (454). Others have shown inflammatory cytokines to peak in the first trimester, decrease in the second trimester and increase again towards term (455, 456).

Local cytokine production at the maternal-fetal interface is likely to be of more relevance than peripheral changes (437). Trophoblasts, decidua and amnion produce IL-4, IL-6, IL-10 and IL-13, adding to the Th2 environment (457-459). Co-culture of trophoblasts with T cells leads to an increase in transcription factors that mediate Th2 cytokine production whilst downregulating Th1 transcription factors (460). *In-vitro* experiments reveal individual cell function, however, disregard *in-vivo* immune influences exerted by other cell types.

Changes in CVF cytokines have been studied across pregnancy. Analysis of CVF cytokine levels in 30 women, including IL-6, IL-8, IL-1β and TNF-α showed IL-6 and IL-8 to be lower in pregnancy, but other cytokines to be similar in pregnant and non-pregnant women (461). Trimester specific analysis has shown IL-6 and IL-8 production to plummet during mid-pregnancy and normalise to pre-pregnancy levels towards term (461). A larger study assessed CVF cytokine concentrations throughout pregnancy and also showed IL-8 and TNF-α to be significantly lower in the second trimester (462). CVF concentrations for IL-1α, IL-1β, IL-6, IL-10 and MMP-8 did not change across the trimesters (462). CVF IL-1α and IL-1β concentrations remain stable between the second and third trimester, however levels peak 4-14 days prior to labour, as does the concentration of IL-1ra (IL-1 receptor antagonist) (463). When CVF concentrations were compared to serum concentrations during pregnancy, IL-1β, IL-6, IL-8 and MMP concentrations were significantly higher in CVF, whereas IL-1α and IL-10 were significantly higher in serum (464).

1.8.6 Immune changes in labour

A number of pro-inflammatory cytokines play a role in the onset of labour, including IL-1ß, IL-6, IL-8 and TNF-α. Labour associates with a significant increase in mRNA expression of IL-1ß, IL-6, IL-8 in the cervix and myometrium, and infiltration of the cervix with neutrophils and macrophages occurs (465).

The peripheral immune response during labour has been studied. As labour is described as a pro-inflammatory state, it is unsurprising to see lowered IL-4 levels and increased TNF-α levels peripherally during labour (466). Others have also demonstrated increased concentrations of IFN-γ and IL-1β during labour (467). The study of Th1 and Th2 cells in peripheral blood however, show no alteration in the Th1:Th2 ratio during labour (468).

During labour local immune cell changes have been reported in the myometrium, cervix and fetal membranes (469). Pre-labour caesarean-section myometrial biopsies were compared to in-labour caesarean-section myometrial biopsies, taken in the absence of infection. Analysis showed a significant influx of neutrophils and macrophages at the upper and lower segments of the myometrium in labouring women, but not in non-labouring biopsies (470). Further analysis showed that the influx of leucocytes associated with an up-regulation of pro-inflammatory cytokine mRNA expression, including IL-6, IL-8 and IL-1ß (471). Others have reproduced similar results demonstrating increased expression of IL-6, IL-8 and IL-1ß mRNA at the lower uterine segment with cervical dilatation (472). Additionally, myometrial expression of intercellular adhesion molecule-1 (ICAM-1), an endothelial adhesion molecule involved in the adhesion and migration of leukocytes, is higher during labour when compared to pre-labour (473). In cultured human myometrial cells, IL-1ß triggers entry of calcium into myometrial smooth muscle cells, contributing to contractility (474). IL-1ß also stimulates phosphodiesterase activity, which in turn stimulates uterine contractions (475). Overall, such studies suggest that infiltration of leucocytes into the myometrium and local release of pro-inflammatory cytokines are involved with the process of labour.

The likening of cervical ripening to an inflammatory process was first described in the 1980s (476). It has since been established that increased IL-6, IL-8 and IL-1ß mRNA expression occurs at the cervix as well as the myometrium (471). Histological analysis shows that during labour, neutrophils and macrophages infiltrate the cervix (471). Nitric oxide, a signalling molecule involved in inflammation, also contributes to cervical ripening (477).

In the absence of histological inflammation, labour alters gene expression in chorioamniotic membranes, in keeping with an acute inflammatory response (478). Examination of fetal membranes shows increased expression of pro-inflammatory mediators in term labour without infection (479). Increased levels of IL-6, IL-8 and IL-1ß have been reported in amniotic and chorionic-decidual tissues in spontaneous labour at term (480, 481). Analysis of amniotic fluid protein levels as a reflection of products synthesised by the chorioamniotic membranes also supports an inflammatory response within the fetal membranes; concentrations of IL-6 , IL-8, IL-1α and IL-1ß within the amniotic fluid increase with the onset of labour (482-486).

1.8.7 Aberrant immune responses and preterm birth

Dysregulated immune pathways are associated with the pathogenesis of sPTB. Aberrant signalling of the innate response predisposes to sPTB. For example, TLR-2 and TLR-4 polymorphisms cause abnormal innate immune responses and sPTB (487). An absence of cervical macrophages has also been correlated with increased risk of delivery prior to 34^{+0} weeks, corresponding to reduced protection against ascending infection (488).

Abnormalities of the complement system also associate with sPTB risk. Elevated plasma C3a levels in the first trimester appear to predict PTB and PPROM; women with raised C3a levels are three times more likely to have adverse outcomes (489). Higher concentrations of complement proteins C3a, C4a and C5a have been reported in women with sPTB and intact membranes (490). C5a within the uteroplacental tissue has been shown to trigger pro-inflammatory cascades that initiate labour (491).

In-vitro work has shown peripheral blood mononuclear cells (PBMCs) in women that deliver preterm to produce significantly higher levels of Th1 type cytokines, including IFN-γ and IL-2, when compared to maternal PBMCs in women that deliver term (492). *In-vitro* environments may not reflect *in-vivo* happenings and are limited in their exclusion of immune effects from other cell types present *in-vivo*. Multiplex assays measuring cytokine concentrations in serum of pregnant women have shown

significantly higher concentrations of IL-1β, IL-2, IL-12, IFN-γ, IL-4 and IL-6 in women delivering preterm with histological chorioamnionitis (493).

Increases in peripheral cytokines have not consistently been shown to occur in sPTB (494). A case-control study measuring plasma cytokine concentrations, showed limited value in circulating cytokine levels in sPTB prediction. Mid-pregnancy elevations in IL-2, TNF-α and GM-CSF did not relate to sPTB risk and elevated IFN-γ and IL-6 were only weakly associated with sPTB (495). The limited value of serum cytokines has been reiterated by others; serum IL-6, IL-8 and TNF-α do not differ in women delivering term or preterm (496). Inconsistent results may relate to differing study methodology such as assessment of cytokines produced by isolated cell groups versus in whole blood.

If peripherally detected cytokines originate more locally from within the maternal-fetal interface, the study of CVF cytokines may provide more understanding into the role of cytokines in association with sPTB. Furthermore, by the time circulating cytokines are elevated, local inflammatory changes may already have initiated labour. Factors such as BMI, maternal age, smoking status, fetal gender and parity alter circulating cytokine concentrations in pregnancy (455, 497). The exaggerated release of IL-6 and TNF-α seen in obese women has been attributed to adipose release of cytokines (456). A systematic review comparing peripheral and local cytokines in sPTB, concluded that CVF and amniotic fluid cytokines strongly associate with sPTB, in comparison to systemic cytokines, implicating local inflammation in the aetiology of prematurity, rather than systemic inflammation (494).

Several studies have investigated the cervicovaginal immune milieu in response to the vaginal microbiome in pregnancy. Increased bacterial diversity relates to an elevated pro-inflammatory cytokine profile within the CVF. Fetweiss et al (2019) demonstrated that elevated CVF cytokines (eotaxin, IL-1β, IL-6 and macrophage inflammatory protein (MIP)-1β) negatively correlated with *L. crispatus* abundance and associated with sPTB (370). The association between the vaginal microbiome and cervicovaginal cytokine profile can further be influenced by cervical cerclage placement. Braided sutures increase bacterial diversity and subsequently amplify CVF pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and MMP-1), leading to cervical remodelling and

increasing the risk of sPTB and non-viable pregnancy (269). Recent work has shown that vaginal depletion of *Lactobacillus* species and high bacterial diversity increases CVF IL-1β, IL-8, IL-6, immunoglobulin (IG) M , IgG, C3b and C5 concentrations (498). *L. iners* dominance relates to increased CVF IL-6, IgM, C3b, C5 and C5a. Overall, the vaginal microbiota influences the cervicovaginal immune profile, impacting the risk of cervical shortening and sPTB.

The interaction between the vaginal microbiome and host immune response is complex. β-defensin-2 is an anti-microbial peptide associated with innate immunity, and high levels have been identified to protect against sPTB (499, 500). In African American women, the risk of sPTB was high even in the presence of a *L. crispatus* dominated vaginal microbiome, when β-defensin-2 levels were low (501). In African American women, sPTB rates reduced with increased β-defensin-2 levels, irrespective of *Lactobacillus* spp. abundance (501). Immune factors, such as β-defensin-2 levels can moderate the risk of sPTB associated with depletion of *Lactobacillus* spp.

1.9 Live biotherapeutics

Live biotherapeutics are defined as live organisms, designed to treat, cure or prevent human disease (502). Although terminology is often used interchangeably, probiotics are defined as regulated dietary supplements and cannot make claims to treat or prevent disease (503).

1.9.1 Live biotherapeutics in reproductive health

The female body is colonised by approximately 44 x 10^{12} individual microorganisms across several niches, each with a unique microbial signature (504). The influence of the vaginal microbiome on reproductive health has led to the exploration of live biotherapeutics as a treatment strategy for adverse reproductive health states.

Recurrent UTIs

Recurrent UTIs affect one third of women and are defined as at least two UTIs within six months or three within one year (505, 506). The threat of antibiotic resistance has presented live biotherapeutics as an appealing alternative. Women with recurrent UTIs have an altered vaginal microbiota particularly at the time of active UTI, including persistent *E. coli* vaginal colonisation (507, 508). As lactobacilli produce hydrogen peroxide (H_2O_2), which inhibits *E. coli* growth, an absence of *Lactobacillus* spp. may predispose to UTI recurrence. The study of live biotherapeutics in prevention of recurrent UTIs has focused on strains with ability to produce H_2O_2, adhere to uroepithelial cells and prevent attachment of pathogens (509).

Clinical trials have produced conflicting results. A placebo-control RCT showed that vaginal *L. Casei v rhamnosus* did not alter UTI incidence in intervention versus placebo groups (510). Two studies have however, shown a significant reduction in UTI recurrence. One study trialled vaginal *L. crispatus* GAI 98322 and compared the number of UTIs before and after treatment. Authors showed a significant reduction in recurrence over a one year period when given to nine women (p=0.0007) (511). In another trial, 21 women were given vaginal *L. crispatus* GAI 98322, two to three times a week for one year. The incidence of cystitis during treatment and one year after *L. crispatus* use was significantly reduced and amplicon sequence analysis showed vaginal *Lactobacillus* colonisation (512).

BV

Antibiotic treatment of BV with metronidazole or clindamycin is limited in that dysbiosis can persist. Recurrence occurs in up to 69% of women within 12 months of antibiotic treatment (513). Clinical trials have assessed the role of *lactobacilli*-containing probiotics in their ability to treat and prevent BV.

Women with confirmed BV (Nugent score of 7 to 10) were treated with oral clindamycin, following which they were randomised to receive vaginal *L. casei rhamnosus* or no intervention (514). Participants were re-swabbed at 4 weeks following vaginal probiotic use. Re-assessment of Nugent scoring revealed that 69/83

(83%) of women receiving *L. casei rhamnosus* showed a reduction in their Nugent score by at least 5 grades in contrast to only 31/88 women (35%) in the non-intervention group (514). The improvement in Nugent scoring was significantly greater in women receiving vaginal *L. casei rhamnosus*.

Following vaginal clindamycin treatment of BV (Amsel criteria), Larsson and colleagues (2008) randomised women to receive vaginal probiotics (*L. gasseri* and *L. rhamnosus*) or a placebo vaginal capsule (515). Although the one-month cure rate was significantly higher in the placebo group, analysis at 6 months showed the time to relapse was significantly lengthened in women supplemented with *lactobacilli*.

A placebo-control RCT randomised women with BV to receive placebo or vaginal Physioflor (*L. crispatus* IP 174178) immediately after metronidazole treatment (516). There was a significantly lower incidence of BV recurrence (Nugent score and Amsel criteria) in women taking Physioflor when compared to placebo.

A systematic review concluded that *lactobacilli*-containing vaginally applied probiotics have potential to cure and prevent BV, but that no particular superior *lactobacilli* strain was identified (517). Studies to date are limited by a lack of molecular-based assessment of the vaginal microbiota following probiotic use.

Group B *Streptococcus* in pregnancy

Streptococcus agalacticae, also known as Group B *Streptococcus* (GBS) is a beta-haemolytic gram-positive facultative anaerobe. GBS is regarded as a commensal with colonisation seen in the gastrointestinal tract and vagina. In the UK, approximately 29% of pregnant women are colonised with GBS (518). During vaginal birth, vertical transmission of GBS causes EOS in 1-2% of neonates (519). As a result, intrapartum antibiotic prophylaxis with intravenous benzylpenicillin is recommended for women with known GBS (520). GBS colonisation is associated with depleted vaginal lactobacillus populations (521). *In-vitro* studies have shown *Lactobacilli* to have inhibitory effects on GBS growth (522, 523). There is therefore scope to extend probiotic use to manage GBS.

1.9.2 Live biotherapeutics and preterm birth

Current PTB prevention strategies are limited, none of which currently target microbial driven sPTB. Therefore, an urgent need exists for the development of new therapeutics. The vaginal microbiota and its ability to shape PTB risk raises the question of whether live biotherapeutics can modulate the microbiome.

Oral probiotics have been explored in their ability to prevent sPTB. Questionnaire-based research exploring dietary *lactobacilli* ingestion has been conducted. A Norwegian questionnaire assessed intake of milk-based *lactobacilli* products and categorised participants into none, low and high intake. Authors identified that women consuming regular milk-based *lactobacilli* had a reduced risk of sPTB (524). Consumption in early pregnancy was particularly protective (525).

Luoto et al (2010) randomised women to receive dietary counselling and oral probiotics (*L. rhamnosus* GG and *Bfifidobacterium lactis* Bb12) or dietary counselling and a placebo in the first trimester (526). There were no differences in sPTB rates between the two groups.

Nishijima and colleagues (2005) randomised women at $\geq 35^{+0}$ weeks' gestation to receive orally ingested fermented milk containing 10^9 CFU/mL of *L. johnsonii* La1 for two weeks, versus a placebo fermented milk (527). Vaginal samples were collected and following incubation on De-Man-Rogosa-Sharpe (MRS) agar plates, *lactobacilli* were grown and counted. Probiotic milk intake correlated with a significantly increased number of counted vaginal *lactobacilli*.

The combination of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 is a commonly studied oral probiotic. Oral use of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 during pregnancy showed a significantly reduced incidence of PPROM and operational taxonomic unit (OTU) analysis revealed a decreased abundance of vaginal *Enterococcus*, *Staphylococcus* and *Streptococcus* species, but no significant change in *lactobacillus* species. (528).

Others have shown oral *L. rhamnosus GR-1* and *L. fermentum RC-14* use to significantly increase vaginal *lactobacilli* abundance when compared to a placebo (529).

A UK-based clinical trial randomised women between 9^{+0} and 14^{+0} weeks' of pregnancy to receive oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or a placebo capsule (530). Metataxonomic assessment using 16S rRNA analysis showed no alteration in the vaginal microbiota, no colonisation with administered strains, no difference in the presence of *E. coli*, GBS or BV and no differences in alpha diversity between the groups.

Women at low-risk of sPTB were randomised to receive oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or a placebo capsule, during pregnancy (531). Vaginal samples were collected and cytokine assays performed. There were no differences in CVF cytokine concentrations between the two groups and 16S rRNA metataxonomic analysis of the vaginal microbiota once again showed no difference in microbial composition.

Other *lactobacilli* strains have been explored. Strains of *L. plantarum* and *L. crispatus* were consumed orally for 10 days, with vaginal and fecal sampling performed on 0, 10 and 7 days after course completion (532). Strains were identified using PCR and restriction endonuclease analysis. *L. plantarum* strains were identified in both vaginal and faecal samples, whereas *L. crispatus* was only detected in faecal samples.

Non-*lactobacillus* containing strains have also been explored. A retrospective analysis of women at high-risk of sPTB who took probiotics containing *Streptococcus faecalis*, *Clostridium butyricum* and *Bacillus mesentericus*, showed a longer gestational duration and lower rates of chorioamnionitis when compared to women who did not consume oral probiotics (533). Amplification of 16S rRNA genes from fecal samples have shown a lower abundance of *Clostridium* and *Bacteroides* in women that deliver preterm when compared to women that deliver term (534). Although Kirihara et al (2018) associate oral intake of *Streptococcus faecalis*, *Clostridium butyricum* and *Bacillus mesentericus* with term delivery, gut and vaginal microbiota analysis were not performed (533).

A Cochrane review conducted in 2018 concluded that there was insufficient evidence to support probiotic use for PTB reduction (535). Notably, the twelve included studies assessed oral probiotic use in low-risk populations. If probiotics are to reduce sPTB risk, vaginal *lactobacilli* colonisation is required. Studies trialling oral probiotics have not convincingly shown vaginal colonisation, proposing vaginal probiotics to be a more suitable option. To determine whether probiotics have potential to reduce PTB risk, women at high-risk of PTB should be targeted in the first instance. The study of probiotics requires careful consideration of strain selection with an appropriate dosing regimen.

A trial conducted in 1993 explored intravaginal application of yoghurt containing *Lactobacillus* (100 million/mL) on vaginal pH in pregnancy. Although authors emphasise that future work should explore intravaginal probiotics, the focus of recent research has been on oral probiotics (536). Since this conclusion made in 1993, vaginal probiotics in pregnancy have been sparsely studied.

Probiotics have been considered in the context of PPROM to improve clinical outcomes. Vaginal dysbiosis characterised by depletion to *Lactobacillus* spp. is seen prior to PPROM, in one third of cases (371). Although erythromycin is widely recommended in the clinical management of PPROM, erythromycin has been shown to exacerbate vaginal dysbiosis, correlating with an increased risk of funisitis and early onset neonatal sepsis (371). Oral probiotics are being assessed in their ability to prolong pregnancy following PPROM. Sandeford et al (2021) outline a protocol involving the randomisation of women with PPROM to receive oral *L. fermentum* or a placebo intervention (537).

Studies assessing vaginal probiotics in PPROM have been conducted, with conflicting results. Yadav et al (2022) randomised women with PPROM to receive a vaginal placebo or a vaginal probiotic containing *L. crispatus, L. rhamnosus, L. gasseri* and *L. jensenni*, twice daily for 10 days in addition to routine antibiotics (intravenous ampicillin and erythromycin for 48 hours, followed by oral amoxicillin and erythromycin for 5 days) (538). Authors found no benefit in prolonging latency period with vaginal probiotic use. Vaginal sampling was not performed and impact on the vaginal microbiota was not explored.

In contrast, Luzi et al (2018) demonstrate that daily vaginal *L. rhamnosus* in PPROM cases, prolonged time to delivery by 26.5 days when compared to 11.6 days in placebo groups (539). Once again, impact on the vaginal microbiota was precluded from assessment. Kavak et al (2014) also compared outcomes of women with PPROM when receiving ampicillin alone, versus ampicillin plus vaginal *L. casei rhamnosus*. Latency periods were significantly prolonged in women receiving vaginal *L. casei rhamnosus*. The mean time to delivery was 41.4 days in women receiving vaginal *L. casei rhamnosus* and 12.3 days in those receiving ampicillin alone (540). Overall, studies exploring vaginal probiotics in PPROM are insightful but sparse.

1.9.3 *Lactobacillus crispatus* CTV-05 (LACTIN-V)

Research associating *L. crispatus* with favourable reproductive outcomes, has led to the consideration of *L. crispatus* as a live vaginal biotherapeutic to explore potential clinical benefit (541). Whilst most commercially available probiotic *Lactobacilli* strains are not commonly isolated from the vagina, *Lactobacillus crispatus* CTV-05 (LACTIN-V) has been isolated from the vagina of a healthy woman (542).

Lactobacilli are the predominant microorganisms within the vaginal microbiota and exhibit bactericidal and viricidal properties through lactic acid and H_2O_2 production (543). *L. crispatus* adheres to vaginal epithelial cells and blocks attachment of pathogenic bacteria (544, 545). *In-vitro* studies show *L. crispatus* to be superior to other *Lactobacillus* spp. in its ability to produce both D and L lactic acid isomers, that not all *Lactobacilli* spp. produce (546, 547). D lactic acid production occurs with *L. crispatus,* but not with *L. iners,* and provides protection from pathogens such as *C. trachomatis* (547). LACTIN-V inhibits vaginal and urinary pathogens, including *N. gonorrhoea, B. fragilis, S. agalacticae, S. pyogenes, G. vaginalis, Dialister* spp., *E. Coli* and *Staph. aureus* (548). LACTIN-V is capable of vaginal colonisation in non-pregnant women (discussed in more detail in Chapter 4) (542). Antibiotic susceptibility of *L. crispatus* CTV-05 is comparable to other *L. crispatus* strains and importantly is resistant to metronidazole (548).

Administration of live vaginal biotherapeutics containing *L. crispatus* have been shown to be safe, with no adverse events when tested in non-pregnant women (511). More specifically, LACTIN-V use in healthy non-pregnant women is safe (549). Reports of probiotic lactobacilli causing bacteraemia and endocarditis exist, however no such events have been described with LACTIN-V use (550).

The ideal probiotic should have a unique DNA signature to allowing for the study of its colonisation. LACTIN-V has a unique DNA fingerprint when compared to all other *lactobacilli* (542). Mass production of LACTIN-V has shown genetic stability; when compared to the original strain of *L. crispatus* CTV-05, reproduced strains have shown comparable genetic fingerprinting (542).

1.10 Project justification

PTB is a global health concern, impacting women and children worldwide. Current preventative measures such as progesterone use, antibiotics and cervical cerclage are limited. There is incentive to explore alternative preventative strategies. Ascending infection and the vaginal microbiota are determinants for sPTB. Whether live biotherapeutics can improve the vaginal microbiota to confer protection is unknown.

Studies assessing live biotherapeutics in the prevention of PTB are of limited quality. Most trials consist of small sample sizes, trial the use of oral probiotics and perform mixed-strain analysis. Furthermore, targeting low-risk populations runs the risk of underestimating benefits in high-risk women.

This research targets a gap in the literature and aims to explore the role of vaginally administered *L. crispatus* CTV-05 in women at high-risk of sPTB. The first aspect of this research focuses on LACTIN-V safety, tolerability and acceptability among pregnant women (Chapter 3). Subsequently, the impact of LACTIN-V on the vaginal microbiota (Chapter 4) and local host immune response (Chapter 5) are assessed. Cytokine concentrations within CVF were studied, to provide a representation of the local immune response. The study of IL-6, IL-8, IL-1β and IL-10 were chosen in view of their well-established role in PTB.

1.11 Hypotheses

- *Lactobacillus crispatus* CTV-05 (LACTIN-V) will be safe and tolerable in pregnancy.
- Women at high-risk of sPTB will regard LACTIN-V as acceptable to use in pregnancy.
- LACTIN-V use will lead to vaginal colonisation with *L. crispatus* CTV-05.
- Successful *L. crispatus* CTV-05 colonisation will modify the vaginal microbiota.
- Successful *L. crispatus* CTV-05 colonisation will modulate the immune milieu at the cervicovaginal interface.

1.12 Aims

- To explore the safety, tolerability and acceptability of LACTIN-V use during pregnancy.
- To determine whether vaginal use of LACTIN-V leads to vaginal colonisation with *L. crispatus* CTV-05.
- To assess the impact of *L. crispatus* CTV-05 colonisation on the vaginal microbiota.
- To assess whether vaginal *L. crispatus* CTV-05 is detected in the neonatal gut microbiome.
- To assess the impact of *L. crispatus* CTV-05 colonisation on the host immune response.
- To observe the rate of sPTB in women at high-risk of sPTB using LACTIN-V.

2 Materials and Methods

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2.1 Materials

	Supplier	Notes
16S rRNA		
Kits and equipment		
Liquid Amies swab	BBL™ CultureSwab™, Becton, Dickinson & Company	
QIAmp® DNA Mini Kit	Qiagen (Catalogue No 51304)	
QIAmp® PowerFecal® Pro DNA Kit*	Qiagen (Catalogue No 51804)	
Glass beads	Sartorius Stedim (Catalogue No BBI-8541400)	
Qiagen Tissue Lyser LT	Qiagen (Catalogue No 69980)	
Illumina MiSeq platform	Illumina Inc. Texas, USA	
Enzymes		
Lysozyme	Sigma L6876, chicken egg white	Prepared to concentration: 10mg/mL in filter sterilised 10mM Tris Hydrochloride (HCl) pH 8.0
Mutanolysin	Sigma M9901/10Ku	Prepared to concentration: 25 U/µL by dissolving 10,000 units in 400µL sterile water
Lysostaphin	Sigma L9043	Prepared to concentration: 4000U/mL by dissolving 23 915 units in 20mM Sodium acetate (NaOAc)
Buffers and solutions		
Filter sterilised phosphate-buffered saline (PBS)	In-house	
NaOAc	Sigma Aldrich	
Tris HCl	Sigma Aldrich	pH 8.0 10mM
TE50	Sigma Aldrich	10mM Tris HCL, 50mM ethylenediaminetetraacetic acid (EDTA), PH 8.0
Triton (12%)	In-house	
Ethanol (100%)	Sigma Aldrich	
Diethyl pyrocarbonate (DEPC)-treated water	Sigma Aldrich	
Protease inhibitor (PI)/PBS mix**	Sigma Aldrich P8340	5µL of PI per 1mL PBS
Filter sterilised Tween 10%*	Sigma Aldrich	

**Meconium and neonatal stool DNA extraction*
*** Placenta and membranes DNA extraction*

	Supplier	Notes
PCR		
NEB One*Taq*® DNA polymerase	BioLabs	
dNTP 10mM (deoxyribonucleotide phosphate)	Sigma (Catalogue No D7295)	
5x buffer	BIO-37045	
6x loading dye	PROMEGA (G190A 1006034)	
Agarose	Invitrogen (Catalogue No 15510-019)	
SYBR Safe DNA stain	Invitrogen (Catalogue No S33102)	
Molecular weight marker	Bioline Hyperladder 100 base pairs (bp) (Catalogue No BIO-33056)	
GBS freeze-dried	ATCC	Used as a control
TBE buffer (Tris/Borate/EDTA)		
16S rRNA Universal Forward primers Sequence: 27F-5'AGAGTTTGATCCTGGCTCAG-3'	Sigma Aldrich	10 pmol/µL
16S rRNA Universal Reverse primers Sequence: 338R-5'GCTGCCTCCCGTAGGAGT-3'	Sigma Aldrich	10 pmol/µL
L. crispatus CTV-05 Forward primer Sequence: 5'-GCTGTTGCAGCCAGACAGTT-3'	Sigma Aldrich	10mM
L. crispatus CTV-05 Reverse primer Sequence: 5'-GCTGTTGCAGCCAGACAGTT-3'	Sigma Aldrich	10mM

Microbiology		
MRS agar plates (MRS = De Man, Rogosa and Sharpe)	Fisher Scientific Ltd	
Oxoid Anaerogen compact sachets	Thermo Scientific	
Inoculation wire	TS/2B Scientific Laboratory Supplies Ltd	
Cell scrapers	TPP cat no 99002 Scientific Laboratory Supplies Ltd	
Preparation of *L. crispatus* CTV-05 DNA		
Lyophilised *L. crispatus* CTV-05	Osel Inc. Ca, USA	
Anaerobic cabinet (Whitley A35 Workstation)	Don Whitley Scientific	
Filter sterilised PBS	In-house	
Omega E.Z.N.A.® Bacterial DNA Kit	Omega Bio-Tek (Catalogue No D3350)	
Sodium hydroxide (NaOH) (3M)	Sigma Aldrich	
Distilled water	In-house	
Qubit™ Protein and Protein Broad Range Assay Kit	Thermo Fisher, Cat. No. Q32853	
Bacterial load		
StepOne© qPCR machine	Thermo Fisher	
Optical 96-Well Reaction Plate	Starlab (Catalogue No E14037700)	
Plate seal EU Opti-Seal Optical Disposable Adhesive	Appleton Woods (Catalogue No BP231)	
Escherichia coli DNA	Sigma, D4889	
DEPC-treated water	Sigma Aldrich	
Platinum PCR Supermix UDG	Life Tech (Catalogue No 11730-017)	
Rox (100µmol/µL)	LifeTech (Catalogue No 11730-017)	
BACTQuant Forward primers (at 100µM) Sequence: 5'-CCT ACG GGA GGC AGC A-3'	Sigma Aldrich	0.025 µmol synthesis scale, desalted
BACTQuant Reverse primers (at 100µM) Sequence: 5'-GGA CTA CCG GGT ATC TAA TC-3'	Sigma Aldrich	0.025 µmol synthesis scale, desalted
BACTQuant Probe (10µmol/µL) Sequence: 5'-6FAM-CAG CAG CCG CGG TA-MGBNFQ-3'	LifeTech, Catalogue No 4316034	6000 pmol scale
Bacterial load (*L. crispatus* CTV-05)		
StepOne© qPCR machine	Thermo Fisher	
Optical 96-Well Reaction Plate	Starlab (Catalogue No E14037700)	
Plate seal EU Opti-Seal Optical Disposable Adhesive	Appleton Woods (Catalogue No BP231)	
L. crispatus CTV-05 DNA	Osel Inc. Ca, USA	
DEPC-treated water	Sigma Aldrich	

QuantiTect® SYBR® Green RT-PCR Master Mix (real time-PCR)	Qiagen	
L. crispatus CTV-05 Forward primer Sequence: 5'-GCTGTTGCAGCCAGACAGTT-3'	Sigma Aldrich	10mM
L. crispatus CTV-05 Reverse primer Sequence: 5'-GCTGTTGCAGCCAGACAGTT-3'	Sigma Aldrich	10mM
Immune assays		
Kits and equipment		
Transwab® MW170 with rayon bud type	Medical Wire & Equipment, Corsham, UK	
Human pre-mixed multi-analyte kit	R&D Systems (Catalogue No LXSAHM)	
MAGPIX® analyser	Luminex®	
Hand-held microplate magnet		
Microplate (96-well plate)		
Foil plate sealer		
Horizontal orbital microplate shaker		800 +/- 50 revolutions per minute (rpm)
Buffers and solutions		
Filter sterilised PBS	In-house	
PI	Sigma Aldrich P8340	
Distilled water		

2.2 Methods

2.2.1 Study overview

Study design

This study was a non-randomized pilot clinical trial, where participants received a course of *Lactobacillus crispatus* CTV-05 (LACTIN-V). At 14^{+0} weeks gestation, women commenced LACTIN-V, self-administered with a vaginal applicator (Figure 2.1). Each applicator contained LACTIN-V powder containing 2 x 10^9 colony-forming units (CFU). The intervention consisted of a loading phase, followed by a maintenance phase (Figure 2.2). The trial was named the FLIP-1 study as it aimed to assess the feasibility of LACTIN-V in pregnancy.



Figure 2.1 - LACTIN-V applicator. Pre-filled applicators contained 2 x 10^9 CFU of *L. crispatus* CTV-05.

LACTIN-V product

LACTIN-V is the first vaginal live biotherapeutic aimed at treating disorders of the female reproductive tract and has already been used as an adjunctive treatment in the prevention of recurrent UTIs and BV. LACTIN-V is unique in that it consists of a unique strain of *L. crispatus* that is naturally occurring and isolated from the vagina of a healthy woman (548). Other benefits include its ability to adhere to epithelial cells, colonise the vagina and antagonise pathogens (548).

Since its first trialled use, the administration technique and dose of LACTIN-V has been optimised. Initially LACTIN-V was formulated as a vaginally delivered gelatin

capsule with a dose of 5 x 10^8 CFU/capsule. In a phase 1 trial, this low dose gelatin capsule of LACTIN-V was shown to be well-tolerated and safe (509). A subsequent phase 2 trial, using the same gelatin capsules demonstrated *L. crispatus* vaginal colonisation (551). Authors concluded that the gelatin capsule did not dissolve as expected in the vagina, limiting *L. crispatus* CTV-05 colonisation and suggested that efficacy could be improved by using a vaginal applicator and increasing the dose to 2 x 10^9 CFU/dose. This modification was trialled in a phase 1 trial whereby women were randomised to receive either 5 x 10^8, 1 x 10^9 or 2 x 10^9 CFU/dose of LACTIN-V by a vaginal applicator. Use of LACTIN-V with a vaginal applicator was safe and well accepted by women at all three doses (549). However, all these studies have been conducted in non-pregnant women. This is the first in-pregnancy clinical trial offering LACTIN-V to pregnant women.

Dosing regimen

The vaginal microbiota during the early second trimester is a risk factor for sPTB (353, 372). Importantly, women surveyed in pregnancy report drug avoidance in the first trimester, due to concerns regarding teratogenicity (552). To pre-emptively avoid this concern, whilst attempting to modulate the microbiota at a stage known to correlate with PTB risk, LACTIN-V was commenced at 14^{+0} weeks' gestation.

The dosing regimen included a loading dose (once daily LACTIN-V for five consecutive days) followed by a maintenance dose (once weekly LACTIN-V for six weeks). Five consecutive days of LACTIN-V use followed by once weekly LACTIN-V for two weeks has shown to be safe in non-pregnant women, with evidence of colonisation seen 28 days after last use of LACTIN-V (553). Longer maintenance phases lasting for ten-weeks show colonisation 24 weeks after LACTIN-V use (554).

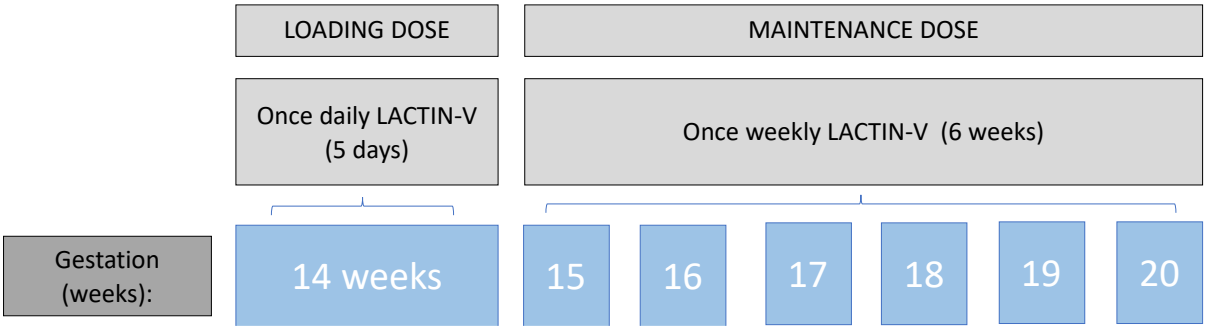


Figure 2.2 – LACTIN-V dosing regimen. The loading dose consisted of five consecutive daily doses of LACTIN-V. During the maintenance phase, women administered LACTIN-V once weekly for six weeks.

Patient and public involvement (PPI)

A PPI group consisting of historical patients from the PTB prevention clinic were involved in the study design. Members of the PPI group meet annually with researchers to discuss on-going and new research ideas. Seven members of the PPI group contributed to the FLIP-1 study design. In advance of the PPI meeting, draft copies of the study protocol, patient information leaflet and consent form were emailed to women. At the PPI meeting, the study design was discussed with women in detail, including logistics of self-administration and LACTIN-V storage, which required refrigeration. PPI members reported that LACTIN-V self-administration appeared straight-forward, and that refrigeration would not be problematic. Two members of the PPI group suggested the incorporation of a paper diary, to remind women of the dosing regimen and follow-up appointments. Advice for study participants to avoid lubricant use, vaginal douching and sexual intercourse was discussed. The group deemed these to be achievable.

Members of the PPI group also contributed to the written language within the patient information leaflet and consent form. A common point emphasised by women of the PPI group was to highlight the safety profile of LACTIN-V from previous non-pregnancy studies. The use of jargon was also highlighted. For example, it was suggested to describe the assessment of ‘colonisation’ as ‘whether *Lactobacillus crispatus* remains in the vagina’.

It was proposed by the PPI group to arrange double appointment slots in the PTB prevention clinic, to allow participants time to ask questions and collect clinical samples. There was a detailed discussion regarding the use of lay man's terminology when describing the study design to women at recruitment. It was suggested to refer to the 'microbiome' as 'flora'. One woman also highlighted the importance of using sensitive language when referring to previous miscarriages, severe PTB, and avoidance of the phrase 'cervical incompetence'. Another woman suggested describing women as 'at risk' of PTB rather than 'high-risk'.

Pre-study questionnaire

Members of the PPI group also contributed to the design of a pre-study questionnaire. Prior to the study start, feasibility of the FLIP-1 trial was assessed through a pre-study questionnaire. Women from the PPI group and existing patients from the PTB prevention clinic were given this questionnaire, aimed to assess willingness to trial live vaginal biotherapeutics as part of a clinical trial in pregnancy (Appendix 3.1).

Ethical approval

This study was sponsored by Imperial College London. The FLIP-1 study received ethical approval from the London-Surrey Borders Research Ethics Committee (IRAS 262987). Health Research Authority (HRA) and Health and Care Research Wales Approval was given for the FLIP-1 study. The trial is registered at www.clinicaltrials.gov (NCT03992534). *Lactobacillus* species were extracted from vaginal swabs of pregnant women enrolled in VMET2 (Vaginal Microbiome and Metabolome in Pregnancy 2), with ethics approval provided by the NHS HRA (London-Stanmore Research Ethics Committee, REC 14/LO/0328). The incidence of PTB was compared to control cohorts (Chapter 3), who were enrolled in VMET2.

Funding

This study was funded for by the March of Dimes European Prematurity Research Centre at Imperial College. LACTIN-V was supplied by Osel at no cost. The Imperial

Health Charity and Parasol Foundation Partnership funded the final year of my fellowship.

Target study population

Participants were recruited from the PTB prevention clinic at Queen Charlotte's and Chelsea Hospital (QCCH), Imperial College NHS Trust. Women identified as high-risk of PTB at their booking appointment were subsequently referred to the PTB prevention clinic. This included women with a history of previous sPTB (<37^{+0} weeks), previous PPROM (<37^{+0} weeks), previous MTL (>16^{+0} weeks), previous cervical shortening, previous cerclage, history of cervical FDCS, congenital uterine anomaly, cone biopsy, LLETZ or Ehlers-Danlos syndrome.

Women were referred to the PTB prevention clinic by 12^{+0} weeks' gestation, where TVUS assessment of cervical length was performed every 2-4 weeks, with surveillance until 28^{+0} weeks' gestation. The frequency and duration of surveillance was greater than that recommended by the saving babies' lives care bundle (version 2) in view of the research activity within the clinic, as well as patient-led requests for regular reassurance.

Study eligibility

Women defined as being at a higher than background risk for PTB were approached in the PTB prevention clinic for study participation.

Inclusion and exclusion criteria

Women with the described risk factors for PTB were eligible if they were aged ≥18, between 11^{+0} and 15^{+6} weeks' gestation and able to give informed consent. Women were excluded from recruitment if aged <18 or if unable to provide informed consent. Women with HIV were excluded from recruitment due to the impact of HIV and anti-retroviral therapy use on the risk of PTB (555). Women receiving antibiotics within one week of recruitment were also excluded due to alterations in the vaginal microbial composition that could ensue (556, 557).

Consent and counselling

Women meeting the inclusion criteria were approached for recruitment. As part of the consent process, women were provided with an in-depth consultation outlining the study design and were provided with a patient information leaflet. All participants were shown how to self-administer LACTIN-V as well as how to store it in a refrigerator.

Recruitment

A total of 123 women meeting the inclusion criteria were approached for participation, of which 73 (59%) consented to study involvement and 50 (41%) declined. Further details on study participation are outlined in Chapter 3.

Research timeline

Following recruitment at 14^{+0} weeks' gestation, participants were followed longitudinally throughout pregnancy. Women were followed up at 15^{+0}, 18^{+0}, 20^{+0}, 28^{+0} and 36^{+0} weeks' gestation as well as at delivery for sample collection and assessment of compliance, adverse events and tolerability (Figure 2.3). Women were asked to avoid sexual intercourse throughout the study. Participants were given a diary to aid compliance, record adverse events and antibiotic use. Postnatally, participants completed a questionnaire to determine overall experience of LACTIN-V.

Sample collection and storage

At the point of recruitment, pre-intervention clinical samples were collected (T0). On the following day participants commenced LACTIN-V. During the maintenance dose, to ensure sampling was not performed immediately after LACTIN-V administration, women were asked to administer LACTIN-V after collection of vaginal samples (Figure 2.4). Vaginal sampling was always performed prior to TVUS, to avoid contamination with lubricant jelly.

At each timepoint, the following vaginal swabs were collected:

- Two BBL™ MaxV Liquid Amies swabs (Becton, Dickinson and Company, Oxford, UK) for 16S rRNA gene sequencing, quantitative real-time polymerase chain reaction (qPCR) and microbiology.
- A Transwab® MW170 with rayon bud type (Medical Wire & Equipment, Corsham, UK) for cytokine analysis.

High vaginal swabs were taken by trained doctors or midwives in the PTB prevention clinic. An additional BBL™ MaxV Liquid Amies swab vaginal was collected at T4 (28^{+0} weeks' gestation) to determine whether live *L. crispatus* CTV-05 could be cultured. At T6, in addition to vaginal swabs, delivery samples were taken. These included placental tissue, fetal membranes and first-pass meconium. At 14 days postnatal, neonatal stool was collected (Figure 2.3). The placenta and fetal membranes were collected wearing sterile gloves and placed into sterile specimen pots. Using sterile gloves and a sterile scalpel, the placenta (sized 1cm x 1cm x 1cm) and fetal membrane (2cm long) samples were dissected, 4cm from the cord insertion site in an open-room environment. Samples were aliquoted into one cryo-tube and snap-frozen at -80°C. Placental samples encompassed both maternal and fetal sides.

Meconium and neonatal stool were collected from diapers. Using a sterile spatula, the outer layer in contact with the diaper was removed. With a second sterile spatula, a sample was taken and placed in a sterile tube. Samples were snap frozen and stored at -80°C in the Imperial College London research tissue bank.

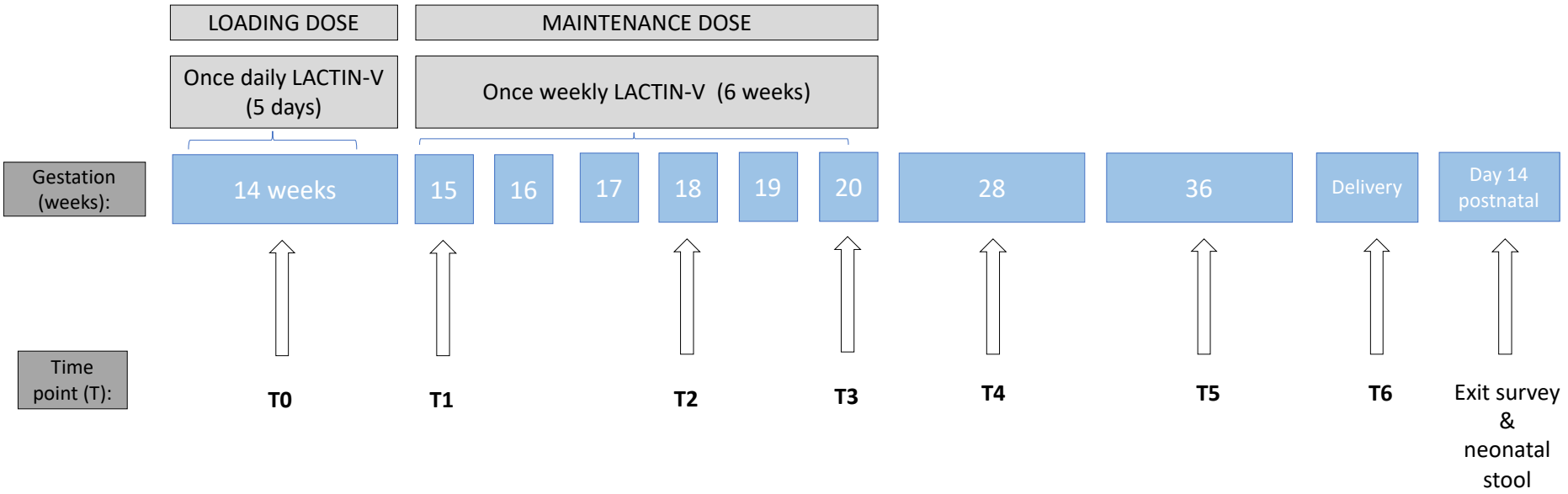


Figure 2.3 – Study design. Participants were recruited at 14^{+0} weeks' gestation and provided with LACTIN-V. Pre-treatment samples were taken at timepoint (T) 0 after which, samples were collected at 15^{+0}, 18^{+0}, 20^{+0}, 28^{+0} and 36^{+0}-weeks' gestation which corresponded to T1, T2, T3, T4 and T5. Samples were also collected at delivery (T6), including placenta, fetal membranes and first-pass meconium. Postnatally participants were asked to complete an exit questionnaire and provide neonatal stool.

Week/GA	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Week 1 **14^{+0}**	Recruitment **T0 samples**	LOADING DOSE Day 1 LACTIN-V	Day 2 LACTIN-V	Day 3 LACTIN-V	Day 4 LACTIN-V	Day 5 LACTIN-V	
Week 2 **15^{+0}**	MAINTENANCE DOSE	**T1 samples** Maintenance dose LACTIN-V					
Week 3 **16^{+0}**		Maintenance dose LACTIN-V					
Week 4 **17^{+0}**		Maintenance dose LACTIN-V					
Week 5 **18^{+0}**		**T2 samples** Maintenance dose LACTIN-V					
Week 6 **19^{+0}**		Maintenance dose LACTIN-V					
Week 7 **20^{+0}**		**T3 samples** Maintenance dose LACTIN-V					
Week 15 **28^{+0}**		**T4 samples**					
Week 23 **36^{+0}**		**T5 samples**					

Figure 2.4 – Sample collection and LACTIN-V regimen. Following consent and recruitment, participants were taught how to self-administer LACTIN-V. Pre-treatment samples (T0) were collected and participants commenced LACTIN-V the following evening. T1 samples were taken after completion of the loading dose. To minimise contamination of samples with LACTIN-V administration, T1 – T3 sample collection was performed during the day prior to evening LACTIN-V use. Clinical samples were collected before TVUS, to avoid contamination with lubricant jelly.

Metadata

Detailed participant metadata was recorded by the research team including maternal age, BMI, ethnicity, obstetric history, gynaecological history, smoking status, and blood group.

TVUS

Each research visit coincided with a clinical consultation at the PTB prevention clinic. In accordance with local and national guidance, TVUS cervical length measurements were taken by trained clinical staff (558). Following emptying of the woman's bladder, the transvaginal probe was inserted into the anterior fornix of the vagina. The endocervical canal was visualised in a sagittal view with visibility of both the internal and external os. Care was taken to avoid excessive pressure which may inadvertently lengthen the cervical length. Measurements were then taken by following the course of the cervical canal between the internal and external os (Figure 2.5). Three separate measurements were taken and the shortest of all was recorded.

Transvaginal cervical length assessment is more sensitive than transabdominal measurements, is safe and produces low-inter observer variation when performed by trained operators (200, 207, 211). A cervical length ≤25mm (<10th centile) predisposes women to sPTB (559). Cervical length is inversely proportional to the likelihood of sPTB and 90% of women with a cervical length >25mm deliver at term (560). The saving babies' lives care bundle (version 3) recommends assessing cervical length every 2-4 weeks between 16^{+0} and 24^{+0} weeks' gestation in women at high-risk of sPTB (246). In the PTB prevention clinic at QCCH, women are seen earlier, more frequently, and beyond 24^{+0} weeks to provide continuous and specialised antenatal care for women whilst facilitating research activity.

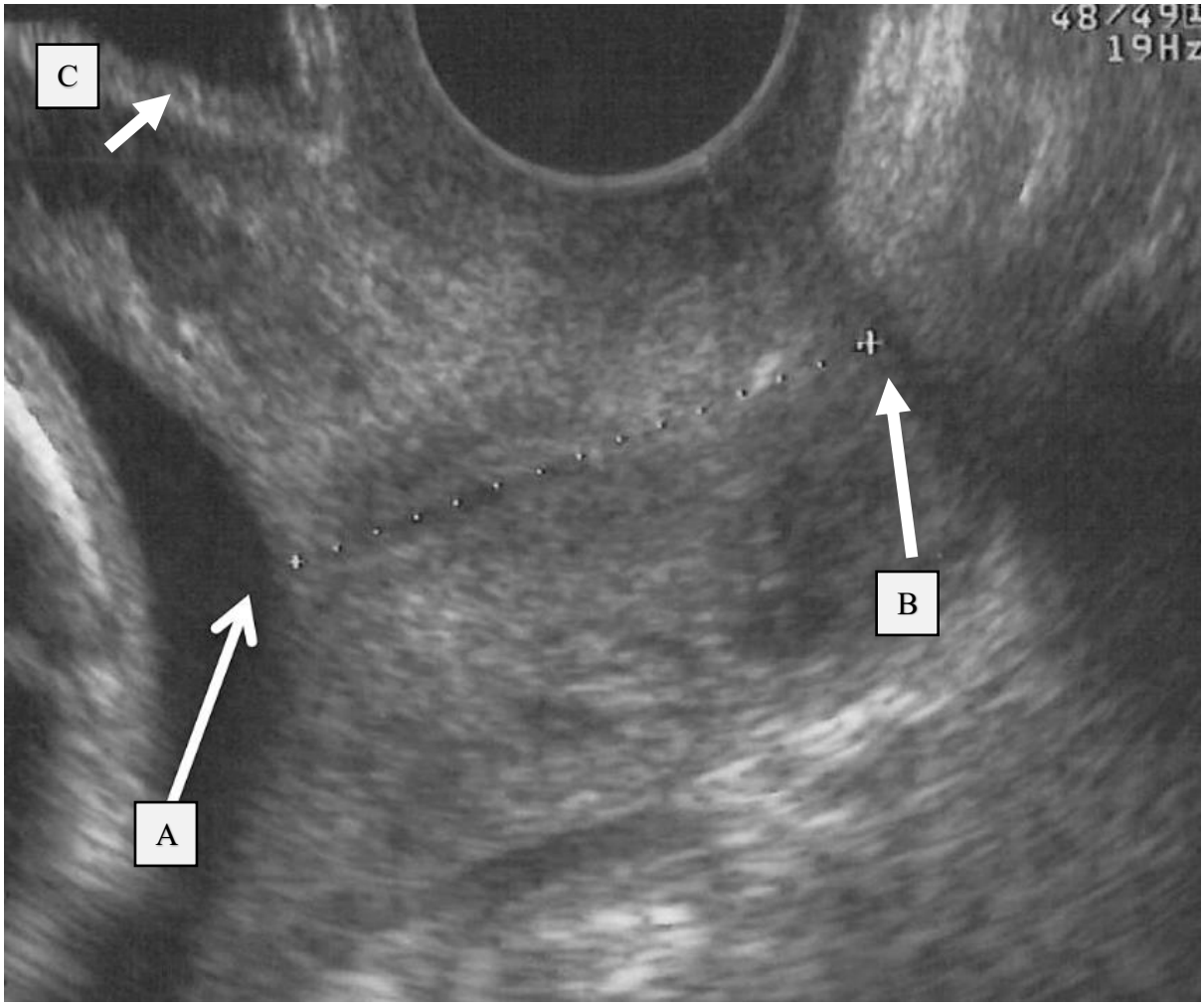


Figure 2.5 – Technique for TVUS cervical length measurement, reproduced from Nott et al (2016) (38). The dotted line measures the cervix from the internal os (A) to the external os (B). The bladder (C) can also be visualised. Women are asked to empty their bladders before scanning to improve visibility and avoid any mechanical pressure and lengthening of the cervix.

Clinical management in the PTB prevention clinic

In line with national guidelines, women <24^{+0} weeks' gestation with a cervical length of ≤25mm were offered a cervical cerclage and/or vaginal progesterone (400mg once daily) (558). Women with a history of a cervical cerclage due to cervical shortening or history of MTL were also offered a cerclage and/or vaginal progesterone (400mg once daily). In women >24^{+0} weeks' gestation where cervical shortening of ≤25mm was detected, vaginal progesterone (400mg once daily) was offered. Once commenced, progesterone was recommended until 34^{+0} weeks' gestation. For consistency it was planned that cervical cerclage placement would be performed by one of three

surgeons within the specialist PTB team at QCCH, using monofilament sutures. Due to the COVID-19 pandemic and restricted availability to operating theatres, this could not be implemented and cerclages were placed by trained consultants at both QCCH and St Mary's Hospital, using either monofilament or braided sutures. Cerclages were removed at approximately 36^{+0} weeks' gestation. In women having an elective caesarean-section (ELCS), cerclages were removed following the caesarean-section.

Clinical outcomes

Clinical outcomes were available through accessing electronic medical notes. Antenatal events such as infections, antibiotic use, cervical length, cervical cerclage or progesterone use were recorded. Obstetric outcomes including gestational age at delivery, presentation of preterm delivery (iatrogenic, spontaneous preterm labour, PPROM and/or chorioamnionitis) and mode of delivery were also recorded. Adverse outcomes including miscarriage, IUD, NND and congenital fetal anomalies were recorded.

Although not powered to assess clinical impact, key pregnancy outcomes in women treated with LACTIN-V were compared to two parallel non-intervention control arms. The first control cohort (group A) included a large population of 2,190 women seen in the PTB prevention clinics between 2002-2020. The second control cohort (group B) included 120 women seen in the PTB prevention clinic between April 2019 to September 2021. Group B was selected to mirror the FLIP-1 study time, to account for potential changes in PTB rates caused by COVID-19. Similarly to FLIP-1 participants, women from groups A and B were at high-risk of PTB and were recruited from the PTB prevention clinic. Metadata for group A was collected by Dr Katie Mountain.

Assessment of safety, tolerability and acceptability

After recruitment, women were followed up in the prematurity prevention clinic at 15^{+0}, 18^{+0}, 20^{+0}, 28^{+0}, 36^{+0} weeks' gestation as well as at delivery for the assessment of safety, tolerability and acceptability of LACTIN-V. Safety was assessed by the incidence and severity of adverse events. Women were asked to record solicited

adverse events on the provided diaries. At follow-up appointments, participants were asked to describe any unsolicited adverse events. Clinical staff reviewed whether unsolicited adverse events were related to LACTIN-V. Severity of adverse events were graded according to the National Institute of Health (NIH) Division of AIDS grading criteria. This is a well-established grading system created by a panel of experts and reviewers. The NIH have released a specific grading system relating to genitourinary adverse events secondary to trials involving new microbicide interventions (561). As the FLIP-1 cohort consisted of a population already at high-risk of miscarriage, MTL, PTB, PPROM and chorioamnionitis, these were not considered as adverse events. Fetal adverse events were assessed by reviewing medical notes for the occurrence of fetal malformations, IUD and NND. Tolerability was assessed by recording discontinuation of LACTIN-V due to adverse events. Acceptability was determined by reviewing study uptake and compliance.

Exit questionnaire

Acceptability was also assessed using an exit questionnaire. As there are no existing relevant questionnaires, a validated questionnaire was designed to assess experience of LACTIN-V use in pregnancy (Figure 2.6). The Medication Acceptability Questionnaire is a newly developed tool to assess adherence to oral medications by assessing convenience, taste, appearance, efficacy and tolerability. Although not applicable to the vaginally administered LACTIN-V, a similar approach was used to design and implement a patient-friendly questionnaire yielding relevant answers (562). Questions were structured to be concise and short, using either Likert scaling or close-ended questions. Questions aimed to gauge ease of use, storage, comfort, convenience and future use. The draft questionnaire was reviewed by two independent groups including relevant stakeholders for validity and reliability (563). The questionnaire was firstly reviewed by the multi-disciplinary research team including senior researchers, doctors and midwives involved in the study and secondly, by the PPI group. The PPI group suggested exploring partner acceptability, which may influence the decision to participate in some women. Language was amended to clarify questions, remove medical jargon and ensure unbiased questioning. The revised questionnaire was piloted by ten patients in the prematurity prevention clinic prior to the study start and the research team for final review.

In view of the COVID-19 pandemic and restrictions on hospital attendance, the questionnaire was conducted by telephone. This was done by members of the research team that were not involved in the provision of clinical care to aid honest answering and avoid answers to appease doctors and midwives.

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NHS Trust

On a scale of 0 to 10 please answer the following questions:

1. How easy was LACTIN-V to administer? (10 = very easy, 1 = very difficult)

2. How comfortable was LACTIN-V? (10 = very comfortable, 1 = very uncomfortable)

3. How convenient was the schedule of use? (10 = very easy, 1 = very difficult)

4. How messy would rate the product? (10 = not messy, 1 = very messy)

5. How easy was the product storage (refrigerated)? (10 = very easy, 1 = very difficult)

6. Would you use the product again in pregnancy?

Yes No Unsure

7. Would you use the product again when not pregnant (for example to help reduce urine infections or vaginal infections)?

Yes No Unsure

8. Was your partner comfortable with you using LACTIN-V? (10 = very comfortable, 1 = very uncomfortable)

Figure 2.6 – Validated exit questionnaire. The exit questionnaire consisted of eight questions and aimed to determine overall experience and acceptability of LACTIN-V in pregnancy.

2.2.2 Extraction of DNA for microbiota analysis

Extraction of DNA for microbiota analysis was performed for vaginal samples, placenta, fetal membranes, meconium (defined as a neonate's first-pass of stool) and neonatal stool. Optimised protocols for each sample type were used as detailed below.

DNA extraction from vaginal swabs

Vaginal swabs were thawed on ice and swabs were re-suspended in liquid Amies by vortexing. Vaginal swab solutions were transferred to DNase/RNase free 2mL tubes

and centrifuged at 6,200g for 10 minutes at 4°C. The supernatant was aspirated, and the residual pellet was used.

Enzymatic and mechanical lysis

For enzymatic lysis, 300µL of master enzyme mix was added to the pellet (Table 2.1). Samples were vortexed and incubated in a water bath for 60 minutes at 37°C. After enzymatic lysis was complete, samples underwent mechanical bead lysis. This was performed by adding 0.1mm diameter acid washed glass beads. Samples were oscillated at 25Hz for 1 minute using the Qiagen Tissue Lyser (Qiagen, Catalogue No 69980). Samples were micro-centrifuged for 15 seconds to allow for separation of beads from the supernatant.

Chemical lysis

Into a new 1.5mL tube, 200µL of supernatant was added along with 20µL of proteinase K and 200µL of Buffer AL. Samples were incubated for 30 minutes at 56 °C, following which, 200µL of ethanol was added and pulse spun. 620µL of lysate was transferred to the QIAamp Spin Column and centrifuged at 6,080g for 1 minute at room temperature. The QIAamp Spin Column was placed in a clean 2mL collection tube and the filtrate was discarded. 500µL of Buffer AW1 was added and samples were centrifuged at 6080g for 1 minute at room temperature. The QIAamp Spin Column was placed in another clean 2mL collection tube, 500µL of Buffer AW2 added and samples were centrifuged at 186,020g for 3 minutes at room temperature. A dry run was performed to eliminate residual buffer by centrifuging samples at 186,020g for 1 minute at room temperature. The filtrate was discarded and the QIAamp Spin Column was placed into a clean 1.5mL microcentrifuge tube. Finally, 100µL of Buffer AE was added and incubated for 5 minutes at room temperature. Samples were centrifuged at 186,020g for 1 minute at room temperature and stored at -20°C.

Table 2.1 – Components of the enzyme master mix. Volumes listed per sample.

Enzyme master mix	Volume
Lysozyme (Sigma L6876)	50µL
Mutanolysin (Sigma M9901)	6µL
Lysostaphin (Sigma L9043)	3µL
TE50 (Sigma Aldrich)	41µL
Triton 12% (in-house)	30µL
PBS (in-house)	170µL

DNA extraction from placenta and fetal membranes

Tissue samples were thawed on ice and a sterile scalpel was used to mince samples over ice. To minimise contamination, efforts were made to maintain sterility whilst weighing tissue samples. Tissue samples were placed onto foil treated with 70% ethanol and ultraviolet (UV) radiation, which was then placed onto the weighing scale. Forceps and scalpels were sterilised with 70% ethanol between the handling of different samples. To each 1.0g of sample, 400µL of protease inhibitor/PBS mix was added (5µL PI/1mL PBS). Samples were lysed using a Tissue Lyzer at 50Hz for two minutes. Samples were transferred to ice and 130µL of enzyme master mix was added. The enzyme master mix was prepared as outlined in Table 2.1, except for PBS. Samples were vortexed and then incubated for 60 minutes at 37°C in a water bath during which, samples were vortexed at 15-minute intervals. Following incubation, samples underwent enzymatic, mechanical and bead lysis as previously described for vaginal swabs.

DNA extraction from meconium and neonatal stool

Processing of meconium is challenging due to its low microbial biomass, tar-like consistency, and high concentrations of PCR inhibitors. Therefore, the QIAmp ® PowerFecal ® Pro DNA Kit (Qiagen, Catalogue No 51804) was used for DNA extraction of meconium and neonatal stool (564).

Meconium and neonatal stool samples were thawed and weighed on ice. Use of Tween 10% has been shown to aid meconium solubilization and liquefaction (565). Therefore, 1mL of filter sterilised Tween 10% (Sigma Aldrich) was added to each

sample (0.10g). Samples were subsequently vortexed horizontally for 20 minutes and centrifuged at 17,000g for 15 minutes at room temperature. The supernatant was discarded, and pellets were re-suspended in 1mL of DEPC-treated water (Sigma Aldrich). Samples were vortexed and centrifuged again at 17,000g for 15 minutes at room temperature. The supernatant was removed and the remaining pellet was used for DNA extraction.

A total of 800µL of CD1 was added to the pellet and re-suspended. This was then transferred to the PowerBead Pro Tube and vortexed horizontally at maximum speed for 15 minutes. PowerBead Pro Tubes were centrifuged at 15,000g for 1 minute. Supernatants were transferred to clean 2mL Microcentrifuge Tubes and 200µL of solution CD2 was added and vortexed for 5 seconds. Samples were centrifuged at 15,000g for 1 minute at room temperature and 700µL of supernatant was transferred to a clean 2mL Microcentrifuge Tube. 600µL of CD3 Solution was added and vortexed for 5 seconds. 650µL of lysate was added onto a MB Spin Column and centrifuged at 15,000g for 1 minute. The flow-through was discarded and this step was repeated to ensure all the lysate had passed through the MB Spin Column. The MB Spin Column was placed into a clean 2mL Collection Tube and 500µL of Solution EA was added and centrifuged at 15,000g for 1 minute. The flow-through was discarded and 500µL of Solution C5 was added to the MB Spin Column and centrifuged at 15,000g for 1 minute at room temperature. The flow-through was discarded again and the MB Spin Column placed into a new 2mL Collection Tube. Tubes were centrifuged at 16,000g for 2 minutes and MB Spin Columns placed into new 1.5mL Elution Tubes. 75µL of Solution C6 elution buffer was added and samples were centrifuged at 15,000g for 1 minute. Spin Columns were discarded and DNA was stored at -20°C.

DNA extraction of commercially available probiotics

DNA extraction was performed on 18 commercially available probiotics (Appendix 3.2) and LACTIN-V. Preparations were either in pessaries, capsule or tablet forms and were suspended in 1-2mL of PBS. Samples were spun at 2400g for 5 minutes at 4°C. The supernatant was aspirated and pellets were used for DNA extraction. Enzymatic, mechanical, and chemical lysis were performed as previously described for vaginal swabs.

PCR amplification

To confirm whether bacterial DNA extraction was successful, PCR amplification was performed using 16S rRNA universal primers. To 5µL of extracted DNA sample, 45µL of PCR master mix was added (Table 2.2A). The PCR cycle conditions were set to hold at 95 ^{o}C for 5 minutes. After this, the PCR cycle was set to 95°C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 90 seconds and 72 ^{o}C for 7 minutes; these steps were repeated 30 times. The plate was held at 4°C until retrieval.

Amplification of bacterial DNA was confirmed by gel electrophoresis. Agarose gels (1.5%) were made by mixing 1.5g of dry agarose powder with 150mL 1xTBE (Tris/Borate/EDTA) buffer and 12µL of SYBR safe DNA stain (Invitrogen, Catalogue No S33102). Once the gel had set, 9µL of loading dye (PROMEGA, G190A 1006034) was added to each sample and the gel was loaded with 3.5µL of molecular weight marker (Bioline Hyperladder 100bp, Catalogue No BIO-33056) and 22µL of PCR mix. Each gel consisted of positive and negative controls. Negative controls included DEPC-treated water, AE Buffer and blank swabs, which were included in the extraction process. Previously isolated *L. crispatus* CTV-05 DNA was used as a positive control. Freeze-dried GBS was purchased from ATCC cells products and DNA was extracted following cell culture. GBS DNA was also used as a positive control. Electrophoresis was performed at 140 volts for 15 minutes and gels were trans-illuminated under UV light and fluorescent bands visualized.

Design of *L. crispatus* CTV-05 primers

The *L. crispatus* CTV-05 genome consists of 2,299 genes. Forward and reverse primers specific to *L. crispatus* CTV-05 were designed by Osel. Gene targets were chosen using genes identified in *L. crispatus* CTV-05, absent in other sequenced bacterial strains and present in low numbers of homologs within vaginal metagenome datasets (542, 554). In brief, this was performed by identifying *L. crispatus* CTV-05 genes without homologues in an additional 52 *L. crispatus* strains. A total of 28 genes unique to CTV-05 were identified which were then searched against 63 vaginal metagenome datasets, available from the Human Microbiome Project. From this, two genes were identified to exist in a low number of metagenomes (≤5%). These two

genes included LBKG_01471 (846 bps, 281 amino acids) and LBKG_01477 (300 bps, 99 amino acids). When assessed against the 63 metagenomes available from the Human Microbiome Project, LBKG_01471 had only one hit, whereas LBKG_01477 had two hits identified from two subjects. Therefore, in the design of the *L. crispatus* CTV-05 primers, LBKG_01471 was selected as the target gene. The final primer sequences targeted the LBKG_ 01471 gene and are outlined in Table 2.2B The specificity of this primer sequence was tested by Osel, against 50 strains of *L. crispatus* (using 10ng of genomic DNA) from the Osel collection. Using qPCR, the targeted gene was absent from 86% of assessed strains.

PCR amplification was also performed using *L. crispatus* CTV-05 specific primers, with the aim of determining *L. crispatus* CTV-05 presence. The PCR methodology was repeated, with the replacement of 16S rRNA Universal primers with *L. crispatus* CTV-05 primers (Table 2.2B). For this, negative controls included DEPC-treated water, AE buffer, blank swabs and GBS DNA. Isolated *L. crispatus* CTV-05 DNA was used as a positive control.

The accuracy of *L. crispatus* CTV-05 primers were assessed by performing PCR against DNA obtained from 24 isolates of *L. crispatus* from patient swabs provided by the laboratory of Julian Marchesi as part of the March of Dimes European Prematurity Research Centre activities (VMET2 cohort). Vaginal swabs were placed in freezing media, vortexed for 2 minutes and frozen at -80ºC. A loopful of freezing media was placed onto MRS, Columbia blood and Vegetative Minimal Medium agar and streaked out. The plate was incubated anaerobically for two days. Morphologically distinct colonies were then isolated and re-plated onto the agar from which they were isolated. Plates were anaerobically incubated for a further two days. Half of the culture was used for DNA extraction, using the Omega ENZA bacterial DNA kit. The DNA was used to perform PCR and amplify the 16s rRNA gene. PCR products were also run on the gel to confirm successful PCR and clean negative controls. PCR products were then cleaned using the Gene Jet PCR cleanup kit. PCR products were sent for sanger sequencing with Genewiz. Sequences were then analysed and bacterial species were identified by running the sequence through the BLAST database. These steps were performed by Lauren Roberts from the Marchesi laboratory.

Table 2.2A – Master mix for PCR with 16S rRNA universal primers. Volumes listed per sample.

PCR master mix	Volume
10mM dNTP (Sigma, catalogue no D7295)	1µL
16S rRNA Universal Forward primers: 27F-5'AGAGTTTGATCCTGGCTCAG-3' (Sigma)	1µL
16S rRNA Universal Reverse primers: 338R-5'GCTGCCTCCCGTAGGAGT-3' (Sigma)	1µL
TBE buffer (Tris/Borate/EDTA)	10µL
NEB One*Taq*® DNA polymerase (BioLabs)	0.25µL
DEPC-treated water (Sigma Aldrich)	31.75µL

Table 2.2B – PCR primers used for detection of *L. crispatus* CTV-05. Volumes listed per sample.

***L. crispatus* CTV-05 primers used in PCR mix**	**Volume (per sample)**
16S rRNA *L. crispatus* CTV-05 Forward primers (Sigma Aldrich): 5' CCT ACG GGA GGC AGC A	1µL
16S rRNA *L. crispatus* CTV-05 Reverse primers (Sigma Aldrich): 5' CCT ACG GGA GGC AGC A	1µL

DNA amplicon sequencing

For vaginal, placental, fetal membrane, meconium and neonatal stool samples, the V1-V2 hypervariable regions of 16S rRNA genes were amplified. The V1-V2 hypervariable regions can distinguish bacterial species to the genus level (335). Forward primers consisted of 28F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG pre-mixes at a 4:1:1:1 ratio. The reverse primer consisted of 388R TGCTGCCTCCCGTAGGAGT. Sequencing of amplicons was performed on a MiSeq platform at Research and Testing (Texas, USA).

2.2.3 Microbiology

Ex-vivo culture of *L. crispatus* CTV-05

L. crispatus CTV-05 was cultured *ex-vivo* to assess viability. At T4 (28^{+0} weeks' gestation), an extra BBL™ MaxV Liquid Amies vaginal swab was collected. This swab was streaked onto MRS plates and incubated for 48 hours in an anaerobic environment (10% H_2, 10% CO_2, 80% N; 37°C; 70% humidity). Following this, 2mL of filtered PBS was added to the surface of the plate. Colonies were scraped off and the 2mL of PBS was transferred into an Eppendorf. Contents were centrifuged at 6200g for 10 mins at 4°C. Supernatants were aspirated and pellets were stored at -80°C.

Clinical microbiology

At each timepoint one high vaginal sample was collected (BBL™ MaxV Liquid Amies swab) and sent to the Imperial College NHS Trust laboratory for clinical microbiology assessment of BV, yeasts and GBS. A description of the methods used for determination of BV, yeasts and GBS presence as per NHS guidelines are provided below. In addition, swabs were streaked onto a blood agar plate and cultured for 14-18 hours allowing for the detection of *Lactobacillus* spp. Colony formation in the third and fourth quadrants indicated moderate or heavy growth.

BV

Gram staining was performed by dabbing the swab onto a glass slide and then heat fixed. Slides were viewed under a microscope and the diagnosis of BV was made using the Hay/Ison criteria as recommended by The British Association for Sexual Health and HIV (BASHH) (Table 2.3) (356, 357).

Yeasts

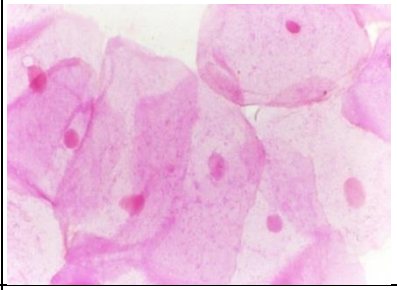
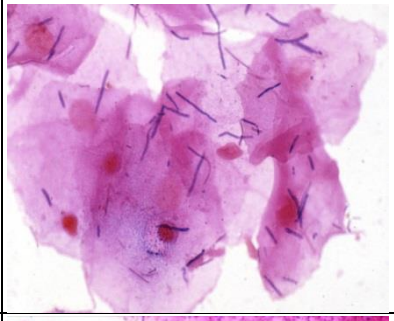
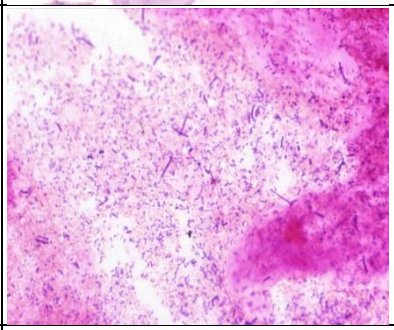
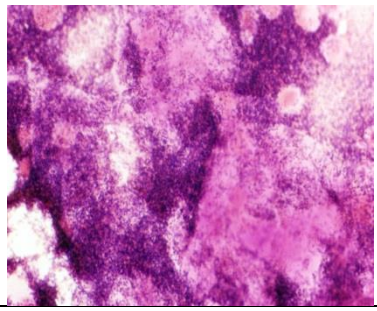
Vaginal swabs were assessed for the presence of yeasts by streaking swabs onto a Sabouraud agar plate and culturing for 40 to 48 hours (566). If no growth occurred after 48 hours, the Sabouraud plates were incubated for a further three days at 30°C.

In cases where yeasts were isolated, colony formation in the third or fourth agar plate quadrants defined moderate or heavy growth.

GBS

A chromogenic agar was used to detect the presence of *Streptococcus agalactiae*, or GBS. Swabs were streaked onto ChromID Strepto B (STRB) agar plates (567). Development of purple or mauve coloured colonies indicated GBS. As Group A and C/G streptococci can appear similar, in the presence of purple or mauve colonies GBS was identified using MALDI-TOF (matrix-assisted laser desorption/ionisation coupled to time-of-flight) mass spectrometry, according to published methodology and Public Health England standards (568-570). There are two steps to the MALDI-TOF technique. Samples are coated with an energy-absorbent matrix. The first step is known as the ionisation step whereby samples are exposed to an ionising laser beam, causing molecules to be vaporised and ionised. A high voltage is then applied which aims to accelerate the charged particles. The second step is known as the time-of-flight mass spectrometry step and measures the flight time of molecules. High mass molecules take more time to travel, allowing for different molecules to be differentiated.

Table 2.3 – Grading system used for gram stain diagnosis of BV, reproduced from North West London NHS Pathology guidance (571). This criterion is based on the *Lactobacillus* morphotype. Grade II–III are indicative of BV.

Grade	Definition of flora	Description	Example
0	Normal	No bacteria seen Epithelial cells only **Gram stain does not indicate BV**	
I	Normal	*Lactobacilli* morphotype only **Gram stain does not indicate BV**	
II	Intermediate	Reduced *Lactobacillus* morphotype with mixed bacterial flora **Gram stain indicates BV**	
III	Bacterial vaginosis	Mixed bacterial flora with few or absent *lactobacillus* morphotype **Gram stain indicates BV**	

2.2.4 Bacterial load

Vaginal bacterial load was quantified for participants at each timepoint. Using the BactQuant method, a broad-coverage qPCR assay was performed to quantify 16S rRNA gene copy numbers and estimate bacterial load (572). Real time qPCR is beneficial as it allows the absolute numbers of gene targets in a sample to be calculated (573). A ten-fold standard curve was created using a known concentration of *Escherichia coli* DNA (Sigma, D4889) from 3,030.397 (S0) to 303,039,700 (S5) copies of 16S DNA.

A qPCR master mix was created (Table 2.4). Sample DNA template was extracted using the QIAmp® DNA Mini Kit (Qiagen, Catalogue No 51304) extraction kit (described in section 2.2.2) and eluted into a 100µL volume. Sample DNA template was diluted at a 1:10 dilution factor. Either 5µL of *E. coli* standard or 5µL of sample DNA template (at 1:10 dilution) was added to 15µL of master mix and loaded in duplicates. The plate was sealed, spun for 1 minute and then transferred to the StepOne© qPCR machine. The PCR cycle conditions were set to hold at 50°C for 2 minutes, 95 °C for 10 seconds, 40 cycles of 95 °C for 15 seconds followed by 60°C for 60 seconds. The plate was held at 4°C until retrieval. The StepOne© software was used to calculate the 16S DNA copy number against the *E. coli* standard curve (Figure 2.7 and 2.8). Negative controls consisted of 5µL of PCR grade water and were run alongside each plate to exclude contamination of PCR reagents.

Table 2.4 – Master mix for qPCR with BACTQuant primers and probe. Volumes listed per sample.

qPCR master mix	Volume
Ultraviolet treated DEPC water (Sigma Aldrich)	4.195µL
Rox 100µmol/µL (LifeTech, catalogue no 11730-017)	0.04µL
Platinum Supermix (Life Tech, catalogue no 11730-017)	10µL
BACTQuant Forward primers 100µM (Sigma Aldrich): 5'-CCT ACG GGA GGC AGC A-3'	0.36µL
BACTQuant Reverse primers 100µM (Sigma Aldrich): 5'-GGA CTA CCG GGT ATC TAA TC-3'	0.36µL
BACTQuant Probe 10µmol/µL (LifeTech, catalogue no 4316034): 5'-6FAM-CAG CAG CCG CGG TA-MGBNFQ-3'	0.045µL

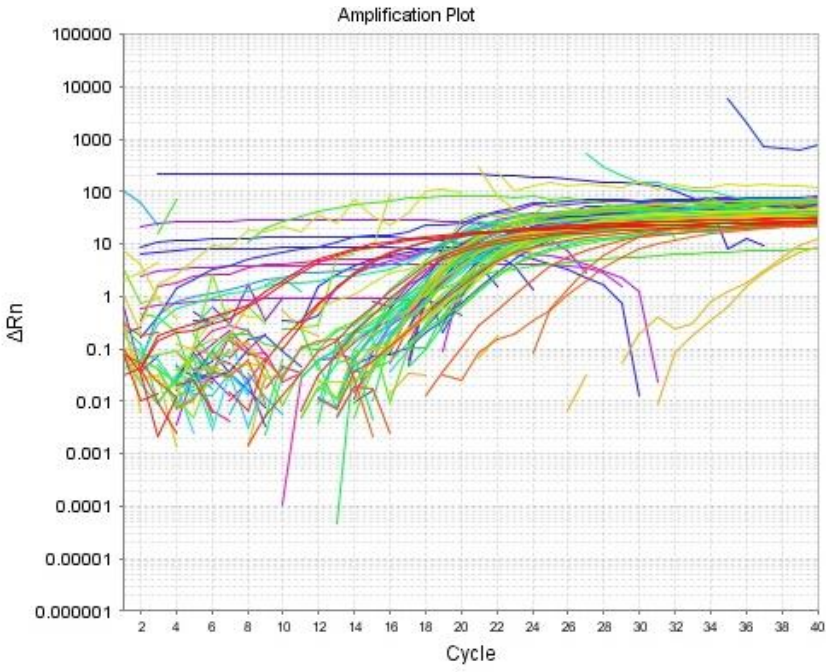


Figure 2.7 – BactQuant amplification plot. An amplification plot was produced by the StepOne© qPCR machine for samples with unknown bacterial quantity and known concentrations of *E. Coli.*

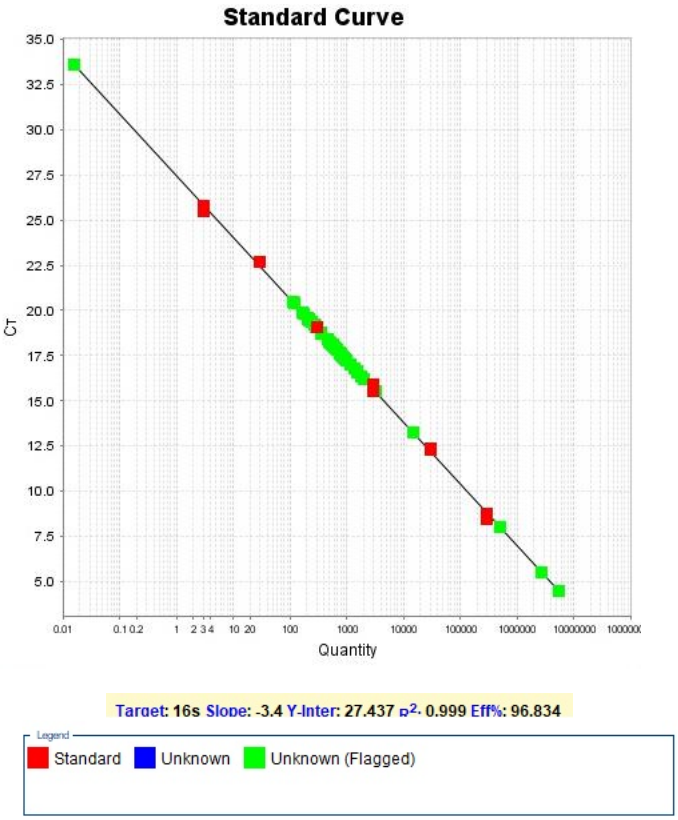


Figure 2.8 - BactQuant standard curve plot. A standard curve was produced by the StepOne© qPCR machine using known concentrations of *E. Coli* used as standards (red plots). This was used to calculate the number of 16S genomes within tested samples, containing an unknown concentration (green plots).

Analysis of bacterial load data

Analysis of the number of 16S RNA genomes present within 5µL of sample was performed using the StepOne (Version 2.0, Thermo Fischer Scientific) software (Figure 2.9 and 2.10). As each swab was eluted in 100µL, results were multiplied by twenty to determine the amount of 16S RNA genome copies per swab. Different bacteria species will have different numbers of 16S genomes. In consideration of this, the relative abundance of bacterial species comprising >95% of sequence reads were calculated. The number of 16S genes within each of these bacterial species was then determined. The Michigan rrn database (https://rrndb.umms.med.umich.edu/) was used to confirm the 16S gene copy number in each bacterial species and was evaluated for relative abundance (574). In cases where the relative abundance of bacterial species did not compromise >95% of sequence reads, proportions were calculated accordingly. When the operon number was not available at the species level, the average copy number at the genera level was used.

Bacterial load of *L. crispatus* CTV-05

L. crispatus CTV-05 load was quantified at each timepoint using qPCR. For this, *L. crispatus* CTV-05 DNA was extracted from lyophilised *L. crispatus* CTV-05 and resuspended in degassed MRS broth (Millipore) made as per the manufacturers recommendation and incubated for 48 hours in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N; 37°C; 70% humidity). The CFU of the culture was determined using the Miles and Misra method (575). This involved taking 1mL of the culture and making a serial dilution with PBS. Dilutions were spotted onto MRS plates and incubated under the described conditions. The number of colonies were counted and the CFU was calculated (1.43×10^8). DNA was extracted using the Omega E.Z.N.A.® Bacterial DNA Kit (Cat. No. D3350) according to the manufacturer's protocol. DNA was quantified using Qubit with the BR Assay Kit (Thermofisher, Cat. No. Q32853) and was quantified to be 65.2ng/µL. Lauren Roberts performed the extraction of *L. crispatus* CTV-05 DNA from lyophilised *L. crispatus* CTV-05 and DNA quantification. Bacterial concentration was calculated from mean sample Ct values using a standard curve based on serial dilutions of CTV-05 strain of *L. crispatus* (Figures 2.8 and 2.9).

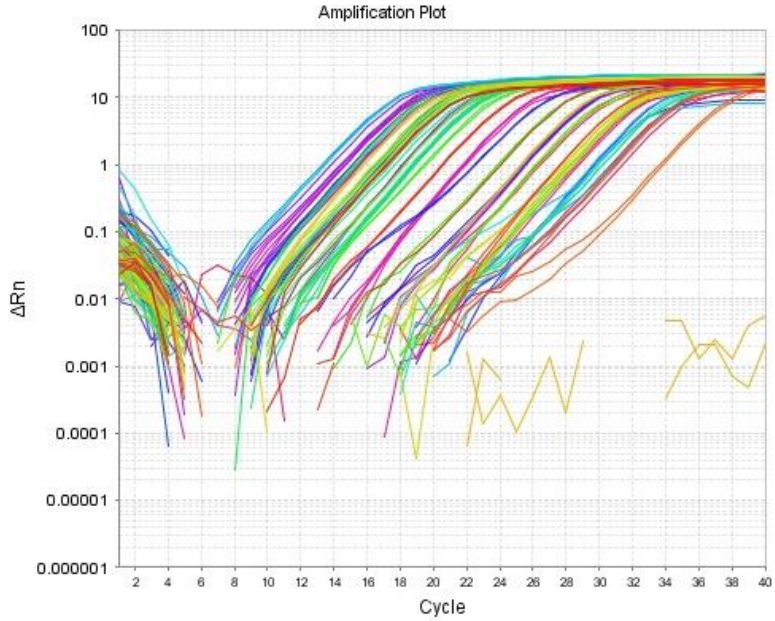


Figure 2.9 – *L. crispatus* CTV-05 quantification BactQuant amplification plot. An amplification plot was produced by the StepOne© qPCR machine for samples with unknown bacterial quantity and known concentrations of *L. crispatus* CTV-05.

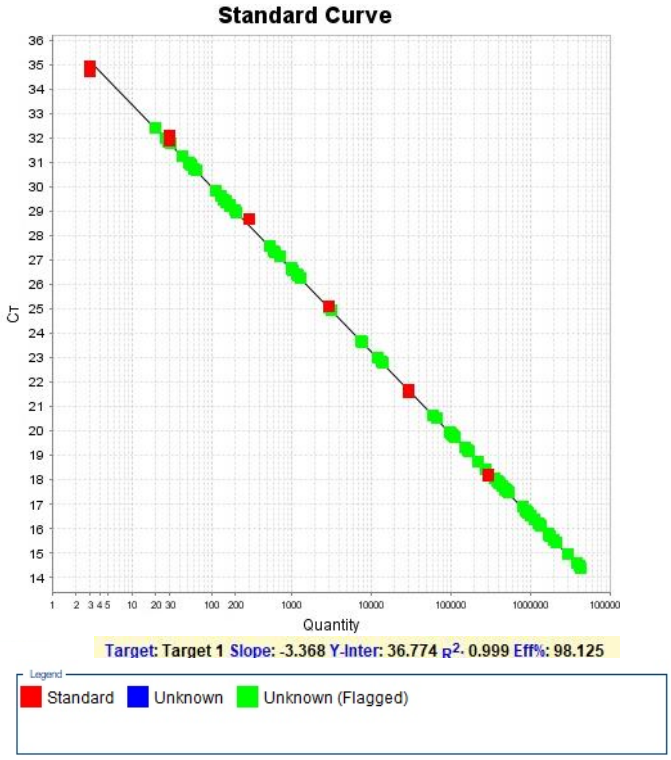


Figure 2.10 - *L. crispatus* CTV-05 quantification BactQuant standard curve plot. A standard curve was produced by the StepOne© qPCR machine using known concentrations of *L. crispatus* CTV-05 used as standards (red plots). This was used to calculate the number of 16S genomes within tested samples, containing an unknown concentration (green plots).

A master mix was made as described in Table 2.5. Strain specific qPCR was performed using primer sequences targeting the LBKG_ 01471 gene. Sample DNA template was diluted by 1:10. A 45µL master mix volume was added to either 5µL of standard or 5µL DNA template. Samples were loaded in duplicates. The plate was sealed, spun for one minute and then transferred to the StepOne© qPCR machine. PCR parameters were set to hold at 95°C for 15 minutes followed by PCR cycling consisting of 40 cycles at 95°C for 30 seconds, 58°C for 60 seconds and 72°C for 60 seconds. The plate was held at 4ºC until retrieval. The StepOne© software was used to calculate the 16S DNA copy number against the *L. crispatus* CTV-05 standard curve. Negative controls were run alongside each plate to exclude contamination of PCR reagents.

Table 2.5 – Master mix for *L. crispatus* CTV-05 qPCR. Volumes listed per sample.

qPCR *L. crispatus* CTV-05 master mix	**Volume**
QuantiTect® SYBR® Green RT-PCR Master Mix (Qiagen)	25µL
L. crispatus CTV-05 forward primer 10mM (Sigma Aldrich) 5'-GCTGTTGCAGCCAGACAGTT-3'	2.5µL
L. crispatus CTV-05 reverse primer 10mM (Sigma Aldrich) 5'-GCTGTTGCAGCCAGACAGTT-3'	2.5µL
DEPC treated water	15µL

Bacterial load data analysis

As samples were run in duplicates, the average numbers were used to calculate 16S rRNA gene copies. Since each swab was eluted in 100µL, results were multiplied by twenty to determine the amount of 16S RNA genome per swab.

2.2.5 Magnetic Luminex® assays

Multiplex bead assays allow for rapid, precise and cost-effective measurements of analytes. Traditional enzyme-linked immunosorbent assay (ELISA) based techniques have been the standard for quantitative analysis of biomarkers but rely on individual antibodies to detect target antigens using specific antibody-antigen interactions.

Multiplex bead assays are advantageous in that they can precisely measure multiple analytes in one sample and therefore measure multiple analytes with smaller sample volumes (576). Multiplex bead assays have also shown to be more accurate than ELISA when samples have low concentrations of the analyte (576). The principles are outlined in Figure 2.11. In the first step, samples were added to a mixture of colour coded beads, pre-coated with antibodies specific to the studied analytes. Secondly, biotinylated detection antibodies, specific to the studied analytes were added to form an antibody-antigen complex. Addition of phycoerythrin-conjugated streptavidin leads to binding to biotinylated antibodies. In the third step, samples are analysed using the MAGPIX® Instrument with xPONENT 4.3 software. A dual-laser detection system is used, whereby the first laser determines the studied analyte and the second laser calculated the magnitude of the phycoerythrin-derived signal. The magnitude of the measured phycoerythrin-derived signal represents the quantity of bound analyte.

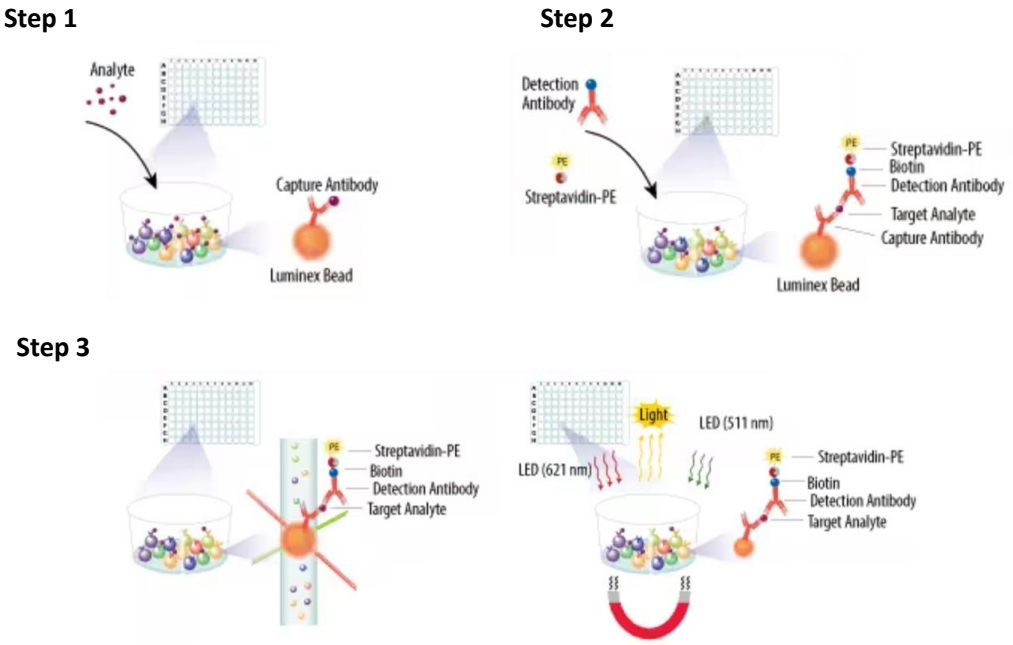


Figure 2.11 –Luminex Assay principles. Step 1) CVF is added to a mixture of colour coded beads. These beads are pre-coated with analyte specific antibodies. The antibodies bind to the analyte being assessed. Step 2) Biotinylated detection antibodies specific to the analytes being measured are added and form an antibody-antigen complex. Phycoerythrin-conjugated streptavidin is added which binds to the biotinylated detection antibodies. Step 3) The MAGPIX® analyser reads beads using a dual-laser flow-based detection system. One laser classifies the bead and determines which analyte is being measured. The second laser detects the magnitude of the phycoerythrin-derived signal, representing the amount of analyte bound in a directly proportionate trend. Taken from R&D Systems Technical Resources (577).

Justification of selected analytes

Analytes were selected for study based on the previously reported associations between CVF concentration, microbiota composition and PTB outcomes.

Interleukin-1ß (IL-1ß)

During parturition, levels of IL-1ß increase in the cervix, myometrium and amnion (465, 578). Histological analysis has shown this to associate with cervical infiltration of neutrophils and macrophages (465). In animal models, intra-amniotic injection of IL-1ß causes infiltration of neutrophils, significant increases in pro-inflammatory cytokines and subsequent sPTB (579, 580). Human studies indicate high levels of IL-1ß associate with sPTB (484, 581).

Interleukin-8 (IL-8)

IL-8 also plays a role in cervical remodelling (582, 583). Acting as a chemoattractant, IL-8 attracts and activates neutrophils. CVF IL-8 concentrations are higher in women delivering spontaneously preterm when compared to those delivering term (584, 585).

Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine that plays a role in cervical re-modelling (582, 583). CVF IL-6 concentrations strongly associate with sPTB (494, 586).

Interleukin-10 (IL-10)

Produced by gestational tissues, IL-10 inhibits the production of pro-inflammatory cytokines including IL-1ß, IL-8, IL-6 and TNF-α by monocytes and macrophages (587-589). IL-10 expression is higher in women with spontaneous preterm labour when compared with term labourers (590).

Justification of selected timepoints for immune profiling

Assay plates were chosen to quantify cytokine concentrations in cervicovaginal swabs. This was performed at T0 (14^{+0} weeks'), T1 (15^{+0} weeks'), T3 (20^{+0} weeks') and T4 (28^{+0} weeks'). T0 was selected to determine cervicovaginal cytokine levels before LACTIN-V use. At T1 cytokine concentrations were measured to assess changes following the LACTIN-V loading dose. Similarly, cytokine measurements at T3 were calculated to determine the impact of the LACTIN-V maintenance dose. Cervicovaginal cytokines concentrations at T4 were measured to gauge changes following an 8-week period without LACTIN-V administration.

The selected timepoints also represent clinically relevant gestations. At 20^{+0} weeks, although women are still at risk of MTL and extreme PTB but remain amenable to interventions, for example cervical cerclage placement and progesterone. At 28^{+0} weeks' gestation, women will have passed the point of extreme prematurity. Neonatal mortality and morbidity are highest in those born before 28^{+0} weeks' gestation (591). Therefore, understanding the CVF immune milieu in response to LACTIN-V at this timepoint is of clinical importance.

Overall, analysis of cervicovaginal cytokines at T0 (14^{+0} weeks'), T1 (15^{+0} weeks'), T3 (20^{+0} weeks') and T4 (28^{+0} weeks') were selected to explore the host immune response to LACTIN-V throughout pregnancy, at clinically relevant timepoints. Analysis at the selected timepoints would also allow opportunity to review the LACTIN-V dosing regimen.

Preparation of CVF samples

Transwab® MW170 swabs were thawed on ice and CVF was re-suspended by vortexing with 350µL PBS solution with PI. The suspended swab was centrifuged, following which the dry swab was discarded and the supernatant was transferred into a new 2mL Eppendorf. The 2mL Eppendorf was centrifuged again, to remove cell debris. The remaining cell-free supernatant was analysed according to the manufacturer guidelines for the Human pre-mixed multi-analyte kits (R&D Systems, Catalogue No LXSAHM) using the MAGPIX® analyser.

Cytokine assay procedure

Standards were prepared as per the manufacturer guidance. After preparation of the standards and reagents (Table 2.6), 50µL of standard or sample was added to each well. When quantifying IL-8, a 10-fold dilution was used using Calibrator Diluent RD6-52. When quantifying IL-1ß, IL-6 and IL-10 dilution was not required, and the assay was performed on a multiplex plate.

Table 2.6 – Preparation of reagents. Reagents were prepared for 96-well plates as per manufacturer guidance. Streptavidin-phycoerythrin was protected from light.

Reagent		
Microparticle Cocktail	500µL of Microparticle Cocktail	5mL of Diluent RD2-1
Biotin-Antibody Cocktail	500µL of Biotin-Antibody Cocktail	5mL of Diluent RD2-1
Streptavidin-phycoerythrin preparation	220µL Streptavidin-phycoerythrin concentrate	5.35mL of the Wash Buffer

A total of 50µL of diluted Microparticle Cocktail was added to each well, incubated for 2 hours at room temperature on a shaker at 800rpm. Prior to each incubation, microplates were covered securely with a foil plate sealer. Wells were washed by removing the liquid from each well, using 100µL of Wash Buffer and removing the liquid. This wash was performed three times. All washes were performed using a handheld magnetic microplate device. 50µL of diluted Biotin-Antibody Cocktail was added to each well. The 96-well plate was incubated for 1 hour at room temperature on a shaker at 800rpm. Wells were washed again using 100µL of Wash Buffer, three times. To each well, 50µL of diluted streptavidin-phycoerythrin was added and incubated for 30 minutes at room temperature on the shaker at 800rpm. Wells were washed using 100µL of Wash Buffer, three times. 100µL of Wash Buffer was added to each well and incubated for 2 minutes at room temperature on the shaker at 800rpm. Plates were read within 90 minutes using the Luminex® MAGPIX®. Microparticles were resuspended immediately prior to reading. Samples were run in

duplicates on 96-well plates and included six standards, one blank and one common pooled sample.

Analyte concentrations were determined by spectral properties of the beads and amount of fluorescence. Standard ranges specified by Luminex® were referred to when analysing analyte detections (Table 2.7). If analytes were detected, but values were beyond the standard range, concentrations were determined from extrapolations off the standard range of the immunofluorescence curve. If analytes were not detected, concentrations were calculated to be half the value of the sensitivity level for the assay. To account for plate-to-plate variability, the coefficient of variation was calculated across all plates.

Table 2.7 – Standard curves and sensitivity values for each analyte (577)

Cytokine	Standard curve (pg/mL)	Sensitivity (pg/mL)
IL-1ß	17.7-4300	0.8
IL-8	4.12-1000	1.8
IL-6	4.53-1100	1.7
IL-10	1.89-460	1.6

2.2.6 Statistical analyses

Unless stated otherwise, statistical analysis was performed using GraphPad Prism version 9.4.1. Statistical significance was defined as $p<0.05$.

Demographics and clinical outcomes

Demographics were assessed using p values calculated by Chi-squared, T-test or Mann-Whitney U, depending on whether data was normally distributed. When comparing the incidence of clinical outcomes in women receiving LACTIN-V versus comparison cohorts, Chi-squared testing with Yate's correction was used.

Microbiome data

Processing of microbiota sequencing data was conducted by Dr Samit Kundu and Dr Sherrianne Ng. Briefly, primer sequence trimming was performed using Cutadapt (592). Amplicon sequence variant (ASV) counts per sample were calculated using the QIME2 bioinformatics pipeline (version 2022.2.1) (593). For denoising DADA2 (version 2022.2.0) was used (594). For the taxonomic classification of ASVs the STIRRUPS data base was used (595). The VALENCIA centroid classification algorithm was used for the classification of samples at species level, into five CSTs (345). These included CST I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), CST IV (*Lactobacillus* deplete) and CST V (*L. jensenii*).

Detection and colonisation of *L. crispatus* CTV-05 at timepoints was analysed by one-way analysis using Chi-squared testing. Analysis of the relative abundance in samples at genus and species level, was performed using Microbiome Analyst version 2.0 (https://www.microbiomeanalyst.ca) (596). Alpha diversity (Shannon Index) between T0 and T6 was also assessed using Microbiome Analyst version 2.0. When assessing alpha diversity, Mann-Whitney U testing was used to assess differences between two groups. The Kruskal-Wallis test with Dunn's correction was performed when three or more groups were analysed.

Transitions of the vaginal microbiota CSTs in women delivering spontaneously preterm was represented using a Sankey Diagram, created by SankeyMATIC (https://sankeymatic.com/). To assess changes in CST throughout the study and factors influencing colonisation, a one-sided Fisher's exact test was used, as some analysed groups had $n<5$.

Bacterial load data

When comparing *L. crispatus* CTV-05 load between two groups, Mann-Whitney U testing was used. To compare bacterial load in three or more groups Kruskal-Wallis testing with Dunn's correction was performed.

Immunoassays

For the analysis of raw data obtained from Luminex Multiplex Immunoassays, the MAGPIX analyser and xPONENT software were used. The coefficient of variation was calculated using Microsoft Excel (version 16.83). As immune data was not normally distributed, non-parametric statistical tests were performed. Mann-Whitney U testing was used to assess differences between two groups. The Kruskal-Wallis test with Dunn's correction was performed when three or more groups were analysed. The assessment of proportional differences was conducted using Fisher's exact or Chi-square testing. Analysis of matched data was performed using a Wilcoxon signed-rank test. As data was not normally distributed, results were plotted as median with interquartile range (IQR).

2.2.7 Impact of COVID-19 pandemic

Within four months of commencing study recruitment, the COVID-19 pandemic hit the UK. The impact of COVID-19 on the FLIP-1 trial is summarised in Figure 2.12 and 2.13. Between September 2019 and March 2020, 32 women had been recruited. A global pandemic was declared by the WHO on 11th March 2020 and the UK government advised pregnant women to isolate from mid-March. The first UK national lockdown was announced on 23rd March 2020. At this point, all clinical research, unrelated to COVID-19, was halted. For the FLIP-1 trial this meant no follow-up sampling of recruited women and no new recruitment. The follow-up of 25 out of 32 women recruited prior to March 2020 was affected by the pandemic, leading to sample attrition. From early June 2020, clinical trials involving essential patient care resumed, and as TVUS cervical length assessment was considered as essential, follow-up sampling of recruits requiring cervical length assessment resumed. During this time, it is likely that some participants were hesitant to attend the hospital, contributing to further sample attrition. Collection of delivery samples and meconium also resumed from early June as women were already inpatients. For the collection of day 14 neonatal stool, arrangements were made to meet participants outside of hospital grounds to reduce potential hospital-based exposure to COVID-19. Recruitment of new participants also resumed in June 2020. The laboratory re-opened in June 2020

with social distancing measures including restrictions on the number of students and staff on site.

During the COVID-19 pandemic members of the research team including midwives and doctors were redeployed to provide clinical care within maternity or on dedicated COVID-19 wards, to relieve the impact of COVID-19 on healthcare staff sickness and meet the demands of clinical care. Ordering and shipping of laboratory consumables and reagents were delayed in view of the international prioritisation and diversion of consumables for use in PCR testing of COVID-19.

During the peak of the COVID-19 pandemic the PTB care pathway at QCCH was altered. Government guidance was to avoid social mixing to reduce viral transmission, with pregnant women classed as being vulnerable. Concerns regarding our patients included the use of transport to and from appointments and risk of exposure in the clinic waiting room. Although NHS England released guidance during the pandemic on how to maintain recommendations outlined in the saving babies' lives care bundle, advice on how to continue providing PTB prevention services was not specified. Imperial College NHS Trust encouraged care providers to replace face-to face attendance with telephone appointments where possible.

In attempt to minimise hospital attendance, our team revised the PTB referral and care pathways as a qualitative improvement study to reduce surveillance frequency and conduct telephone consultations were possible. This had a substantial impact on the ability to recruit and follow-up existing recruits. As part of this quality improvement study, we published our changes to the PTB clinic care and referral pathway in response to the COVID-19 pandemic for other units to follow (597). These changes were based on a review of published literature and guidelines to refine the referral criteria and include women at the highest risk of sPTB. Risk factors removed from the referral criteria included previous FDCS, congenital uterine anomaly, or trachelectomy. A history of sPTB $<34^{+0}$ weeks was readjusted from $<37^{+0}$ weeks. Clinician vetting of referrals was introduced to minimise unnecessary clinic attendance and telephone consultations were introduced. A joint decision was made with the anaesthetic team to perform cerclages under spinal anaesthetic rather than general anaesthetic, to minimise aerosol-generating procedures. The implementation of these

changes were based on a Plan, Do, Study, Act (PDSA) cycle. Patients from a PPI group and staff members were surveyed regarding the introduced changes. On completion of the PDSA cycle, previous FDCS was re-introduced as one woman with a history of FDCS was not referred to the clinic but required a rescue cerclage. Overall, the number of face-to-face consultations reduced by 54% whilst the number of telephone consultations increased by 65%.

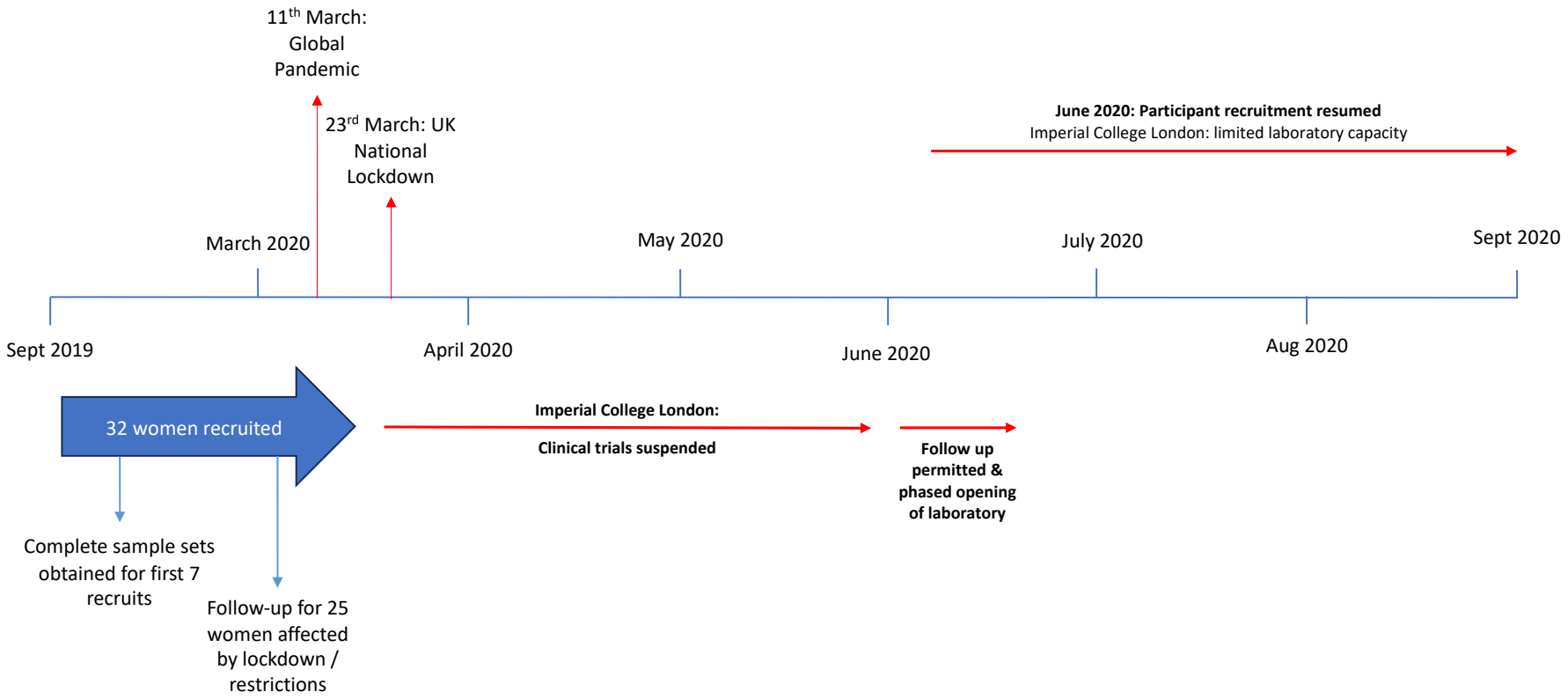


Figure 2.12 – Timeline of COVID-19 and impact on participant follow-up and recruitment. A national lock down was declared in the UK on 23rd March 2020 leading to a suspension of active clinical trials. Prior to 23rd March 2020, 32 women had been recruited. Sample collection was complete for seven women. Sample collection for the remaining 25 women was affected by COVID-19, contributing to missing samples. From early June, follow-up was permitted if coinciding with provision of essential care, such as cervical length measurement. Imperial College London laboratory closure occurred between March and June 2020. From June 2020 the laboratory was re-opened with restricted capacity to facilitate social distancing.

Participant ID	Recruitment Date	T0	T1	T2	T3	T4	T5	T6
F001	16.9.19							
F003	23.9.19							
F004	7.10.19							
F005	14.10.19							
F006	14.10.19							
F007	14.10.19							
F009	14.10.19							
F011	21.10.19							
F012	21.10.19							
F013	28.10.19							
F015	30.10.19							
F017	4.11.19							
F019	2.12.19							
F021	6.1.20							
F022	10.2.20							
F023	6.1.20							
F024	20.1.20							
F025	6.1.20							
F026	20.1.20							
F028	27.1.20							
F029	3.2.20							
F030	3.2.20							
F031	3.2.20							
F032	3.2.20							
F033	3.2.20							
F034	10.2.20							
F035	17.2.20							
F036	17.2.20							
F037	24.2.20							
F038	2.3.20							
F039	2.3.20							
F041	2.3.20							
F042	1.6.20							
F043	5.6.20							
F044	8.6.20							
F045	8.6.20							
F046	8.6.20							
F047	8.6.20							
F048	15.6.20							
F049	15.6.20							
F051	15.6.20							
F052	22.6.20							
F053	29.6.20							
F054	20.7.20							
F055	20.7.20							
F056	27.7.20							
F057	27.7.20							
F058	27.7.20							
F059	27.7.20							
F060	27.7.20							
F061	27.7.20							
F062	10.8.20							
F063	17.8.20							
F064	17.8.20							
F065	18.8.20							
F067	18.8.20							
F068	18.8.20							
F069	18.8.20							
F070	7.9.20							
F071	7.9.20							
F072	7.9.20							
F073	7.9.20							

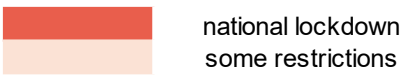


Figure 2.13 – Sampling affected by COVID-19. Participant identification (ID) numbers are listed (for example, F001) with recruitment dates. Dark red represents times of national lockdown and light red represents times when clinical trial activity was permitted at restricted capacity.

3 Safety, Acceptability and Tolerability of LACTIN-V

Information in this chapter published as:

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3.1 Chapter overview

The work described in this chapter set out to test the hypothesis that *L. crispatus* CTV-05 (LACTIN-V), when administered vaginally is a safe, well-tolerable and acceptable therapeutic intervention in pregnant women. Specifically, I aimed to:

- Assess the safety, tolerability and acceptability of LACTIN-V use during pregnancy.
- Quantify and compare key pregnancy outcomes in those treated with LACTIN-V and a parallel non-intervention control arm.

Key results

From 73 recruited women, eight women withdrew and four women were lost to follow-up leaving a sample size of 61. Self-reported compliance was high; 56/60 women (93%) were compliant to the full LACTIN-V course. Adverse events were recorded for both the cohort of 61 women completing LACTIN-V as well as for the 8 withdrawals (n=69). Solicited adverse events were reported by 13 women (19%), the most common of which was altered vaginal discharge. Although one unsolicited adverse event was reported at 38^{+0} weeks consisting of haematuria, this was deemed to be unrelated to LACTIN-V. No serious adverse events occurred. Only one mild adverse event in the form of vaginal burning led to study withdrawal. The exit questionnaire was completed by 31 women. Most women (30/31, 97%) reported LACTIN-V as easy and comfortable to use. Willingness for use in future pregnancies was also shown. Although a quarter of women found the regimen difficult to remember, compliance was not impacted. The incidence of sPTB $<34^{+0}$ weeks in women using LACTIN-V was 3.3%. The incidence of sPTB was higher in women who did not receive LACTIN-V; the incidence of PTB $<34^{+0}$ weeks was 7% and 7.5% in women from groups A and B respectively.

Conclusion

Vaginally administered LACTIN-V is safe and tolerable in pregnancy. Compliance and willingness to use in future pregnancies was impressive. Overall, LACTIN-V is well accepted. A reduced sPTB incidence occurred when compared to high-risk control cohorts.

3.2 Introduction

The vaginal microbiota plays an important role in reproductive health outcomes for both mother and baby. During pregnancy a shift in the vaginal bacterial community structure towards a *Lactobacillus* dominated state occurs (598-601). This shift promotes a protective environment, whereby release of bacteriocins and lactic acid leads to a hostile environment for pathogens (602). *Lactobacillus* deplete vaginal microbiota can increase infection susceptibility by activating immune responses and degrading the host mucosa (603).

The Bennett/MacIntyre group were the first to show that in pregnancy *Lactobacillus* spp. dominance, particularly with *L. crispatus* protects against sPTB (353). In contrast, *L. iners* dominance as early as 16^{+0} weeks of gestation predisposes to sPTB (353). Other authors have reproduced these findings. Vaginal sampling of 936 pregnant women showed that high *L. crispatus*, *L. gasseri* or *L. jensenii* abundance associated with term delivery, whilst *L. iners* abundance related to sPTB (604). Many studies have now been conducted, reiterating the benefits associated with *Lactobacillus* spp. A meta-analysis involving data from 12 different cohorts, involving vaginal 16s rRNA sequencing during pregnancy, concluded that *L. crispatus* abundance negatively correlated with sPTB whilst *L. iners* dominance or high diversity related to sPTB (375). Additionally, early sPTB (<32 weeks) was shown to be more predictable from the vaginal microbiome (375). Other authors have similarly reviewed published vaginal microbiome datasets from pregnant women, showing VALENCIA CSTs to predict early sPTB (<32 weeks) (376). As such, optimising the microbiome to improve clinical outcomes has been considered as a therapeutic intervention.

BV is characterised by vaginal dysbiosis, where the proportion of lactobacilli is reduced and opportunistic bacteria such as *Gardnerella vaginalis, Prevotella* and *Mobiluncus* species dominate (605-607). Meta-analyses have shown BV to be a risk factor for sPTB and late miscarriage (608, 609). Subsequently, antibiotics for the treatment of BV to reduce the risk of sPTB have been explored.

Clinical trials looking at antibiotic eradication of BV to reduce sPTB have produced conflicting results. Pregnant women with a diagnosis of BV were randomised to

receive either metronidazole and erythromycin or placebo (610). Although results showed a lower incidence of sPTB in women treated with antibiotics, this benefit was limited to women with a diagnosis of BV in early pregnancy (610). Similarly, treatment of BV in pregnancy before 20^{+0} weeks' gestation with intra-vaginal clindamycin showed statistically significant reductions in sPTB when compared to a placebo intervention (611). However, antibiotic use for the treatment of BV in pregnancy and sPTB reduction has not been consistently shown. This may be attributed to differences in study design including participant demographics, antibiotic choice, dose and mode of administration, and gestational age at sampling. A Cochrane review including 21 trials concluded that antibiotic treatment of BV in pregnancy does not significantly reduce PTB (359, 612). Over-use of antibiotics in pregnancy will undoubtedly contribute to the growing issue of antibiotic resistance (613). Additionally, harm associated with antibiotic use in pregnancy should not be over-looked. Erythromycin use for PPROM has been associated with chorioamnionitis, funisitis and early onset of neonatal sepsis through *Lactobacillus* spp. depletion (371). Further, a RCT randomising women at high-risk of sPTB with BV and a positive fFN to receive metronidazole or placebo, was terminated early due to a significant increase in sPTB seen with metronidazole use (614). Concerns around antibiotic use have led to the consideration of microbiome-based therapeutics and immune modulators as alternative treatment strategies for the prevention of microbial-associated PTB.

Live biotherapeutics are live organisms designed to treat, cure or prevent human disease and are exclusive of vaccines, oncolytic viruses and organisms used as vectors for the transfer of genes (615). Live biotherapeutics have been explored as an alternative to antibiotics in inflammatory and infectious conditions (616-618). Probiotics vary with respect to their labelling and marketing claims; they are regulated as dietary supplements and cannot make claims to treat or prevent disease (503).

Live biotherapeutics as a PTB prevention strategy have shown inconsistent results. A population-based cohort study involving over 70,000 women showed that oral probiotic intake in late pregnancy led to reduced sPTB (619). Despite its large population sample, this Swedish study was limited in its design, as relied on women self-reporting whether they chose to consume one of two widely available probiotic containing milk products, meaning that strain, dose, and quantity-specific analysis could not be

performed. A Cochrane review involving 12 studies concluded that insufficient evidence exists to determine whether maternal supplementation with probiotics prevents sPTB (535). This Cochrane review was restricted to reviewing oral intake of probiotics and included studies targeting women at low-risk of PTB. Culture-based studies have shown that oral administration of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 can lead to increased vaginal lactobacilli detection (529). However, more recent bacterial DNA sequencing-based work has demonstrated that live oral biotherapeutics do not alter the vaginal microbiota (530, 531, 620).

LACTIN-V is a live vaginal biotherapeutic containing a naturally occurring strain of *Lactobacillus crispatus* CTV-05. In non-pregnant women, vaginal administration of LACTIN-V leads to vaginal colonisation as well as reduced recurrence of UTIs and BV (551, 554). Clinical trials to-date have shown live vaginal biotherapeutics to be safe in non-pregnancy (517). As part of their development, well-designed clinical trials are required to assess the safety and efficacy of live biotherapeutics within the target patient population (615, 621, 622). This is the first safety trial of live vaginal biotherapeutics involving *Lactobacillus crispatus* CTV-05 use in pregnancy. In this chapter, the safety, acceptability and tolerability of LACTIN-V in pregnant women at high-risk of sPTB was explored.

3.3 Results

3.3.1 Pre-study questionnaire

The pre-study questionnaire was completed by 55 women, of which 28 (51%) women had taken probiotics before and 27 (49%) women had not. 48 women (87%) women were very or quite likely to take probiotics during pregnancy if shown to have benefits. Only 5 women (9%) stated that they would not and 2 (4%) were unsure. When asked about mode of administration, only 10 women (18%) were put off by vaginal probiotics as opposed to oral. In total 44 women (80%) would consider taking vaginal probiotics as part of research.

3.3.2 Study population and characteristics

During the study period, 123 women met the inclusion criteria and were approached for recruitment (Figure 3.1). Of these, 50 declined participation and 73 consented. Four women were lost to follow up as withdrew their care from the PTB prevention clinic or transferred antenatal care to alternative hospitals. Eight women withdrew from the study, one of which chose to complete the full course of LACTIN-V but elected to minimise hospital attendance during the COVID-19 pandemic. Overall, data on safety, acceptability and tolerability of LACTIN-V was available for 61 women. Demographics for women receiving LACTIN-V (n=61) are presented in Table 3.1, alongside demographics for the two comparison cohorts, groups A and B. Distribution of age, BMI, risk factor and ethnicity remained consistent between women receiving LACTIN-V and women in groups A and B.

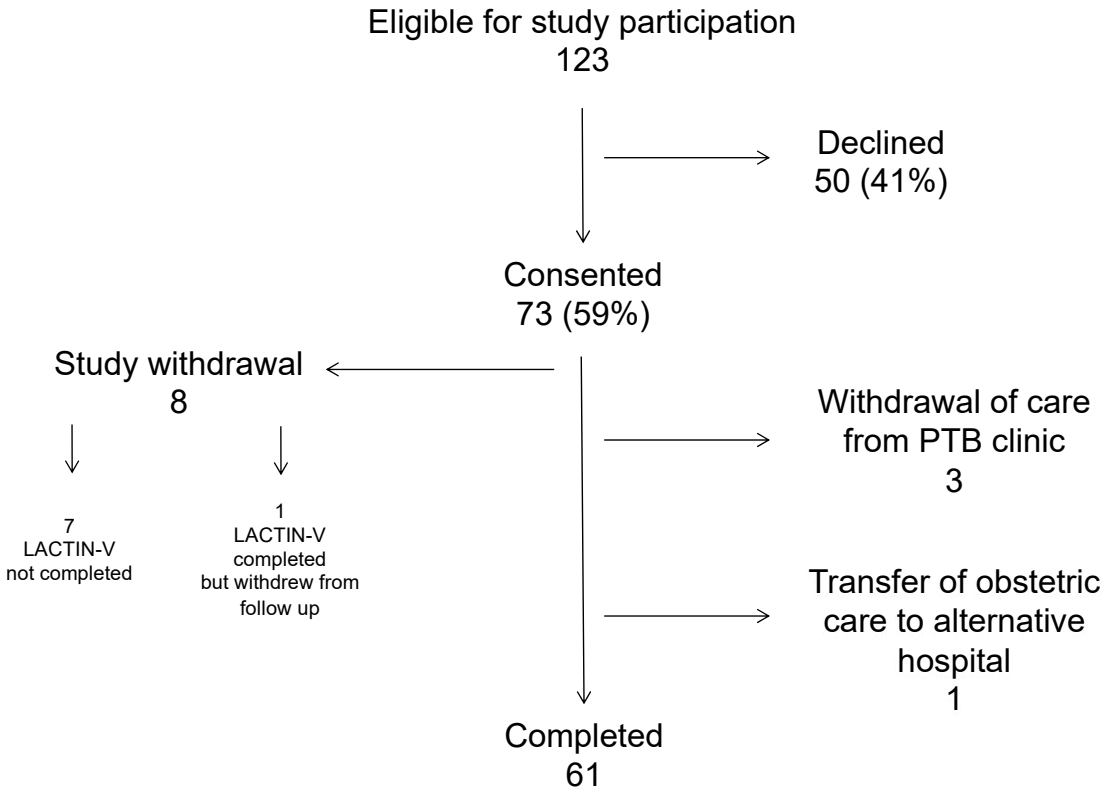


Figure 3.1 – Study recruitment. A total of 123 women at high-risk of sPTB met in the inclusion criteria and 73 women consented to participate. Four women were lost to follow up as either withdrew care from the PTB prevention clinic or from QCCH. Eight women withdrew from the study, one of which chose to complete the course of LACTIN-V but declined input from PTB prevention clinic. Overall, results regarding the safety, tolerability and acceptability of LACTIN-V were available for 61 women.

Table 3.1 – Participant demographics. Demographics are presented for n=61 women receiving LACTIN-V and two comparison cohorts. Group A consists of data for 2,190 women seen in the PTB prevention clinic between 2002-2020. Group B includes data for 120 women seen in the PTB prevention clinic between September 2019 and April 2020. All women were recruited from the PTB prevention clinics at Imperial College NHS Trust and represent women at high-risk of sPTB. Data for age and BMI are presented as median (IQR). Statistics: T-test/Mann-Whitney U testing, depending on the distribution of data or Chi-squared with Yate's correction. P value[a] and p value[b] were determined from comparison of women receiving LACTIN-V with women groups A and B respectively.

	LACTIN-V (n=61)	**Group A (n=2190)**	***p* value[a]**	**Group B (n=120)**	***p* value[b]**
Age (IQR)	34 (3)	34 (7)	0.06	34 (6.5)	0.58
BMI (IQR)	26 (6)	24 (6)	0.89	24 (6)	0.11
Ethnicity (%)					
White	37 (61)	1188 (54)	0.39	66 (55)	0.46
Black African or Black Caribbean	8 (13)	485 (22)	0.13	20 (17)	0.53
Asian or Middle Eastern	10 (16)	312 (14)	0.77	18 (15)	0.81
Mixed or Other	6 (10)	205 (10)	0.92	16 (13)	0.50
Risk Factor (%)					
PTB or MTL	29 (48)	1300 (59)	0.85	47 (39)	0.28
Cervical treatment	27 (44)	703 (33)	0.06	50 (42)	0.74
Other	5 (8)	187 (8)	0.89	23 (19)	0.54

Other (risk factor) = previous cervical cerclage, incidental cervical shortening, FDCS, congenital uterine anomaly or trachelectomy.

3.3.3 Safety

Solicited and unsolicited adverse events were reviewed for all women commencing LACTIN-V (Table 3.2). This included 61 participants who completed the study, as well as the eight women who withdrew (n=69). A total of 13 solicited adverse events were reported (19%). The most common was a change in vaginal discharge, reported by 7 women (10%). Other solicited adverse events included change in vaginal odour (n=1), vulvar or vaginal itching (n=3) and vaginal bleeding (n=1).

One unsolicited adverse event was reported at 38^{+0} weeks as haematuria (Table 3.2). As symptoms and urinary culture results were in keeping with a UTI and developed 18 weeks after last LACTIN-V use, clinical trial staff judged this to be unrelated to LACTIN-V. An urology opinion was sought, which confirmed the likely diagnosis of a UTI. There were no severe or life-threatening adverse events; all were graded as mild and self-resolved. There were no IUDs or NNDs in women completing LACTIN-V (Table 3.3). One fetal anomaly was reported (bronchopulmonary sequestration) but was deemed unrelated to LACTIN-V since administration commenced after complete cardio-respiratory embryogenesis.

Table 3.2 – Solicited and unsolicited adverse events. Adverse events were recorded for n=69 women including all 61 women completing the study and the 8 women who withdrew.

Solicited adverse event	**Number of women (%)**
Change in vaginal discharge	7 (10)
Change in vaginal odour	1 (1)
Change in colour/consistency of urine	1 (1)
Vulvar/vaginal itching	3 (4)
Vaginal bleeding	1 (1)
Unsolicited adverse event	
Macroscopic haematuria	1 (1)

Table 3.3 - Outcomes for women receiving LACTIN-V (n=61) versus women from comparison cohorts, group A (n=2,190) and group B (n=120). Values are given as number of women (%). The diagnosis of chorioamnionitis was based on the Royal College of Obstetricians and Gynaecologists guidance and included a combined clinical assessment including maternal observations, biochemistry and fetal heart rate (623). Patients who experienced cervical shortening are captured within those listed as receiving an ultrasound indicated cerclage. P value[a] and p value[b] were determined to compare outcomes in women receiving LACTIN-V with women from groups A and B respectively. Statistics: Chi-squared with Yates' correction.

	LACTIN-V (n=61)	Group A (n=2190)	*p* value[a]	Group B (n=120)	*p* value[b]
Progesterone (%)	16 (26)	*data not available*		35 (29)	0.81
Cervical cerclage (%)	13 (21)	562 (26)	0.61	38 (32)	0.20
History indicated cerclage (%)	7 (11)	283 (13)	0.97	19 (16)	0.93
Ultrasound indicated cerclage (%)	6 (10)	279 (13)		19 (16)	
Nylon cerclage (%)	11 (85)	432 (77)	0.75	37 (97)	0.32
Mersilene cerclage (%)	2 (15)	130 (23)		1 (3)	
Miscarriage <16/40 (%)	1 (1.6)	23 (1.1)	0.85	0 (0)	
Mid-trimester loss ≥16/40 and <24/40 (%)	1 (1.6)	57 (2.6)	0.95	3 (2.5)	0.87
Spontaneous PTB ≥24/40 and <34/40 (%)	2 (3.3)	153 (7.0)	0.38	9 (7.5)	0.43
Spontaneous PTB ≥34/40 and <37/40 (%)	6 (9.8)	390 (17.8)	0.15	12 (10)	0.65
PPROM (%)	4 (6.6)	160 (7.3)	0.98	13 (11)	0.51
Chorioamnionitis (%)	0 (0)	22 (1.0)		1 (0.8)	
Intra-uterine death (%)	0 (0)	16 (0.7)		1 (0.8)	
Neonatal death (%)	0 (0)	6 (0.3)		1 (0.8)	

3.3.4 Tolerability

Withdrawal reasons were only recorded if volunteered by women (Table 3.4). Only one woman withdrew secondary to an adverse event. In this case, withdrawal was due to vaginal burning and itching associated with LACTIN-V use. The onset of symptoms occurred following immediate use and resolved within 15 minutes. As the symptoms did not require medical treatment, hospital admission or impact quality of life, this adverse event was graded as mild.

Table 3.4 – Withdrawal reasons. Only one woman reported withdrawal to be associated with an adverse event.

Reason	Number of women
No reason specified	4
Anxiety of clinical trial involvement during pregnancy	1
Taking too many medications	1
Vaginismus - preference to avoid vaginal administration	1
Burning and itch associated with use	1

3.3.5 Compliance

Study uptake was high, with 59% of women consenting to involvement. Most women (60/61, 98%) were fully compliant to the 5-day loading dose. One woman was non-compliant to two out of five doses. Three women took an extra dose of LACTIN-V during the loading phase. These women were given one extra dose of LACTIN-V so as not to alter compliance to the maintenance phase. Compliance data for the maintenance dose is reported for 60 women as one woman miscarried before its start. Most women 56/60 (93%) were fully compliant to the 6-week maintenance course of LACTIN-V. Four women forgot to administer one of the weekly LACTIN-V doses. No participants omitted more than one of the weekly doses. Overall, compliance to the full course of LACTIN-V was 93% (56/60).

3.3.6 Questionnaire

The questionnaire assessed ease of administration, comfort, schedule of use, storage, convenience, partner acceptance and future use. Thirty-one women completed the questionnaire and 30 (97%) described the vaginal applicator as easy and comfortable to use (Figure 3.3). Eight women (26%) found the schedule difficult to remember, however compliance was not compromised. Three women (10%) described LACTIN-V as messy to use and one woman reported difficulties storing LACTIN-V in the refrigerator. Two women reported that their partners felt uncomfortable with the use of LACTIN-V in pregnancy, however both described this as being related to partner exclusion from antenatal care during the COVID-19 pandemic and remained compliant. When questioned about future pregnancies, 30 (97%) stated that they would use LACTIN-V in future pregnancies if benefits and safety were confirmed. Similarly, 28 (90%) women would use LACTIN-V outside of pregnancy if suffering from UTIs or BV.

3.3.7 Clinical Outcomes

Clinical outcomes are summarised in Table 3.3 and Figure 3.3. The incidence of sPTB in group A was 17.8% and 7.0% for $<37^{+0}$ and $<34^{+0}$ weeks' gestation respectively. In women receiving LACTIN-V, the incidence of sPTB was lower at 9.8% (p=0.15, Chi-squared with Yate's correction) and 3.3% Interestingly, despite four cases of PPROM among women taking LACTIN-V, there were no cases of chorioamnionitis. In contrast 13.8% and 7.7% of women in groups A and B respectively, developed chorioamnionitis. Group B was included as a comparison group during the same time-period, in view of the impact of COVID-19 pandemic on PTB rates (624, 625). Although there was no reduction in the incidence of PTB $<37^{+0}$ weeks in women receiving LACTIN-V, PTB $<34^{+0}$ weeks was lower in women receiving LACTIN-V (3.3%) versus women in group B (7.5%). To determine whether COVID-19 impacted sPTB incidence in this high-risk cohort, the sPTB incidence was compared between groups A and B. There was no significant difference in the incidence of PTB $<34^{+0}$ or $<37^{+0}$ weeks' gestation (p=0.99 and p=0.95 respectively, Chi-squared with Yate's correction) between the two control groups.

The incidence of sPTB was further assessed according to risk factor (Table 3.5). Women with a previous PTB or MTL were compared to women with previous cervical treatment. In women with previous PTB/MTL, the incidence of PTB <34^{+0} weeks was 13.9% and 13.0% in groups A and B respectively. In women using LACTIN-V, the incidence of PTB was lower at 6.9%. For women with a history of cervical treatment, the incidence of PTB <34^{+0} weeks was 5.0% and 6.0% in groups A and B respectively. In women with previous cervical treatment using LACTIN-V, there were no cases of PTB <34^{+0} weeks.

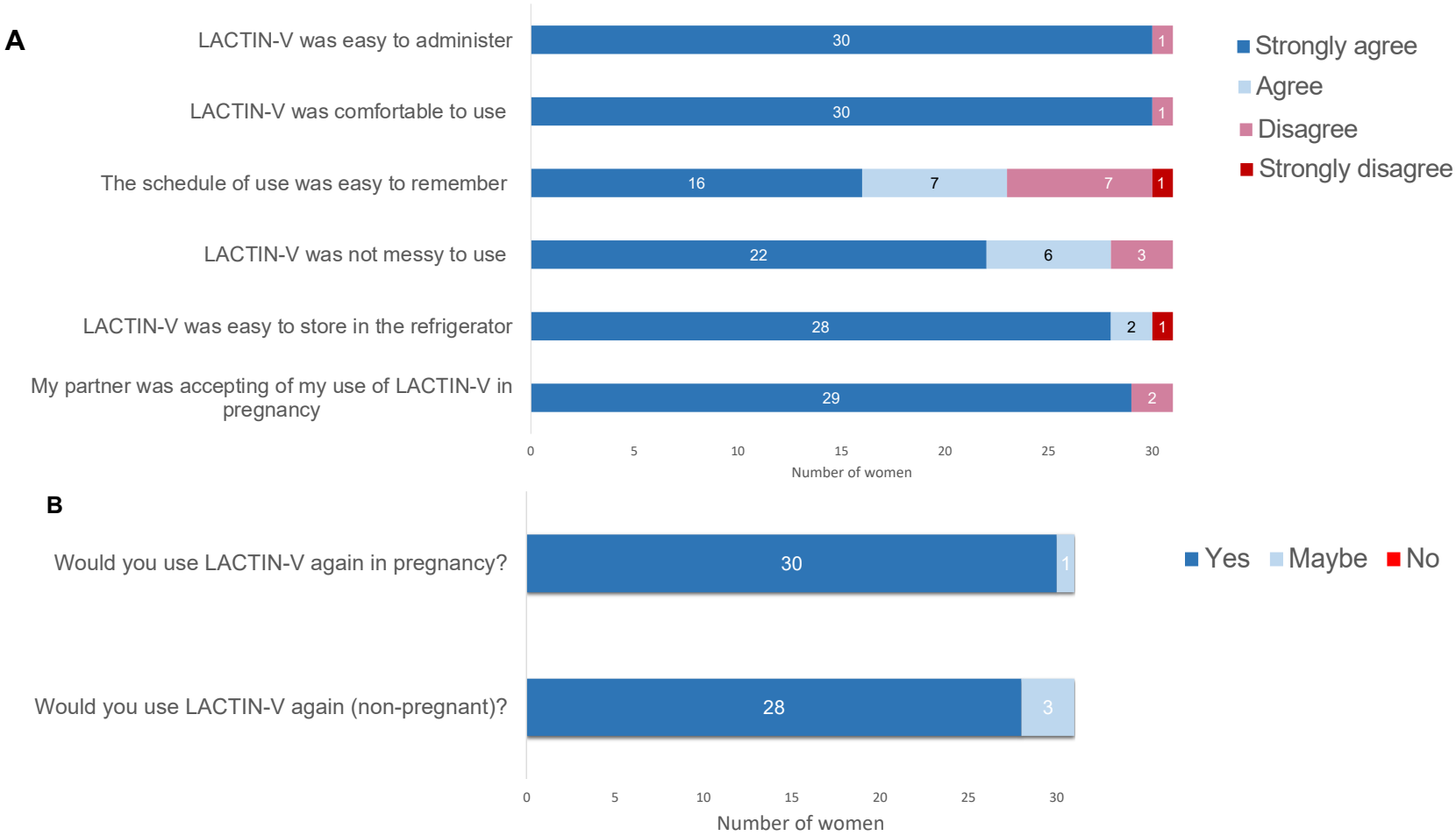


Figure 3.2 – Questionnaire results. A total of 31 women completed the questionnaire. **(A)** Most women found LACTIN-V easy to administer, comfortable and easy to store. LACTIN-V was acceptable by most partners. Eight women found the schedule of use difficult to remember. **(B)** On assessment of future use, most women indicated an interest in future use both in pregnancy (30/31) and outside of pregnancy (28/31).

Table 3.5 – Incidence of sPTB according to risk factor. Gestational age at delivery was described according to risk factor in women receiving LACTIN-V and women from groups A and B. Women with other risk factors for sPTB such as history of cervical cerclage, incidental cervical shortening, FDCS, congenital uterine anomalies or trachelectomy were excluded from analysis. Statistics: Chi-squared with Yates' correction.

LACTIN-V cohort vs Group A	**<37**	***p* value**	**<34**	***p* value**
Previous PTB / MTL				
Group A (n=1300) %	354 (27.2)	0.16	181 (13.9)	0.42
LACTIN-V (n=29) %	4 (13.8)		2 (6.9)	
Previous cervical treatment				
Group A (n=703) %	94 (13.4)	0.24	35 (5.0)	
LACTIN-V (n=27) %	1 (3.7)		0 (0)	
LACTIN-V cohort vs Group B				
Previous PTB / MTL				
Group B (n=47) %	3 (6.4)	0.50	6 (13.0)	0.67
LACTIN-V (n=29) %	4 (13.8)		2 (6.9)	
Previous cervical treatment				
Group B (n=50) %	3 (6.0)	0.92	3 (6.0)	
LACTIN-V (n=27) %	1 (3.7)		0 (0)	

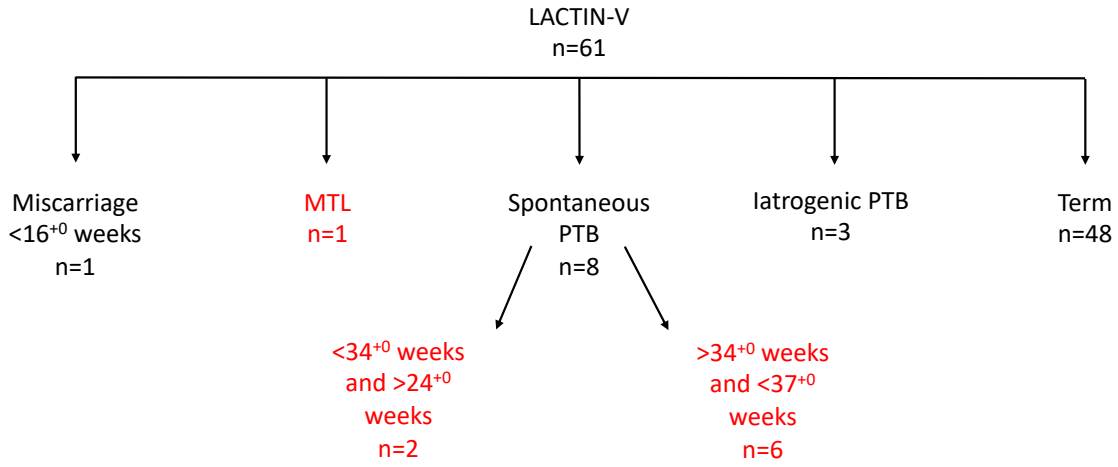


Figure 3.3 - Clinical outcomes for women using LACTIN-V. There was one miscarriage at 14^{+6} weeks' gestation in a dichorionic diamniotic (DCDA) twin pregnancy. MTL was defined as delivery after 16^{+0} weeks. One MTL occurred at 19^{+4} weeks and presented as PPROM. A total of 48 women delivered term. Three women had iatrogenic PTBs, all at >35^{+0} weeks' gestation. Indications included obstetric cholestasis and severe pre-eclampsia. Eight women experienced sPTB, of which the majority (n=6) were between 34^{+0} and 36^{+6} weeks' gestation. Only two were at <34^{+0} weeks' gestation.

3.4 Discussion

The study uptake was high, with two-thirds of women agreeing to trial LACTIN-V. Other trials involving nutritional supplements have shown probiotics to be popular among pregnant women (619). Only one withdrawal was associated with an adverse event, highlighting that LACTIN-V is tolerated well in pregnancy. Despite one woman discontinuing LACTIN-V due to vaginismus, two women with vaginismus completed the full course of LACTIN-V with no reported issues. The ethics committee did not permit questioning women regarding withdrawal reasons, so as not to detriment the patient-doctor relationship. Withdrawal reasons were therefore only recorded if volunteered by participants, meaning that for four women withdrawal reasons were undetermined. Despite this, a commonly reported challenge in clinical trials involving nutritional supplements include time commitments required from participants and wishes to avoid clinical sampling (619, 626). It is probable that without regular clinical sampling the uptake of LACTIN-V would be higher and withdrawals lower. Other authors offering oral probiotics to women in pregnancy report lower withdrawal rates

of 1% (627). The higher withdrawal with LACTIN-V may have been exacerbated by the ambiguity of the COVID-19 pandemic, with patients wishing to minimise contact with healthcare professionals and hospital attendance (628). Equally, it is possible that had there not been a concomitant pandemic, study uptake would have been higher.

Compliance to LACTIN-V was high, with 93% of women being fully compliant. A clinical trial based in Rwanda reported compliance to oral probiotics in pregnancy to be 72% and 85% for placebo and treatment groups respectively (629). The lower compliance in this Rwandan study is likely to reflect differing participant demographics and limited access to healthcare or research staff. A clinical trial in Ireland offering obese women oral probiotics reported compliance to be 80% (627). The higher compliance rates with LACTIN-V may represent a group of women who acknowledge their individual risk of sPTB, which they are motivated to reduce.

Oral routes of administration for drugs and supplements have been indicated to be preferrable by the general public (630). It is reassuring to see that vaginal administration of LACTIN-V did not impact compliance. Although there is a perceived convenience associated with oral intake, vaginal administration of antibiotics, for example to treat vulvovaginal candidiasis or BV, do not impact compliance when compared to oral treatments (631). Studies exploring the impact of vaginal progesterone administration in the prevention of PTB, have demonstrated high compliance levels (96%) in both treatment and placebo groups, despite vaginal use (632). This reaffirms compliance to vaginally administered medication in motivated groups of women such as those aware of their heightened PTB risk. For women at high-risk of PTB, the gestational timepoint whereby women feel a sense of security is at 24^{+0} weeks, where fetal viability is reached. This may support the impressive compliance seen with LACTIN-V. Similarly, studies looking at progesterone compliance in women at high-risk of first trimester miscarriage, have shown compliance to be highest until 12^{+0} to 14^{+0} weeks' gestation (633). This may reflect a trend of women to comply to treatment until the pregnancy is perceived to be secure.

A total of 13 (19%) adverse events were reported, which were all mild and self-resolved after LACTIN-V completion. The most common adverse event was a change in vaginal discharge. It is important to consider that several factors may influence

vaginal discharge in pregnancy. Physiological changes in vaginal discharge occur from the first trimester, with discharge increasing as pregnancy advances (634). Notably, 26% of women administered vaginal progesterone, which is also known to alter vaginal discharge (635). Future work should include RCTs to help elucidate whether changes in vaginal discharge are exclusive to LACTIN-V.

Clinical trials offering *L. crispatus* to non-pregnant women have shown similar safety results. A safety trial assessing *L. crispatus* GAI98322 for the prevention of UTI recurrence (n=9) showed a significant reduction without causing adverse events (511). This trial is currently being continued in a larger sample size to further assess adverse events (636). Randomisation trials with vaginal *L. crispatus* IP 174178 showed no difference in adverse events between intervention and placebo groups and demonstrated a reduced recurrence of BV as well as longer time to recurrence in treatment groups (516).

All trials with LACTIN-V in non-pregnant women have produced reassuring results with respect to safety. A phase 1 safety trial of LACTIN-V for BV prevention in non-pregnant women showed no difference in the number of adverse events between LACTIN-V and placebo groups (549). The succeeding phase 2a trial showed vaginal colonisation with LACTIN-V in 61% of women and reproduced comparable adverse events in LACTIN-V and placebo groups (553). Similar to the results reported in this chapter, a phase 1 trial of LACTIN-V for UTI prevention also showed vaginal discharge to be the most commonly reported adverse event, with no differences seen in women receiving LACTIN-V or placebo (509). The subsequent phase 2 trial demonstrated vaginal colonisation with *L. crispatus* CTV-05, as well as a significant reduction in recurrent UTIs in women receiving LACTIN-V versus placebo (551). Similarly, a phase 2b clinical trial randomising women to receive LACTIN-V or placebo following metronidazole treatment of BV, showed that altered vaginal discharge and odour were the most frequent adverse events with no serious adverse events (554). Interestingly, Cohen et al (2020) report a much higher incidence of solicited adverse events, 87% and 79% in LACTIN-V and placebo groups respectively. This difference may be attributable to the different dosing regimen used, which included four consecutive daily doses of LACTIN-V, followed by twice-weekly administration for 10 further weeks (554).

A systematic review and meta-analysis assessing the safety of oral probiotics containing *Lactobacillus* spp. in pregnancy, showed no adverse impact on caesarean-section rates, birth weight or congenital malformations (637). Studies included assessed probiotic use for a wide range of indications including infant eczema and response to immunisations. Authors were unable to comment on miscarriage rates, as this was unreported by the included studies. Whilst this review assessed oral probiotics in pregnancy, results from our study similarly show no congenital malformations associated with LACTIN-V use. Although LACTIN-V impact on mode of delivery or birth weight was not assessed, results from this chapter demonstrate that miscarriage incidence was comparable to that observed in comparison cohort group A. Another study assessed the safety of orally administered *Lactobacillus* in early pregnancy and in keeping with results from FLIP-1, showed no increase in miscarriage or congenital fetal anomalies when compared to a comparison group (638). It must, however, be considered that rare outcomes may have been missed due to a small sample size. Although reports of lactobacilli containing probiotics causing bacteraemia and endocarditis exist, no such cases occurred with LACTIN-V (550).

Although results in this chapter demonstrate LACTIN-V to be safe to use in pregnancy, further work is needed to confirm its safety in immunocompromised individuals (639). Oral probiotics have been shown to be safe in HIV and cancer populations (640, 641). LACTIN-V safety in individuals with HIV or active cancer has been undetermined from this study.

Results from the exit questionnaire showed LACTIN-V to be both easy and comfortable to use. Although a quarter of women (26%) found the dosing regimen difficult to remember, compliance was not impacted. Similarly, willingness for future use was not impacted as most women (97%) indicated a desire to use LACTIN-V in future pregnancies, if safety and benefits were confirmed. Willingness for future use has been reported to be similarly high at 97% with oral probiotics in pregnancy (627). High satisfaction levels and willingness for future use have also been shown in non-pregnant women using vaginal capsules containing *L. crispatus* (642).

This study was not powered to assess clinical outcomes. The most insightful observation was the incidence of PTB at $<34^{+0}$ weeks' gestation in women with a

history of PTB, PPROM or MTL. The incidence of PTB <34^{+0} weeks' gestation in women receiving LACTIN-V was half that of women from groups A and B. Dysbiosis and a low relative abundance of *Lactobacillus* spp. have been associated with PPROM and PTB (371, 643). Displacement of *Lactobacillus* spp. and increased bacterial diversity can activate local inflammation leading to cervical ripening and preterm labour (498). Meanwhile, dominance of the vaginal microbiota with *L. crispatus* is protective against PTB (644). *Lactobacillus* spp. produce lactic acid and bio-surfactants, thus creating an acidic, hostile environment, inhibiting adhesion of pathogens (645, 646). The pathophysiology predisposing women with previous cervical treatment to PTB is less well understood. A depth-dependent relationship between depth of cervical tissue excised and risk of sPTB has been reported (647). In addition to a mechanical cause, a microbial element is also thought to exist. Cervical intraepithelial neoplasia (CIN) relates to vaginal dysbiosis and decreasing relative abundance of *Lactobacillus* spp. (648). Following cervical treatment, an improved vaginal microbiota composition is seen (649). Thus, vaginal dysbiosis due to CIN may contribute to sPTB risk in women with previous cervical treatment.

The main limitation of this study was the small sample size, limiting statistical analysis particularly for the assessment of clinical outcomes. As this was an initial safety trial, a placebo arm was not included. A placebo-control randomised trial would further investigate adverse events associated with LACTIN-V. The impact of COVID-19 is another limitation of this study. Women seen in the prematurity clinic during the same study period acted as a comparison group (group B, n=120). Although data on the number of exposed and vaccinated women was not collected, there was no significant difference in the incidence of PTB at <37^{+0} or <34^{+0} weeks' between groups A and B. Moreover, the impact of COVID-19 on the incidence of sPTB is conflicted in the literature. Research from Denmark and Ireland have shown lower rates of sPTB following periods of lockdown (624). This trend was more pronounced for extreme preterm deliveries <28^{+0} weeks' gestation (624, 625). The pandemic has revealed possible socio-environmental factors predisposing to PTB, including reduced exposure to pathogens during periods of lockdown, work-related stress, environmental pollution, exercise, exposure to smoking or recreational drugs (625). Other researchers, including work conducted in a London-based hospital, showed an increase in the incidence of stillbirth but no change in sPTB during the lockdown period

(650). It is unsurprising to note an increase in stillbirths, which may relate to delayed hospital presentation, poor staffing levels or reduced access to ultrasound scanning (651). The impact of COVID-19 on stillbirth and sPTB rates should not be considered in isolation as it is possible that delayed presentation in women with threatened preterm labour culminated in stillbirth rather than prematurity. Interestingly, a meta-analysis demonstrated a 3-4% reduction in sPTB rates in high-income countries, unrelated to the increase in stillbirth (652). Published literature focuses on the impact of COVID-19 on sPTB among the general antenatal population. Although results from this chapter show no difference in the incidence of prematurity before and after the pandemic, the impact on women already at high-risk of sPTB remains unclear.

3.5 Conclusion

In accordance with studies conducted in non-pregnant women, results from this chapter show that LACTIN-V is safe with a change in vaginal discharge to be the most common adverse event. All adverse events were graded as mild and self-resolved. Withdrawal due to an adverse event was only reported in one participant, suggesting that LACTIN-V is tolerable. With impressive compliance and willingness for future use demonstrated, LACTIN-V is overall well accepted by pregnant women at high-risk of sPTB, qualifying it to be a promising live vaginal biotherapeutic. Although restricted by sample size, the incidence of sPTB was lower in women using LACTIN-V when compared to control cohorts. Upcoming chapters explore colonisation potential and host immune response to LACTIN-V.

4 The Vaginal Microbiota in Women using LACTIN-V

4.1 Chapter overview

Having shown that LACTIN-V was safe, well tolerated and accepted by pregnant women at high-risk of sPTB, I next sought to investigate the impact of LACTIN-V on the vaginal microbiota composition. It was hypothesised that LACTIN-V would promote *L. crispatus* CTV-05 vaginal colonisation during pregnancy and in doing so, reduce the prevalence of sub-optimal CSTs associated with PTB risk.

Aims of the research conducted in this chapter were to:

- Optimise and validate methods for detection of *L. crispatus* CTV-05 in vaginal samples.
- Determine and characterise whether LACTIN-V use leads to viable and stable colonisation of *L. crispatus* CTV-05.
- Explore how LACTIN-V use leads to dynamic changes in the vaginal microbiota composition throughout pregnancy.
- Examine endogenous and exogenous factors associated with colonisation response.
- Assess whether *L. crispatus* CTV-05 is detected in the neonatal gut microbiome.

Key results

L. crispatus CTV-05 colonisation was assessed using PCR, qPCR and metataxonomics approaches. Persistent colonisation, defined as *L. crispatus* CTV-05 detection at T4 and T5, reflecting eight and 16 weeks since last use of LACTIN-V, revealed that 37 (65%) and 28 (58%) women achieved long-term colonisation ($p<0.0001$). High bacterial load colonisation (HBLC) was defined as a *L. crispatus* CTV-05 load $>10^6$/swab and was observed in 29 (52%) and 21 (44%) of women at T4 and T5 ($p<0.0001$). At T4, 32/55 women (58%) demonstrated viable *L. crispatus* CTV-05 detection, following its culture and cultivation. *L. crispatus* relative abundance increased throughout the study. Despite exogenous use of *L. crispatus* CTV-05 and its subsequent colonisation, the total bacterial load remained unchanged suggesting colonisation concomitantly displaces other bacterial commensals and pathogens, including *G. vaginalis*. This was evidenced by a significant reduction in alpha diversity

(measured using the Shannon Index) between T0 to T4, before slightly increasing at T5. The prevalence of CST I increased from 44% at T0 to 86% and 88% by T4 and T5 respectively. A high *L. crispatus* CTV-05 load associated with protection from BV. Women with CST III or IV at T0 were more likely to colonise when compared to women starting with CST I. Factors prohibiting colonisation included pre-study *L. crispatus* dominance and antibiotic use. Nine women delivered before 37^{+0} weeks' gestation, of which seven displayed colonisation, suggesting non-microbial PTB aetiologies in these women. *L. crispatus* CTV-05 was not detected in placenta and fetal membrane samples obtained at ELCS indicating that colonisation is localised to the cervicovaginal niche.

Conclusion

LACTIN-V administration in early pregnancy leads to successful and long-term colonisation of *L. crispatus* CTV-05 in most women. Colonisation of *L. crispatus* CTV-05 leads to a shift in microbiota composition away from sub-optimal, high-risk *L. iners* and high diversity compositions towards a protective CST I vaginal microbiota.

4.2 Introduction

Vaginal colonisation of lactic acid producing *Lactobacillus* spp. is associated with protection against BV, candidiasis, *C. trachomatis*, *N. gonorrhoea* and HIV (339-344). The microbial composition of the vagina is dynamic and can be influenced by hormonal variations of the menstrual cycle (653). During pregnancy, which is a highly oestrogenic state, there is a shift towards *Lactobacillus* spp. dominance, as well as increased stability and decreased diversity (598, 600, 654).

There is now strong evidence that the vaginal microbiome is a risk factor for sPTB. Recent meta-analyses and network analyses of publicly available datasets show that despite differences in study design, patient demographics and methodologies, women harbouring *L. crispatus* have reduced risk of PTB compared to women with *L. iners* or high-diversity *Lactobacillus* spp., deplete microbiota (376). These relationships are particularly evident when considering protection against early sPTB ($<32^{+0}$ weeks) (375). Therefore, a live biotherapeutic that could modulate the vaginal microbiota towards *L. crispatus* dominance may provide a novel strategy for reducing PTB risk.

Molecular-based studies show that orally administered live biotherapeutics do not lead to vaginal colonisation in pregnancy (530). However, vaginal administration of *L. crispatus* CTV-05 leads to vaginal colonisation in non-pregnant women. Hemmerling and colleagues reported that 2 x 10^9 CFU/dose of *L. crispatus* CTV-05 for five consecutive days followed by two weekly doses, resulted in colonisation of 50% of women by day 10 and 44% by day 28 (553). Cohen et al (2020) assessed LACTIN-V use in the context of BV prevention (554). Following five days of treatment with vaginal metronidazole, women with BV were randomised to receive LACTIN-V (four daily doses followed by twice weekly doses for ten weeks, 2 x 10^9 CFU) or placebo. Vaginal sampling of women at 4, 8, 12 and 24 weeks after enrolment was performed. Using qPCR, *L. crispatus* CTV-05 was detected in 79% and 48% of participants at the 12 and 24-week follow-ups respectively suggesting that *L. crispatus* CTV-05 colonisation declines in non-pregnant women following LACTIN-V cessation.

L. crispatus CTV-05 colonisation has also been shown to reduce the level of BV-related anaerobes in non-pregnant women. Ngugi et al (2011) randomised women

with BV to receive LACTIN-V (2 x 10^9 CFU/dose for five consecutive days followed by two weekly doses) or a placebo intervention following BV treatment with metronidazole vaginal gel (655). Women with evidence of *L. crispatus* CTV-05 colonisation displayed lowered levels of *G. vaginalis* from $10^{4.5}$ to $10^{4.3}$ rRNA gene copies per swab between enrolment and at day 28 (p=0.19). *Atopobium* spp. also reduced from $10^{2.7}$ rRNA gene copies per swab to below the limit of detection in women with *L. crispatus* CTV-05 colonisation (p=0.04). The ability of *L. crispatus* CTV-05 to displace pathogens within the vaginal microbiome in pregnancy is unknown.

After demonstrating its safety and acceptability in Chapter 3, this chapter explores colonisation of *L. crispatus* CTV-05 and impact on the vaginal microbiome when used by pregnant women at high-risk of PTB.

4.3 Results

4.3.1 Study population characteristics

As described in Chapter 3, safety, tolerability and acceptability of LACTIN-V was assessed for 61 women, with one participant choosing to withdraw from both clinical and research follow-up. This participant did however complete the full course of LACTIN-V and provided consent for the analysis of samples collected prior to her withdrawal (T0, T1 and T2). Consequently, results in this chapter data are represented for 62 participants. Participant demographics are summarised in Table 4.1. Most pregnancies (97%) were singleton with 35% of participants being nulliparous and 65% multiparous. The median age and BMI were 34 (years) and 26 (kg/m^2) respectively. Most women (60, 97%) were non-smokers. Within the studied cohort, the majority were of White ethnicity (37, 60%), 8 (13%) were black African or black Caribbean, 11 (18%) were Asian or Middle Eastern and 6 (9%) were mixed or other ethnicities. The proportions represented from each ethnicity are reflective of the demographics of women attending the PTB prevention clinic. Published work from the within the research team recruiting similarly high-risk women report similar ethnic distributions (371).

Previous sPTB, PPROM or MTL was a risk factor for 29 (47%) women. A history of cervical treatment (LLETZ or cone biopsy) existed in 28 (45%) women. A smaller proportion of women (8%) had other risk factors including history of cervical shortening, FDCS, cervical tear or congenital uterine anomaly. Clinical outcomes are reported in Table 4.2. Progesterone was used by 17 (27%) women and 14 (23%) required a cervical cerclage. Most cerclages were nylon (monofilament) (11, 79%) as opposed to Mersilene (braided) (3, 21%). Eight women (13%) required a history indicated cerclage and 6 women (10%) required an ultrasound indicated cerclage for cervical shortening ≤25mm. Within the studied cohort, one woman miscarried at 14^{+6} weeks and one woman had a 19^{+4} week MTL, presenting as PPROM. Fourty-nine women delivered at term and three women had an iatrogenic PTB (iPTB) due to obstetric cholestasis or severe pre-eclampsia. All iPTBs occurred after 35^{+0} weeks' gestation. A total of eight sPTBs occurred, including two before 34^{+0} weeks.

Table 4.1 – Demographics. 62 women at high-risk of sPTB trialled LACTIN-V in pregnancy. Other risk factors included previous cervical cerclage, incidental cervical shortening, FDCS, congenital uterine anomaly or trachelectomy.

	LACTIN-V
	(n=62)
Singleton (%)	60 (97)
Twin (%)	2 (3)
Parity (%)	
0	22 (35)
1	24 (39)
2	11 (18)
≥3	5 (8)
Smoking (%)	
Non-smoker	60 (97)
Current smoker	2 (3)
Age (IQR)	34 (5)
BMI (IQR)	26 (6)
Ethnicity (%)	
White	37 (60)
Black African or Black Caribbean	8 (13)
Asian or Middle Eastern	11 (18)
Mixed or Other	6 (9)
Risk Factor (%)	
PTB or MTL	29 (47)
Cervical treatment	28 (45)
Other	5 (8)

Table 4.2 – Clinical outcomes. Outcomes for all 62 women completing LACTIN-V are described.

	LACTIN-V
	(n=62)
Progesterone (%)	17 (27)
Cervical cerclage (%)	14 (23)
History indicated cerclage (%)	8 (13)
Ultrasound indicated cerclage (%)	6 (10)
Nylon cerclage (%)	11 (79)
Mersilene cerclage (%)	3 (21)
Term (%)	49 (79)
Miscarriage <16/40 (%)	1 (1.6)
Mid-trimester loss ≥16/40 and <24/40 (%)	1 (1.6)
Spontaneous PTB ≥24/40 and <34/40 (%)	2 (3.2)
Spontaneous PTB ≥34/40 and <37/40 (%)	6 (9.7)
PPROM (%)	4 (6.5)
Chorioamnionitis (%)	0 (0)
Iatrogenic PTB (%)	3 (4.8)
Intra-uterine death (%)	0 (0)
Neonatal death (%)	0 (0)

4.3.2 Detection of *L. crispatus* CTV-05

A total of 384 samples were collected from 62 participants between T0 and T6. Successful extraction of bacterial DNA was confirmed by performing PCR amplification using 16S rRNA universal primers. Each gel also contained a positive and negative control. Positive controls included GBS and *L. crispatus* CTV-05 DNA. Negative controls consisted of DEPC-treated water and AE buffer to exclude contamination from the DNA extraction process. A blank swab also underwent DNA extraction and was included as an additional negative control. The presence of bacterial DNA was confirmed in all 384 samples. Amplification occurred in positive controls but not in negative controls. An example is included in Figure 4.1.

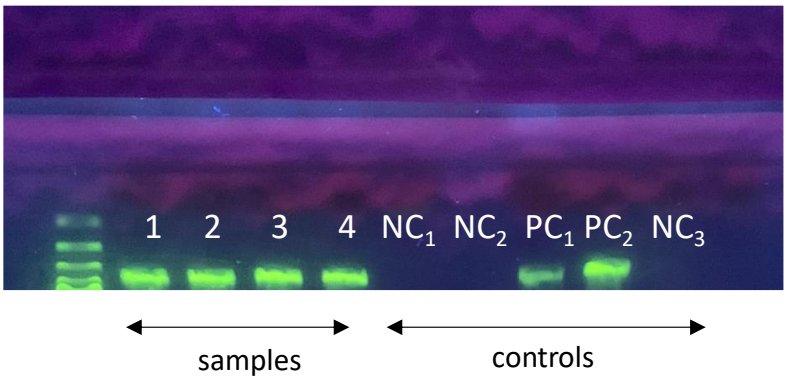


Figure 4.1 – Example of agarose gel of PCR product using universal primers. PCR was performed with a molecular weight marker. Detectable bacterial DNA amplicons of the expected molecular weight (400 bp) was seen in participant samples (1 to 4). Bacterial DNA was not detected in negative controls NC_1 (DEPC-treated water), NC_2 (AE buffer) or NC_3 (blank swab). DNA was detected in positive controls PC_1 (GBS DNA) and PC_2 (*L. crispatus* CTV-05 DNA). NC = negative control, PC = positive control.

After confirmation of successful DNA extraction, detection of *L. crispatus* CTV-05 was determined by using primers specific to *L. crispatus* CTV-05. Negative controls included DEPC-treated water, AE buffer, blank swabs and GBS DNA. The positive control included *L. crispatus* CTV-05 DNA extracted directly from a LACTIN-V applicator. This was performed for all 384 samples with a representative agarose gel of the resulting PCR product presented in Figure 4.2.

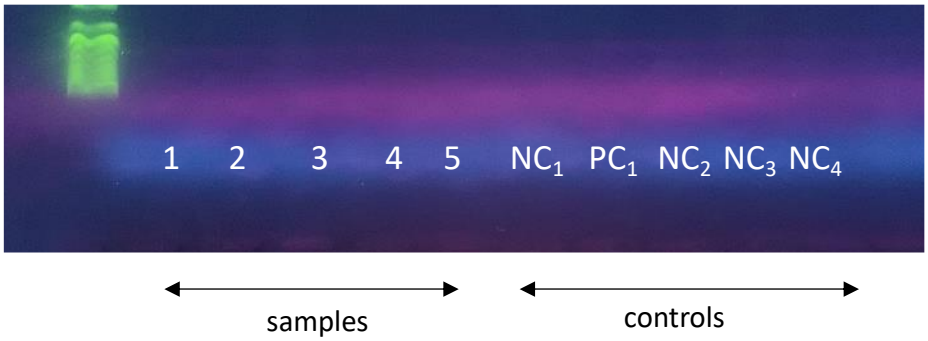


Figure 4.2 – Example of agarose gel of PCR product using *L. crispatus* CTV-05 specific primers. A molecular weight marker was included to confirm size of PCR product. Detectable bacterial DNA amplicons of the expected molecular weight (206 bp) occurred in patient samples 1-4. *L. crispatus* CTV-05 was not detected in sample 5. *L. crispatus* CTV-05 was not detected in the negative controls NC_1 (blank swab), NC_2 (DEPC-treated water) NC_3 (AE buffer) or NC_4 (GBS DNA) but was detected in the positive control (PC_1) where *L. crispatus* CTV-05 DNA was used.

L. crispatus CTV-05 detection within clinical samples was performed in two ways; analysis of metataxonomic results and PCR (targeted and quantitative).

1. Sequence amplicon-based detection

Resulting metataxonomic data was examined to determine if a resulting amplicon sequence variant (ASV) could be used as a marker for LACTIN-V. For this, metataxonomic data from participants sampled between T0-T6, negative controls (blank swabs), positive controls (two patient isolates of *L. crispatus* (VMET-2), LC-1398, LC-824), commercially obtained probiotics (Appendix 3.2) and LACTIN-V (extracted directly or cultured) were compared. Within the dataset, 13 *L. crispatus* ASVs were identified. ASV-1 represented the most prevalent sequence detected within the dataset and was observed in seven samples: *L. crispatus* CTV-05 (from both culture and applicator), LC-1398, LC-824 and commercially available probiotics including Vacramal, Probioscience and Physioflor (Figure 4.3A). The second most detected ASV was ASV-2, which was only present in *L. crispatus* CTV-05 (Figure 4.3B). Therefore, ASV-2 was considered specific for *L. crispatus* CTV-05.

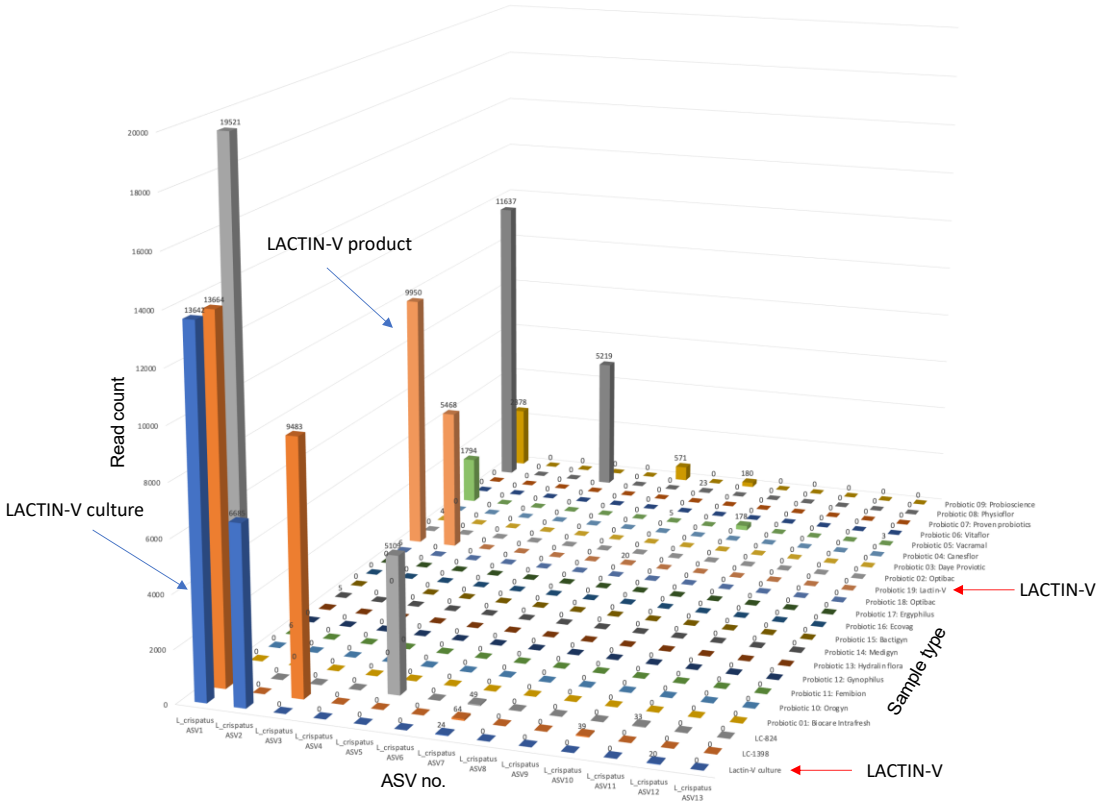


Figure 4.3A – Identification of a LACTIN-V specific ASV. Metataxonomic analysis was performed on extracted DNA acquired from culture of *L. crispatus* CTV-05 as well as directly from a vaginal applicator. The most common ASV, ASV-1, was present in 7 samples including LACTIN-V (both culture and applicator), positive controls (LC-1398, LC-824) and four commercially obtained probiotics. The second most common ASV among analysed samples was ASV-2, unique to LACTIN-V.

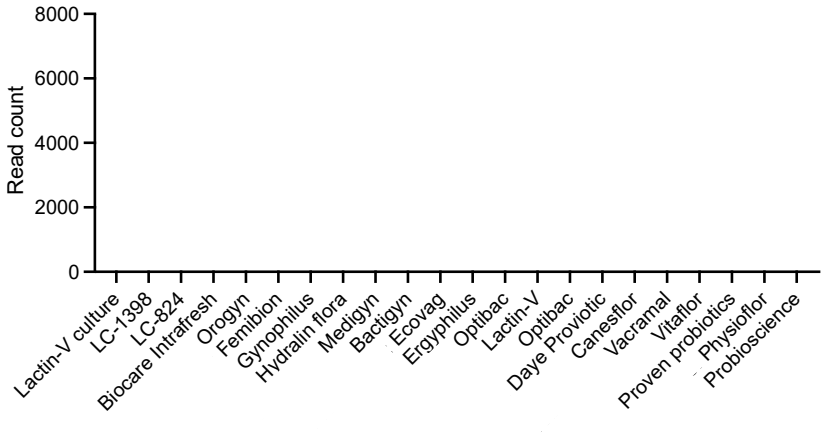


Figure 4.3B – Assessment of ASV-2. ASV-2 was only present in LACTIN-V. ASV-2 was not detected in other assessed *L. crispatus* strains or in commercially available probiotics.

2. PCR-based detection

a) Targeted PCR

To begin with, specificity of *L. crispatus* CTV-05 primers were assessed. Primers were designed to target a 206 bp sequence encoding a hypothetical protein unique to *L. crispatus* CTV-05, as described in Chapter 2 (554). PCR was performed using *L. crispatus* CTV-05 specific primers in a total of 24 *L. crispatus* isolates derived from clinical samples collected from pregnant women seen in PTB prevention clinics (VMET2). Amplicons were not observed in any of these isolates, indicating that the *L. crispatus* CTV-05 primers were specific without cross-reactivity against other *L. crispatus* strains (Figure 4.4).

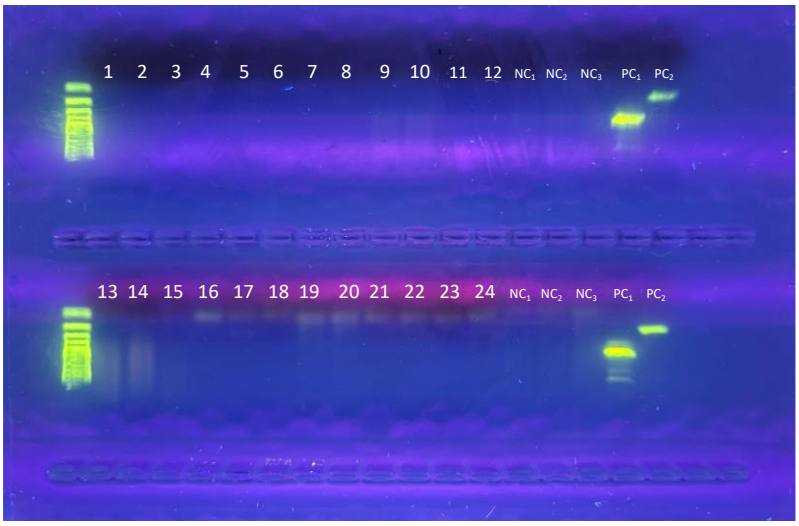


Figure 4.4 – Agarose gel of PCR product from 24 isolates of *L. crispatus*. *L. crispatus* CTV-05 forward and reverse primers were tested on 24 (1-24) *L. crispatus* isolates obtained from swabs provided by pregnant women (VMET-2). *L. crispatus* CTV-05 was not detected in the negative controls NC_1 (DEPC-treated water) NC_2 (blank swab) or NC_3 (GBS DNA). An amplicon was observed in the positive control (PC_1) where GBS DNA was used with universal primers to confirm successful PCR. An amplicon was also observed in the positive control (PC_2) where *L. crispatus* CTV-05 DNA was used with *L. crispatus* CTV-05 specific primers. Amplicons specific to *L. crispatus* CTV-05 were not observed in any of the 24 *L. crispatus* isolates assessed.

b) Real time-quantitative PCR (RT-qPCR)

RT-qPCR was also used to assess the presence of *L. crispatus* CTV-05 in the background population. The range of *L. crispatus* CTV-05 load detected at T0 was between 3-17,613 bacteria/swab, with a mean of 654 bacteria/swab. Examination of metataxonomic results for the participant with the highest *L. crispatus* CTV-05 load at T0 (17,613 bacteria/swab) showed that ASV-2 was not present at T0 suggesting that the RT-qPCR detection was unlikely to represent *L. crispatus* CTV-05 but more likely represent a genetically similar *L. crispatus* strain. The participant with the second highest *L. crispatus* CTV-05 load at T0 (4277 bacteria/swab) had both ASV-1 and ASV-2 detected, thus could represent naturally occurring *L. crispatus* CTV-05.

Comparison of PCR and sequencing based approaches for *L. crispatus* CTV-05 detection

For both PCR methods (targeted and quantitative), 384 samples were analysed including n=62, n=62, n=58, n=57, n=56, n=48 and n=41 at T0, T1, T2, T3, T4, T5 and T6 respectively. Targeted PCR indicated *L. crispatus* CTV-05 detection in 15 women (24%) at T0 (Figure 4.5A). Following completion of the loading dose, this increased to 49 women (79%). Throughout the active dosing period, *L. crispatus* CTV-05 detection was maintained at high levels (T2 = 49 (84%), T3 = 45 (80%)). At T4, 8 weeks after last administration of LACTIN-V, 45 (79%) women still had *L. crispatus* CTV-05 detected. By T5 and T6, *L. crispatus* CTV-05 detection reduced to 31 women (65%) and 23 (56%) women respectively. The increase in *L. crispatus* CTV-05 detection after LACTIN-V administration was significantly higher compared to T0 baseline levels at all study timepoints (T1-T5 $p<0.0001$; T6 $p<0.0005$, Chi-squared).

Consistent with these results, ASV-2 based detection of *L. crispatus* CTV-05, was significantly increased following the loading dose (Figure 4.5B, $p<0.0001$). At T0 only two (3%) women had ASV-2 present increasing to 46 (74%) after the loading dose. At T2 the proportion of women with ASV-2 detected reduced slightly to 36 (62%) women, which was maintained at T3 to 35 (63%) women. A further decline was noted at T4, T5 and T6 where ASV-2 was detected in 31 (54%), 23 (48%) and 20 (49%) respectively. Similar trends in *L. crispatus* CTV-05 detection were observed when

using RT-qPCR and a threshold of 4277 copies/swab (Figure 4.5C) with a significant increase in detection observed after the loading dose at all timepoints (p<0.0001).

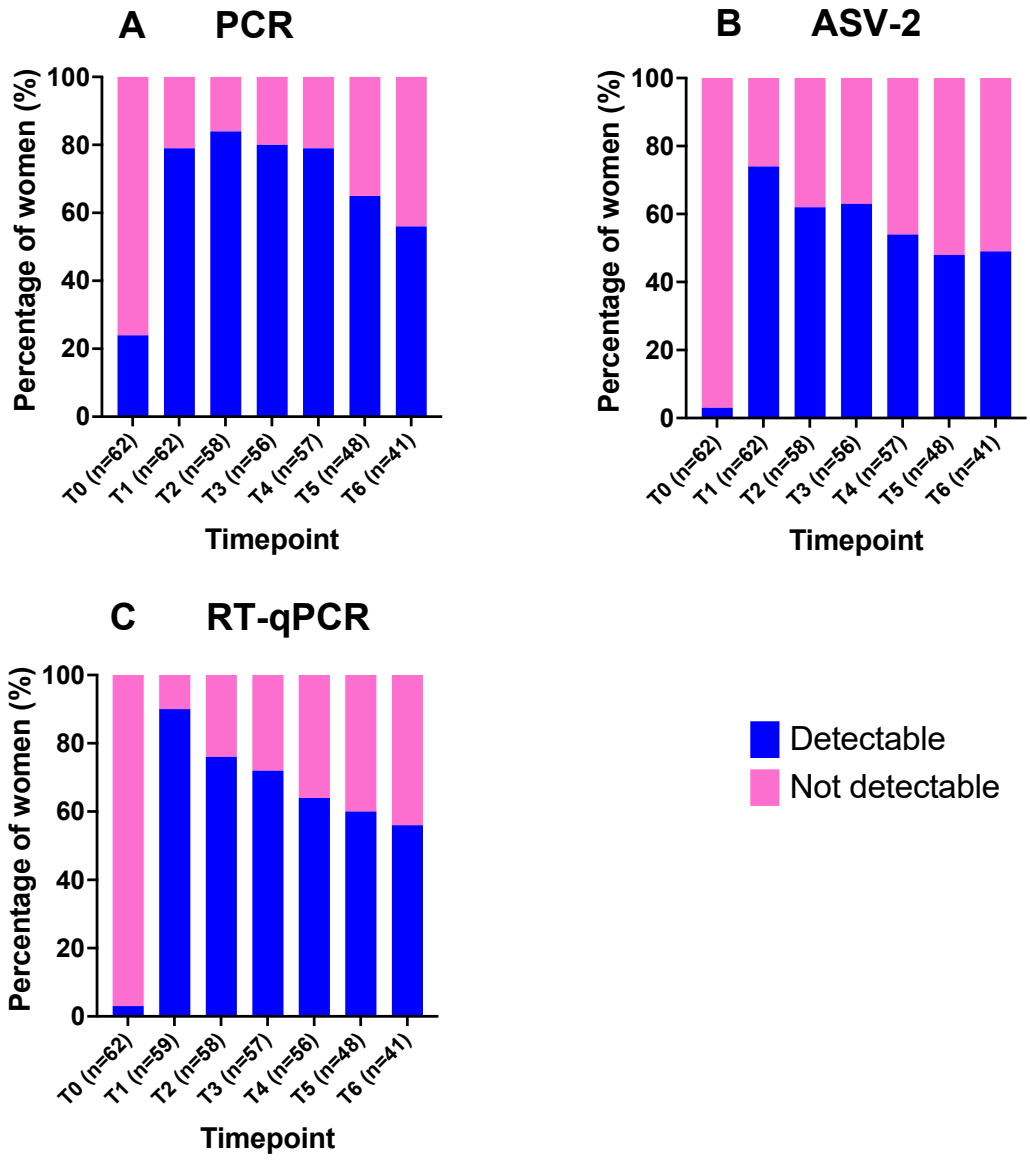


Figure 4.5 – Comparison of different methods for detection of *L. crispatus* CTV-05 in the FLIP-1 cohort. A) PCR detection of *L. crispatus* CTV-05 from T0 to T6 using primers specific for *L. crispatus* CTV-05 indicated a significant increase in detection from 15 (24%) at T0, to 49 (79%), 49 (84%), 45 (80%), 45 (79%), 31 (65%) and 23 (56%) women at T1-T6, respectively (T1-T5, p<0.0001; T6, p<0.0005). **B)** Detection according to the presence of ASV-2 again showed a significant increase in samples positive for *L. crispatus* CTV-05 from 2 (3%) at T0, to 46 (74%), 36 (62%), 35 (63%), 31 (54%), 23 (48%) and 20 (49%) between T1-T6, respectively (T0 versus all other timepoints, p<0.0001). **C)** When a cut-off of ≥4277 bacteria/swab was used as a threshold for detection using RT-qPCR, *L. crispatus* CTV-05 was detected in 2 (3%), 53 (90%), 44 (76%), 41 (72%), 36 (64%), 29 (60%) and 23 (56%) women at T0-T6 respectively (T0 versus all other timepoints, p<0.0001). Statistics: one-way Chi-squared.

To further compare the different techniques, results for individual patient participants were assessed across each time point using targeted PCR results and ASV-2 detection. Table 4.3 summarises participant results, grouping women with congruent PCR and ASV-2 results, patients with *L. crispatus* CTV-05 presence or absence demonstrated by both techniques. For most of the analysed samples, PCR and ASV-2 detection were consistent (n=303, 79%). Across timepoints, congruency was achieved in most samples; 45 (73%), 59 (95%), 45 (78%), 42 (75%), 41 (72%), 40 (83%) and 31 (76%) at T0-T6 respectively. Inconsistent detection usually consisted of positive PCR detection without ASV-2 detection. For results displaying a discrepancy between PCR and ASV-2, quantitative *L. crispatus* CTV-05 load was assessed so as not to prematurely exclude results which may be positive for *L. crispatus* CTV-05.

Table 4.3 - Congruency of targeted PCR and ASV-2 for *L. crispatus* CTV-05 detection. At most timepoints, assessment of *L. crispatus* CTV-05 presence by targeted PCR and ASV-2 were consistent. Congruency was seen for 73%, 95%, 78%, 75%, 72%, 83% and 76% of samples at T0-T6 respectively. Instances of discrepancy between the two techniques were usually due to detection by PCR, without ASV-2 presence.

	T0 N=62 (%)	**T1 N=62 (%)**	**T2 N=58 (%)**	**T3 N=56 (%)**	**T4 N=57 (%)**	**T5 N=48 (%)**	**T6 N=41 (%)**
PCR & ASV-2 congruent	45 (73)	59 (95)	45 (78)	42 (75)	41 (72)	40 (83)	31 (76)
PCR negative ASV-2 positive	2 (3)	0 (0)	0 (0)	2 (4)	1 (2)	0 (0)	4 (10)
PCR positive ASV-2 negative	15 (24)	3 (5)	13 (22)	12 (21)	15 (26)	8 (17)	6 (15)

For samples displaying discrepancies between PCR and ASV-2 results, *L. crispatus* CTV-05 was deemed detected if the quantitative *L. crispatus* CTV-05 load was ≥4277/swab, in the presence of ASV-1. Detection rates using this combined approach are reported in Table 4.4 and Figure 4.6. In the 81 samples whereby PCR and ASV-2 results were inconsistent, 36 samples were identified to have a *L. crispatus* CTV-05 load of ≥4277/swab, of which 34 had ASV-1 detected. Subsequent reporting of detection rates from hereon in are derived from this combined approach.

Table 4.4 - Combined approach to *L. crispatus* CTV-05 detection. Samples were deemed positive for *L. crispatus* CTV-05 presence if samples had visible DNA amplicons of the expected molecular weight using *L. crispatus* CTV-05 specific forward and reverse primers **and** positive for ASV-2. Samples with a discrepancy between the two, were further assessed and regarded as positive for *L. crispatus* CTV-05 if the quantitative bacterial load was ≥4277/swab in the presence of ASV-1. A summary of total samples considered to be positive of *L. crispatus* CTV-05 is highlighted in blue.

	T0 N=62	T1 N=62	T2 N=58	T3 N=56	T4 N=57	T5 N=48	T6 N=41
PCR & ASV-2 positive	0	45	36	33	30	23	16
≥4277 & ASV-1 positive	1	3	6	6	7	5	6
***L. crispatus* CTV-05 detected (%)**	1 (2)	48 (77)	42 (72)	39 (70)	37 (65)	28 (58)	22 (54)

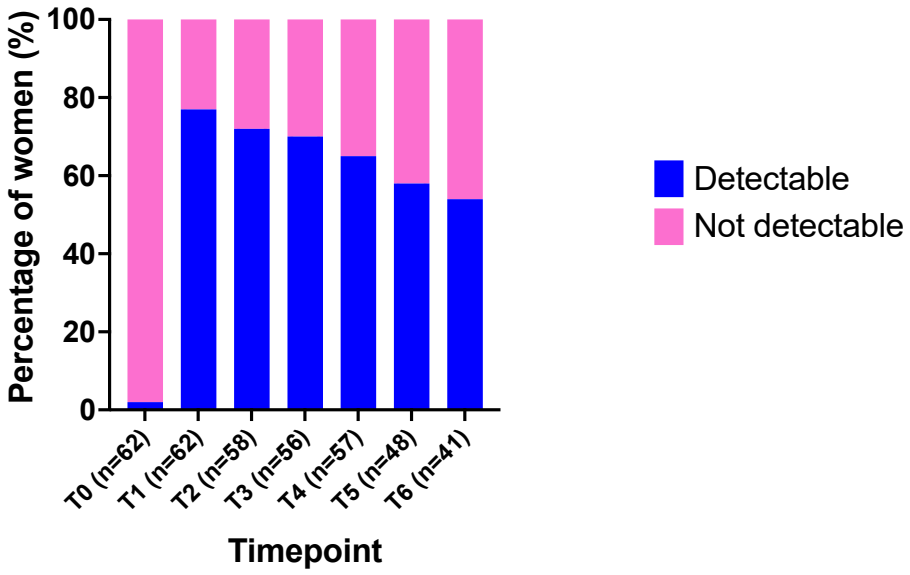


Figure 4.6 - *L. crispatus* CTV-05 colonisation. Colonisation rates of *L. crispatus* CTV-05 from T0 to T6 are represented. Colonisation occurred in 1 (2%), 48 (77%) 42 (72%), 39 (70%), 37 (65%), 28 (58%) and 22 (54%) of participants from T0-T6. *L. crispatus* CTV-05 colonisation was statistically significant at T1-T6 when compared to T0 ($p<0.0001$). Statistics: one-way Chi-squared.

4.3.3 Assessment of *L. crispatus* CTV-05 colonisation persistence

Persistent colonisation was defined as the presence of *L. crispatus* CTV-05 following 8 weeks without LACTIN-V administration (T4, 28^{+0} weeks' gestion). Detection was also assessed at 36^{+0} weeks' gestation, 16 weeks after the last administration of LACTIN-V (T5) (Table 4.5). Using PCR, 45 (79%) and 31 (65%) women demonstrated persistent colonisation at T4 and T5 respectively ($p<0.0001$). Using ASV-2, 31 (54%) and 23 (48%) demonstrated long-term colonisation at T4 and T5 ($p<0.0001$). Analysis of qPCR data with a threshold of ≥4277 bacteria/swab applied, indicated colonisation in 36 (64%) and 29 (60%) women ($p<0.0001$). Overall, using the combined approach 37 (65%) and 28 (58%) had evidence of persistent colonisation at 28^{+0} (T4) and 36^{+0} (T5) weeks respectively ($p<0.0001$). A significant increase in *L. crispatus* CTV-05 detection occurred at 28 and 36 weeks ($p<0.0001$) was seen with all testing modalities.

Analysis of levels required to confer protection against BV in non-pregnancy clinical trials have identified that a bacterial load of *L. crispatus* $>10^6$/swab confers protection against BV and this level has consequently been identified as a threshold for high bacterial load colonisation (HBLC) (548). An additional assessment of HBLC was performed, with a threshold of *L. crispatus* CTV-05 $>10^6$/swab (Table 4.5). At T4 and T5, 52% and 44% displayed evidence of HBLC with *L. crispatus* CTV-05.

Table 4.5 – *L. crispatus* CTV-05 colonisation. *L. crispatus* CTV-05 detection at T4 and T5 was used to determine long-term colonisation. Results according to different detection methodologies are described, including PCR, ASV-2 and RT-qPCR (threshold ≥4277 bacteria/swab). A combined approach as described previously (blue) is also reported. Eight weeks (T4) after last LACTIN-V use, *L. crispatus* CTV-05 was detected in 54-79% according to detection technique. 16 weeks after last LACTIN-V use (T5), detection occurred in 48-65% of samples. Using the combined approach (blue) 65% and 58% of women demonstrated persistent colonisation at 28^{+0} (T4) and 36^{+0} (T5) weeks respectively. HBLC (green) was seen in 52% and 44% at T4 and T5 respectively ($p<0.0001$ when compared to T0). When detection at T4 and T5 were compared to T0, significance was reached ($p<0.0001$). Statistics: one-way Chi-squared. * n=56, **n=48

Timepoint (gestation)	PCR	P value	ASV-2	P value	RT-qPCR (≥4277)	P value	Combined approach	P value	RT-qPCR (>10^6)	P value
T0 (14^{+0}) N=62 (%)	15 (24)		2 (3)		2 (3)		1 (2)		0	
T4 (28^{+0}) N=57 (%)	45 (79)	<0.0001	31 (54)	<0.0001	36 (64)*	<0.0001	37 (65)	<0.0001	29 (52)*	<0.0001
T5 (36^{+0}) N=48 (%)	31 (65)	<0.0001	23 (48)	<0.0001	29 (60)**	<0.0001	28 (58)	<0.0001	21 (44)**	<0.0001

L. crispatus CTV-05 load/swab across the study period was assessed (Figure 4.7). Median values for *L. crispatus* CTV-05 load/swab were 101.5; 1,898,498; 1,961,970; 2,721,899; 1,262,552; 550,802 and 30,247 load/swab for T0-T6 respectively. The *L. crispatus* CTV-05 load/swab significantly increased at each subsequent timepoint, when compared to T0 ($p<0.0001$). In Figure 4.7A, the red line indicates a load $>10^6$/swab, representative of HBLC. The blue line indicates background levels (4277 bacteria/swab) as seen in one participant thought to have pre-existing *L. crispatus* CTV-05 at T0. HBLC was observed in 36 (61%), 33 (57%), 34 (60%), 29 (52%), 21 (44%) and 13 (32%) women at T1-T6 respectively (Figure 4.7B, $p<0.0001$).

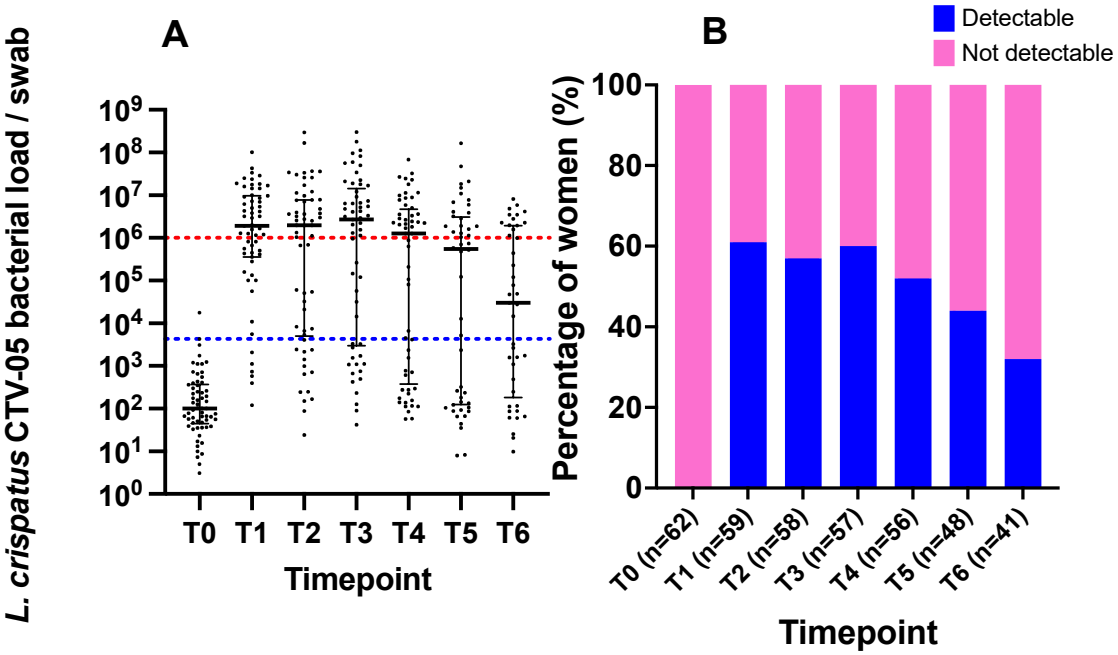


Figure 4.7 – *L. crispatus* CTV-05 bacterial load across study. A) RT-qPCR (n=381) assessment of *L. crispatus* CTV-05 copy numbers per swab at each study timepoint (n= 62, 59, 58, 57, 56, 48 and 41 women at T0-T6, respectively). Median values at T0-T6 were 101.5; 1,898,498; 1,961,970; 2,721,899; 1,262,552; 550,802 and 30,247 load/swab. The load/swab was significantly higher at all timepoints when compared to T0 ($p<0.0001$). Statistics: Kruskal-Wallis. Data is presented as median values with interquartile range. The red line indicates bacterial load of 10^6 and is considered as HBLC. The blue line indicates background levels of *L. crispatus* CTV-05 (4277 bacteria/swab) detected in one participant at T0. **B)** At T0, no women had a *L. crispatus* CTV-05 bacterial load/swab $>10^6$. However, with LACTIN-V use quantities increased $>10^6$ for 36 (61%), 33 (57%), 34 (60%), 29 (52%), 21 (44%) and 13 (32%) women at T0-T6 respectively ($p<0.0001$, T0 versus all time points). Statistics: using one-way Chi-squared testing.

4.3.4 Assessment of *L. crispatus* CTV-05 viability

At T4 (28 weeks' gestation) an extra BBL™ MaxV Liquid Amies vaginal swab was collected for culture. Vaginal swabs were streaked onto MRS plates and incubated for 48 hours in anaerobic conditions (Chapter 2, section 2.2.3). Following this, colonies were removed and resuspended with 2mL of PBS. Samples were then centrifuged and pellets used for DNA extraction (n=55). Using targeted PCR, 47/55 (85%) women had detectable bacterial DNA amplicons of the expected molecular weight when performing PCR with *L. crispatus* CTV-05 primers, indicating the presence of viable *L. crispatus* CTV-05 8 weeks after last administration of LACTIN-V. ASV-2 was detected for 32/55 women (58%).

4.3.5 Impact of LACTIN-V on the vaginal microbial composition

384 vaginal swabs were analysed from T0 to T6, generating a total of 6,919,048 reads for the whole data set. The average read count was 17,878 reads per sample, ranging from 1,300 to 43,325. A total of 1,329 ASVs were identified across the whole dataset however, only 400 ASVs had ≥2 counts. All vaginal samples were analysed on the same run.

At T0, most women were dominated by *Lactobacillus*. With LACTIN-V use, the relative abundance of *Lactobacillus* increased (Figure 4.8). BV associated bacteria including *Gardnerella* spp. and *Prevotella* spp. became less abundant throughout the study. More specifically, *L. crispatus* relative abundance increased with LACTIN-V use (Figure 4.9).

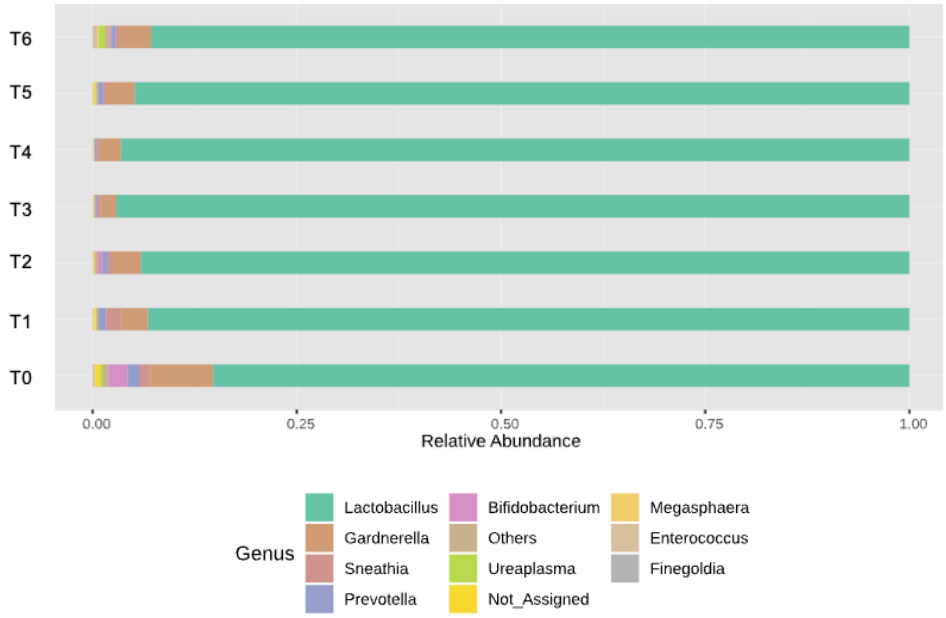


Figure 4.8 – Stacked bar chart showing top 10 taxa across study timepoints. *Lactobacillus* becomes more dominant throughout the study.

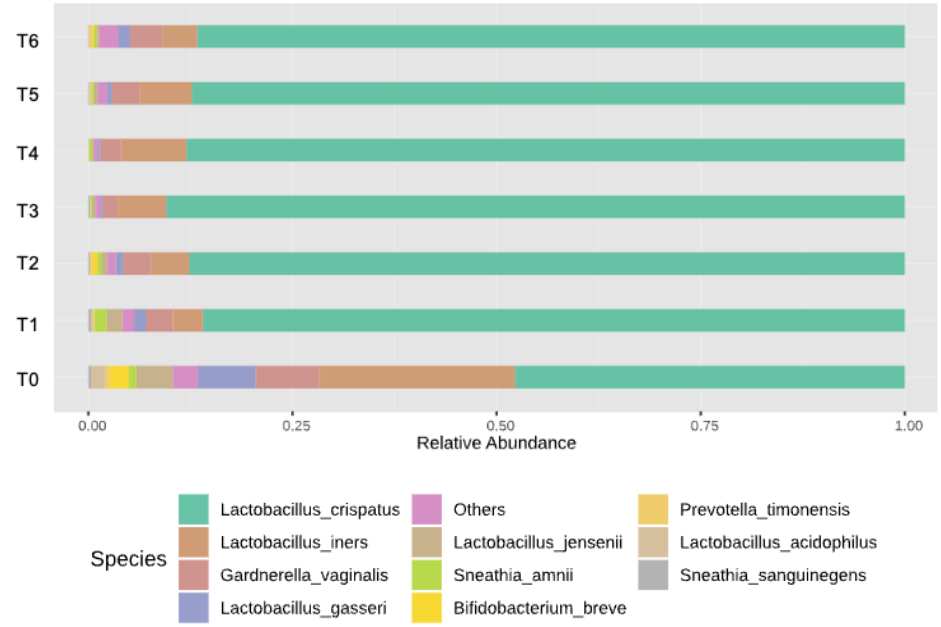


Figure 4.9– Stacked bar chart showing top 10 species across study timepoints. *L. crispatus* relative abundance increases, becoming the more dominant species throughout the study.

Alpha diversity

In view of the increasing relative abundance of *L. crispatus* throughout the study, it was hypothesised that alpha diversity would decrease throughout the study. As shown in Figure 4.10, there was a significant reduction in both the median and mean Shannon Index alpha diversity from T0 to T4 before a slight increase observed at T5 and T6. By T6, the Shannon diversity was comparable to that at T0.

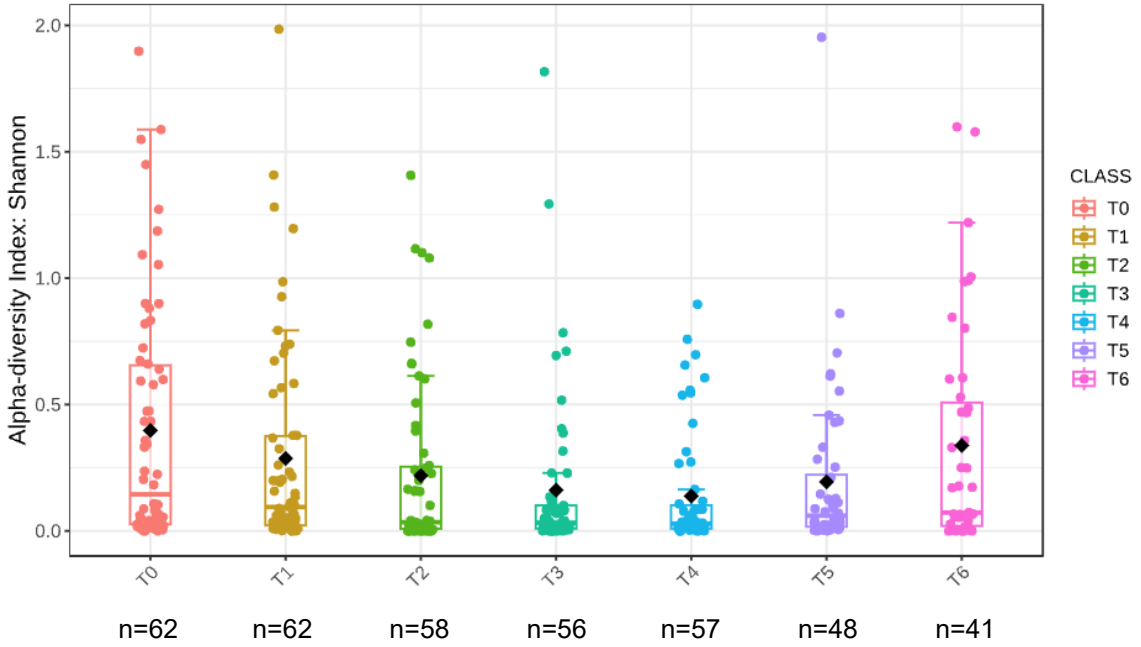


Figure 4.10 – Assessment of Alpha Diversity (Shannon Index) from T0 to T6. Box and whisker plot including median and IQR. Means depicted with black plots. The Shannon Index reduces across the study period with LACTIN-V usage, before increasing from T5. P=0.0025 (Kruskall-Wallis).

Community state type (CST) throughout study

At the start of the study, most women were classified to CST I (27 women, 44%). The remaining women were classified as CST III (15 women, 24%), CST IV (10 women, 16%), CST II (6 women, 10%) and CST V (4 women, 6%) (Figure 4.11). Following the loading dose, 89% of women shifted to CST I. This was maintained across the study, with 86% and 88% of women maintaining CST I by T4 (28 weeks) and T5 (36 weeks), respectively ($p<0.0001$). This was accounted for by a loss of CST II by T1, a loss of CST V by T2 and a reduction in CST III and IV throughout the study period.

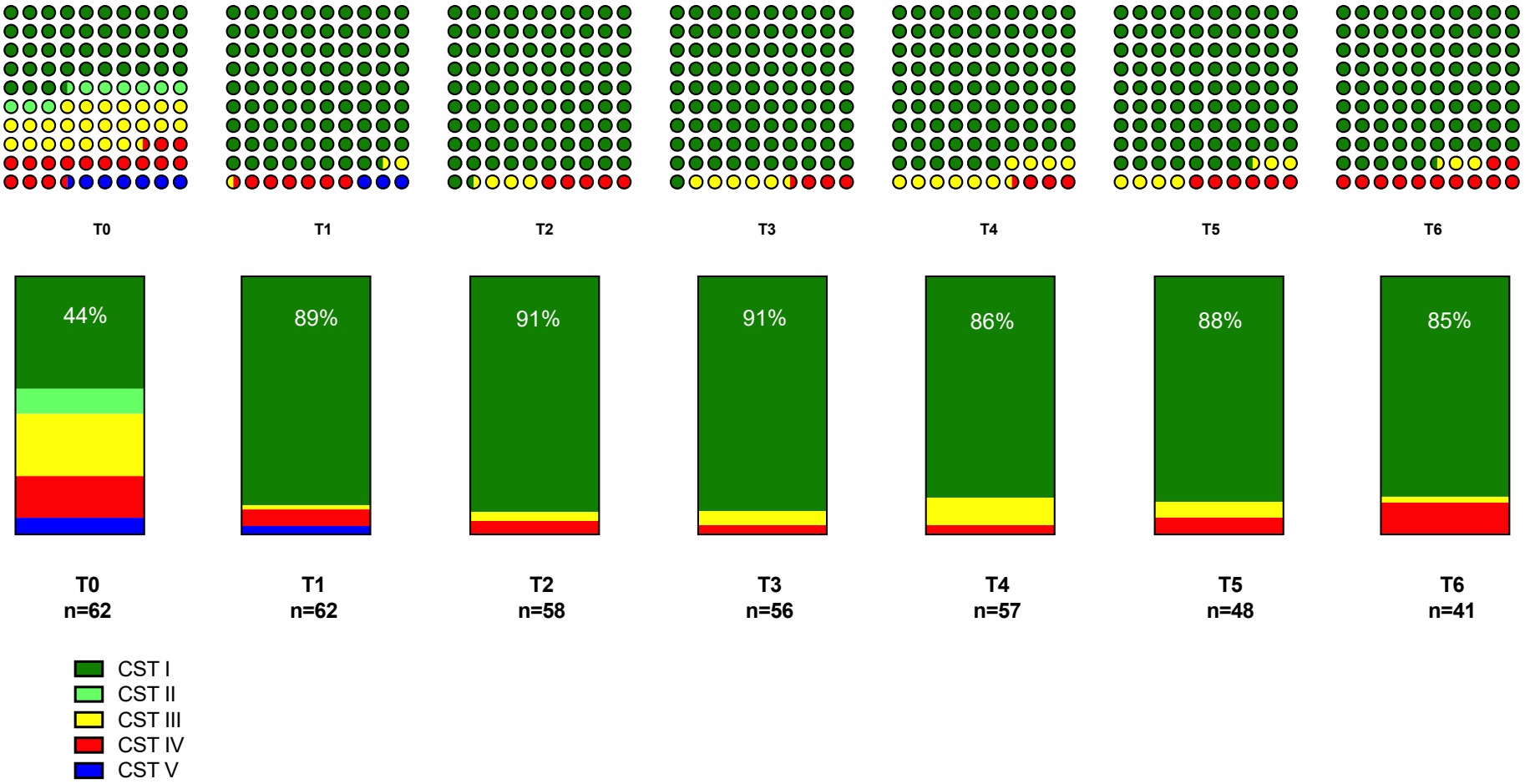


Figure 4.11 – Shifts in CST throughout the study. Throughout the study CST I becomes the over-riding CST. Percentages indicate women with CST I. At T0 44% start with CST I. This increases to 89%, 91%, 91%, 86%, 88% and 85% at T1-T6 respectively. One-sided Fisher's exact testing was used to compare prevalence of *L. crispatus* dominance (CST I) versus *L. crispatus* non-dominance (CST other) at T1-T6, in comparison to T0 ($p<0.0001$).

4.3.6 Assessment of total bacterial load and displacement of pathogens in response to LACTIN-V

Total bacterial load across the study period remained stable (Figure 4.12). The median bacterial load at baseline was 204,680,553 copies/swab compared to 319,521,197 (T0, p=0.0856); 345,989,992 (T1, p=0.0825); 271,434,805 (T3, p=0.7162); 261,232,257 (T4, p>0.9999); 261,232,257 (T5, p>0.9999); 485,886,276 (T6, p= 0.1598).

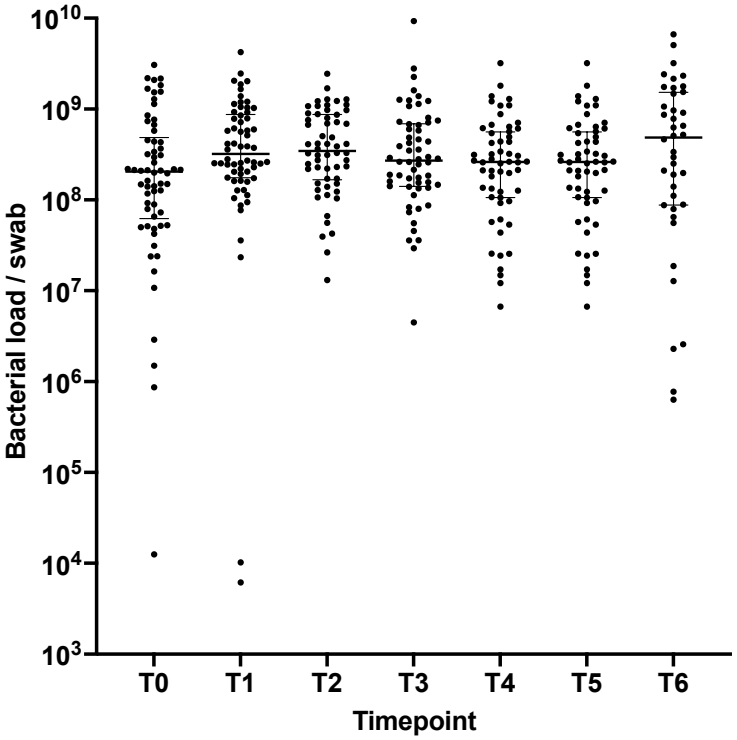


Figure 4.12 – Total bacterial load throughout study. From T0 to T6 the total bacterial load did not significantly change despite LACTIN-V administration. Median values were 204,680,553; 319,521,197; 345,989,992; 271,434,805; 261,232,257; 261,232,257; 485,886,276 at T0-T6 respectively (p>0.05 at all timepoints). Median values with interquartile range are shown. Statistics: Kruskal-Wallis.

At each timepoint a high vaginal swab was collected and sent to the Imperial College NHS Trust laboratory for the identification of GBS, BV and yeasts (Chapter 2, section 2.2.3). Figures 4.13-4.15 represent the observed HVS results for women reported as being positive for GBS, BV and yeasts at least once during the study.

Twelve women (19%) tested positive for GBS on HVS sampling on at least one timepoint (Figure 4.13). T5 (36^{+0}) and T6 (delivery) represent the clinically relevant timepoints regarding GBS status. Of these timepoints taken closest to delivery, GBS was detected in just four women (6%).

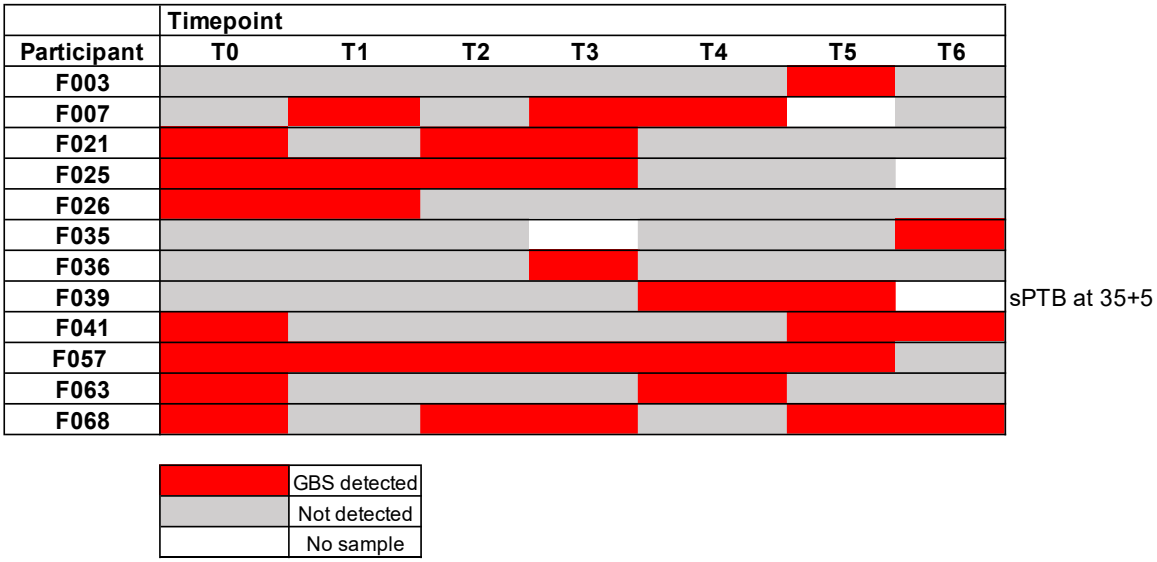


Figure 4.13 – GBS diagnosis using culture and MALDI-TOF (NHS Laboratory). The detection of GBS across the study is shown. Twelve women (19%) out of 62 participants tested positive for GBS at some point during the study period. By T5-T6, eight women (67%) showed clearance of GBS (F003, F007, F021, F025, F026, F036, F057 and F063). Only four women (33%) displayed persistent vaginal GBS colonisation (F035, F039, F041 and F068).

BV was diagnosed in ten (16%) women at least once during the study (Figure 4.14). None of these women received antibiotic treatment for BV as all were asymptomatic and diagnosis was incidental due to frequent sampling. BV was eradicated in eight of these ten women by the end of the study period. Clinical diagnosis of BV using the Hay/Ison criteria was compared to 16S rRNA data. Two women (F041 and F057) with a diagnosis of BV (Hay/Ison) demonstrated *L. iners* abundance on analysis of 16S rRNA results at corresponding timepoints. High diversity was seen in four women (F035, F039, F049 and F059). Interestingly, participant F049 appears to be unresponsive to LACTIN-V as maintains a state of high diversity accompanied by persistent detection of BV (Hay/Ison) throughout the study period, culminating in a sPTB. The remaining participants showed *L. crisptatus* dominance on analysis of 16S rRNA data (F007, F023, F055 and F058) at timepoints where a diagnosis of BV was made.

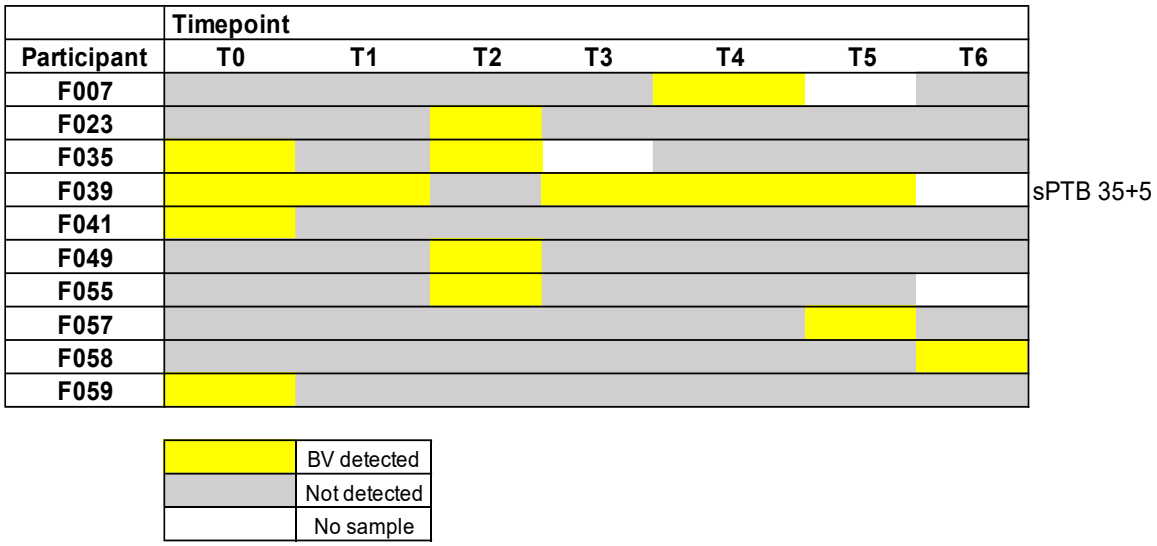


Figure 4.14 – BV diagnosis as determined by the Hay/Ison criteria (NHS Laboratory). The diagnosis of BV across the study is shown. Women did not receive antibiotic treatment for BV clearance. Ten women (16%) of 62 had BV diagnosed at any one timepoint. Towards the study end, spontaneous clearance of BV was seen in eight (80%) women (F007, F023, F035, F041, F049, F055, F057 and F059) and only two (F039 and F058) displayed persistent BV, one of whom delivered preterm.

Yeasts were prevalent among the studied cohort and detected in 23 participants (37%) (Figure 4.15). Women received treatment with clotrimazole pessaries if symptomatic. No change in prevalence of yeasts was observed in response to LACTIN-V administration.

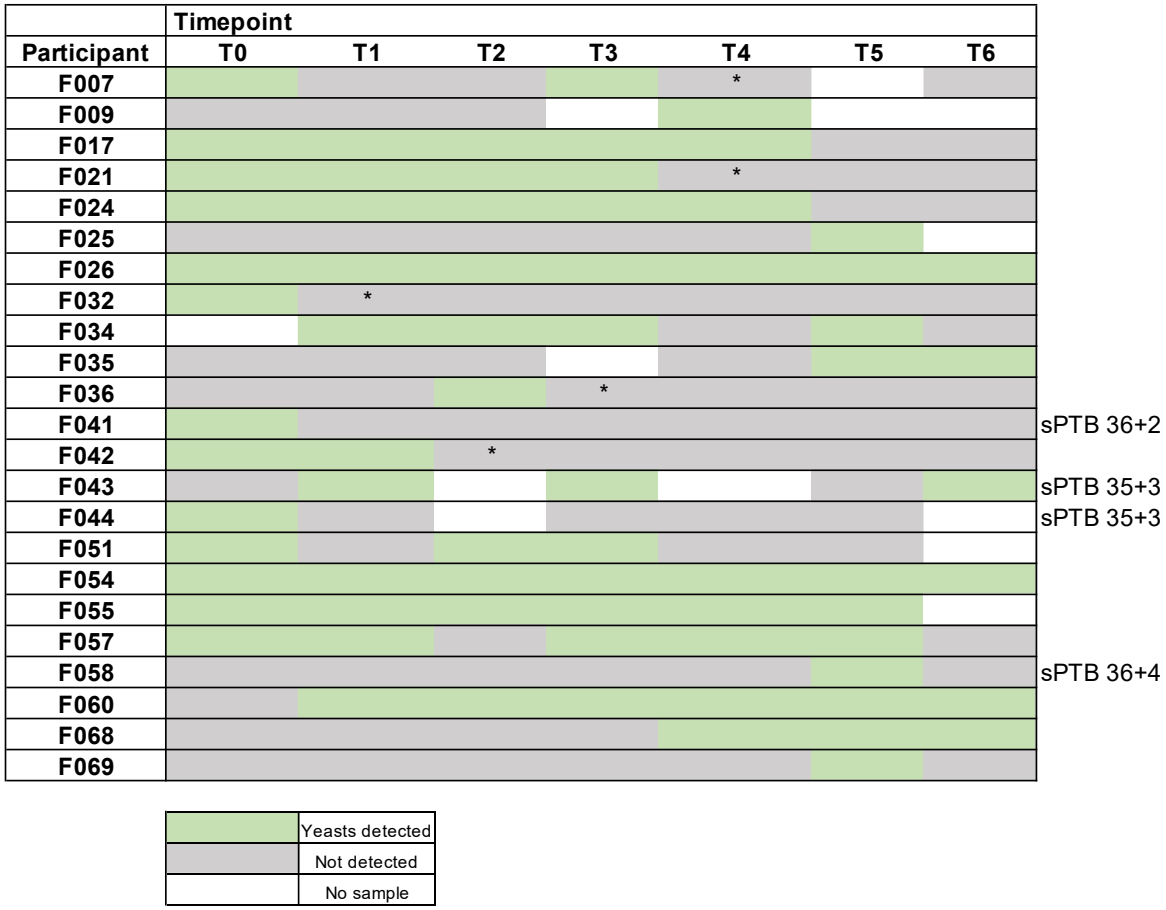


Figure 4.15 – Detection of yeasts as determined by culture (NHS Laboratory). The detection of yeasts across the study is shown. Yeasts were detected in 23 women (37%). *Represents women receiving clotrimazole pessaries for symptomatic treatment.

To further explore the impact of LACTIN-V on pathogen displacement, *L. crispatus* CTV-05 load was assessed according to the presence of GBS, BV and yeasts (Figure 4.16A-C). A lower median *L. crispatus* CTV-05 load was seen in women with GBS (median 54,245 *L. crispatus* CTV-05 load/swab) when compared to women without GBS (median 494,026 *L. crispatus* CTV-05 load/swab), although significance was not reached (p=0.0966) (Figure 4.16A). Women with BV had a significantly lower *L. crispatus* CTV-05 load (p=0.0273). The median *L. crispatus* CTV-05 load was 245 bacteria/swab and 515,252 bacteria/swab for women with and without BV respectively (Figure 4.16B). The median *L. crispatus* CTV-05 load was 32,696 bacteria/swab and 718,344 bacteria/swab for women with and without yeasts detected respectively (Figure 4.16C). Although a lower *L. crispatus* CTV-05 load was observed in women with yeasts, statistical significance was not reached when compared to women without yeasts (p=0.4791).

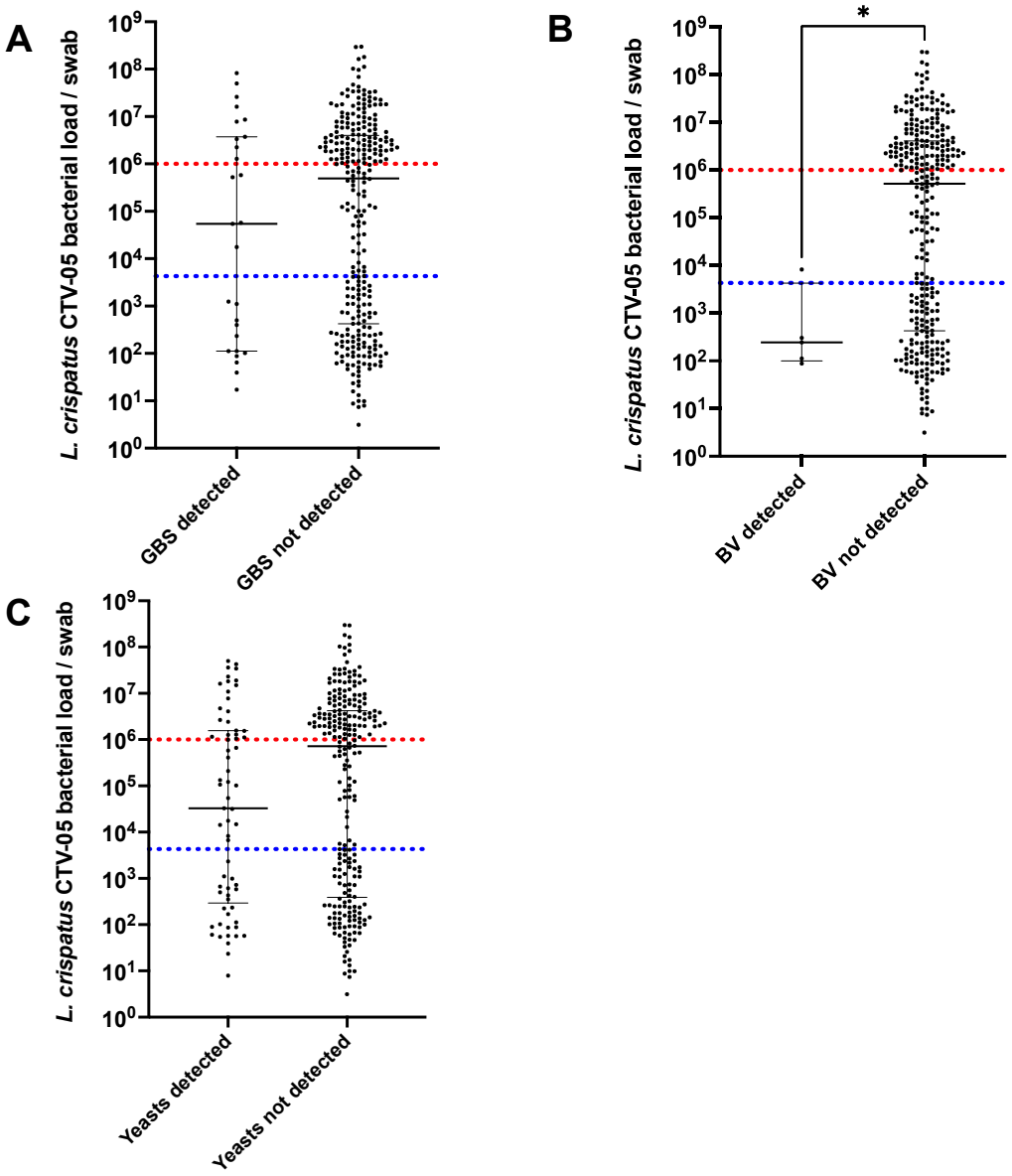


Figure 4.16 - *L. crispatus* CTV-05 load in women testing positive for GBS, BV or yeasts. A) *L. crispatus* CTV-05 load/swab in women with and without GBS identified by culture and MALDI-TOF. The median *L. crispatus* CTV-05 load was lower in women with GBS detected (54,245 *L. crispatus* CTV-05 load/swab) when compared to women without GBS (median 494,026 *L. crispatus* CTV-05 load/swab) (p=0.4791). **B)** *L. crispatus* CTV-05 load/swab in women with (median 245/swab) and without (median 515,252/swab) BV, diagnosed using the Hay/Ison criteria. The load of *L. crispatus* CTV-05 was significantly higher in women without BV, when compared to women with BV (p=0.0273) **C)** *L. crispatus* CTV-05 load/swab in women with and without yeasts. *L. crispatus* CTV-05 load/swab was greater in women without yeasts (median 718,344/swab) when compared to women with yeasts (32,696/swab) (p=0.0966). Statistics: Mann-Whitney U one-tailed testing. Values are presented as median values with IQR. The red line indicates a *L. crispatus* CTV-05 load/swab of 10^6/swab. The blue line indicates a *L. crispatus* CTV-05 load of 4277/swab.

4.3.7 Factors influencing *L. crispatus* CTV-05 colonisation dynamics

Starting CST

A total of 20 women were found to have no detectable *L. crispatus* CTV-05 at T4 and were thus deemed non-colonisers. A significantly higher proportion of these women (15/20) had a *L. crispatus* dominant state observed at T0 compared to colonisers, who were more likely to have a baseline CST III or IV (p=0.023, Fisher's exact test).

Ethnicity

Ethnicity as a confounder was also explored. The studied population represented women from a mostly White (60%) ethnic background. Representations from other ethnicities included eight (13%) women from Black, 11 (18%) from Asian and six (9%) from mixed/other ethnic groups. The prevalence of CST I at baseline in the whole cohort was 27 women (44%). The prevalence of CST I at T0 in White, Black, Asian and other/mixed ethnic groups was 53%, 14%, 36% and 33% respectively (Figure 4.17). Black women had the lowest proportion of baseline CST I at T0. By T4, a shift to CST I was seen in the majority of women from all ethnicities (Figure 4.17), suggesting that ethnicity does not limit LACTIN-V response. The prevalence of *L. crispatus* dominance increased at T4 for women across all ethnicities. This was statistically significant for the whole cohort ($p<0.0001$ and White women ($p<0.0001$), but not for Black (p=0.1515), Asian (p=0.0799) or other/mixed (p=0.1212) women. Colonisation at T4 according to ethnicity was also assessed. Black and Asian women were not more likely to be non-colonisers (p=1, Fisher's exact test). Similarly, White women were not more likely to colonise (p=0.5762).

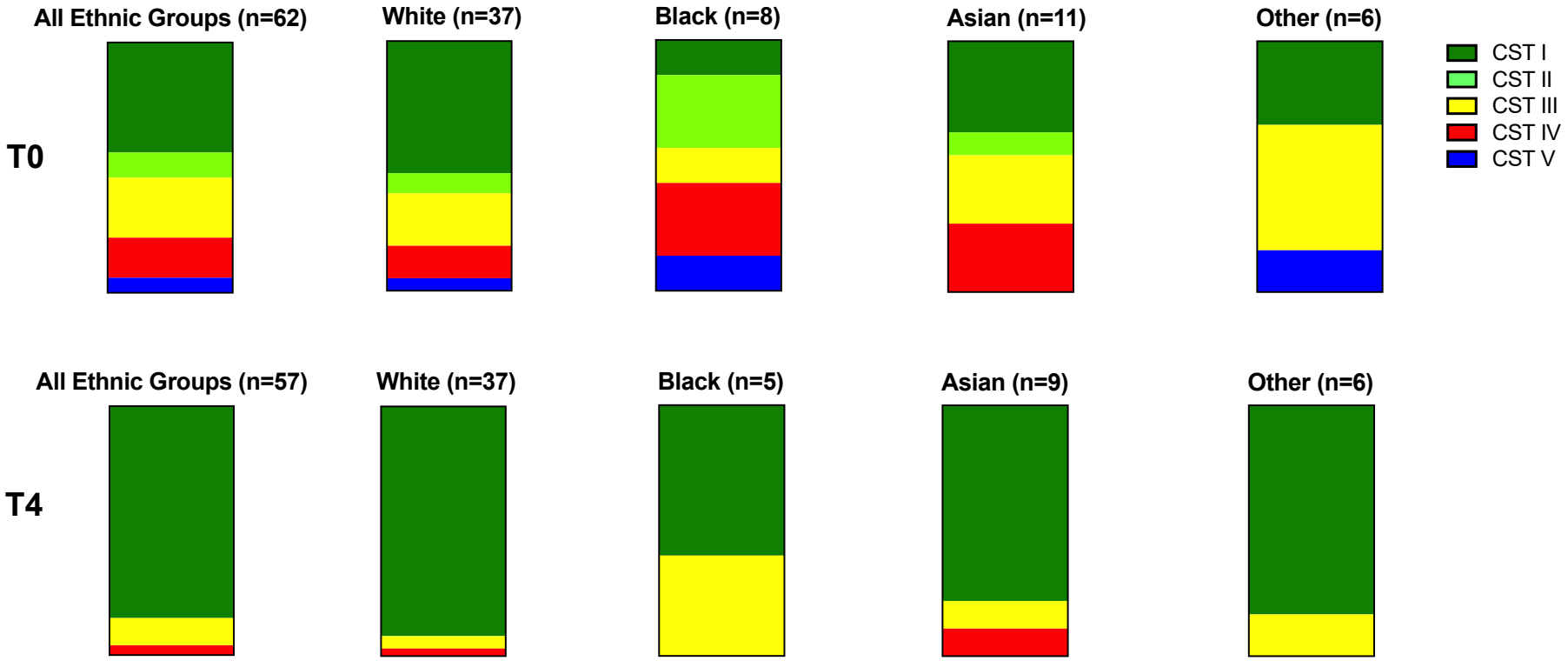


Figure 4.17 – CST dynamics according to ethnicity at T0 and T4. At T0 44% of the whole cohort had CST I observed. When assessed according to ethnicity 53%, 14%, 36% and 33% of White, Black, Asian and other/mixed backgrounds commenced with CST I respectively. At T4 (28^{+0} weeks' gestation) a substantial shift to CST I was seen across all ethnicities. By T4 86% of the whole cohort had CST I observed. This included 92%, 60%, 78% and 83% of White, Black, Asian and other/mixed ethnicities. The increase in *L. crispatus* dominance was significant for the whole cohort ($p<0.0001$) and White (<0.0001) women. A shift to *L. crispatus* dominance was observed for Black ($p=0.1515$), Asian ($p=0.0799$) and other/mixed ($p=0.1212$) women. Statistics: Fisher's exact test.

Antibiotics

Thirteen women received antibiotics between 14^{+0} (T0) and 36^{+0} (T5) weeks' gestation. Nine women received antibiotics for UTI treatment, the most common indication for antibiotics in this cohort. Other indications for antibiotics, included infections of the chest (n=2), ear (n=1) and tooth (n=1). One woman required antibiotic clearance of group C/G streptococcus incidentally isolated in a vaginal swab, under the advice of a microbiologist. Five women received antibiotics for UTI treatment after 36^{+0} weeks and were not included, as subsequent vaginal samples for comparison purposes were limited. Three women receiving erythromycin for PPROM were also excluded from this analysis. The *L. crispatus* CTV-05 load in 13 patients requiring antibiotics is presented in Figure 4.18 with a description of each patient's clinical journey provided below:

- Participant F005 (Figure 4.18A) took nitrofurantoin between T3 and T4. A high *L. crispatus* CTV-05 load/swab is seen at T2, after which the *L. crispatus* CTV-05 load declines. At T3, this participant required antibiotics for a UTI, following which the *L. crispatus* CTV-05 load/swab attempts to increase, but a low *L. crispatus* CTV-05 is acquired. The *L. crispatus* CTV-05 load does not surpass 4277 bacteria/swab following antibiotics.

- Participant F007 (Figure 4.18B) appears to be a non-responder to LACTIN-V. Throughout the study, the *L. crispatus* CTV-05 load is persistently low and appears unaffected nitrofurantoin.

- Participant F013 (Figure 4.18C) may represent a LACTIN-V non-responder as a low *L. crispatus* CTV-05 load is seen across the study. Cefalexin does not impact the *L. crispatus* CTV-05 load, although a slight decline is seen.

- Patient F024 (Figure 4.18D) a high *L. crispatus* CTV-05 load is initially seen at T1 and T2. Between T2 and T3, the *L. crispatus* CTV-05 load falls, following which cefalexin is required for a UTI. After cefalexin use, the *L. crispatus* CTV-05 load continues to fall without recovery.

- Participants F033 and F041 (Figure 4.18E-F) administer cefalexin and amoxicillin between T1-T3 and amoxicillin between T2-T3 respectively. In both women, a high *L. crispatus* CTV-05 load above 10^6 bacteria/swab is maintained despite antibiotic use. At T6, samples collected at delivery, the *L. crispatus* CTV-05 load declines for participant F033.

- For participant F045 (Figure 4.18G), cefalexin between T4 and T5 leads to a slight decline in *L. crispatus* CTV-05 load below 10^6 bacteria/swab at T5.

- F053 (Figure 4.18H) initially has a high *L. crispatus* CTV-05 load at T1. However, after use of amoxicillin between T1 and T2, the *L. crispatus* CTV-05 load reduces without recovery.

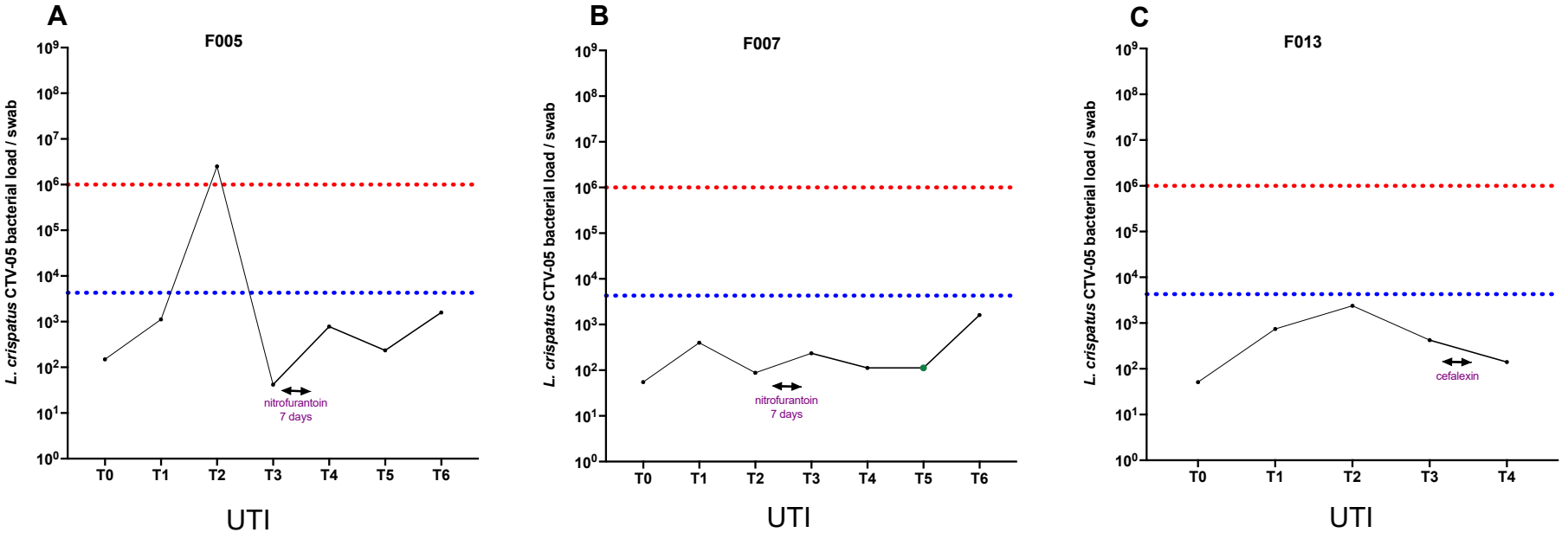
- F055 (Figure 4.18I) appears to be a non-responder, with a slight increase in *L. crispatus* CTV-05 load seen following LACTIN-V use, without attaining a high *L. crispatus* CTV-05 load. Following cefalexin use between T2 and T3, the *L. crispatus* CTV-05 load reduces.

- F057 (Figure 4.18J) responds well to the loading dose. The *L. crispatus* CTV-05 load surpasses 10^6 copies/swab by T1 which is maintained to T3. Antibiotic use after T3, leads to a subsequent steep decline in *L. crispatus* CTV-05 load.

- F063 and F071 (Figure 4.18K-L) display good initial responses to LACTIN-V, with a high *L. crispatus* CTV-05 load measured, reaching $>10^6$ copies/swab. Antibiotic use in these two patients, including penicillin V use between T2-T3 for F063 and intravenous co-amoxiclav once only between T1-T2 in F073, led to a slight reduction in *L. crispatus* CTV-05 load. However, overall high levels of *L. crispatus* CTV-05 load in these two women were maintained.

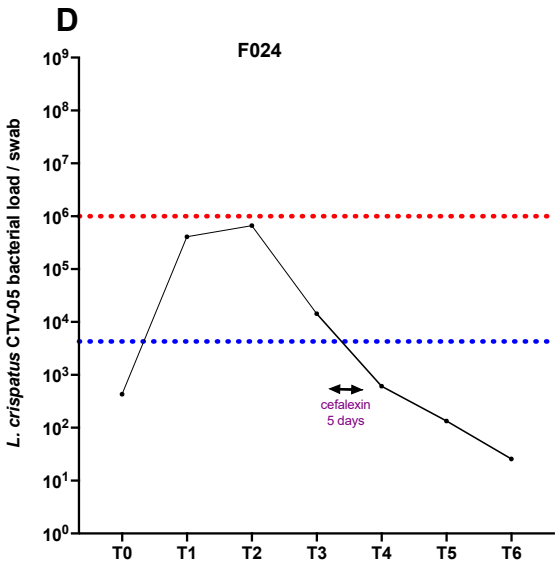
- Participant F073 (Figure 4.18M) also displayed a good response to LACTIN-V, with high *L. crispatus* CTV-05 load measured $>10^6$ copies/swab at all timepoints following T0. Despite the use of two courses of antibiotics, amoxicillin and cefalexin, between T4-T5, a high *L. crispatus* CTV-05 load was maintained.

Figure 4.18 continued

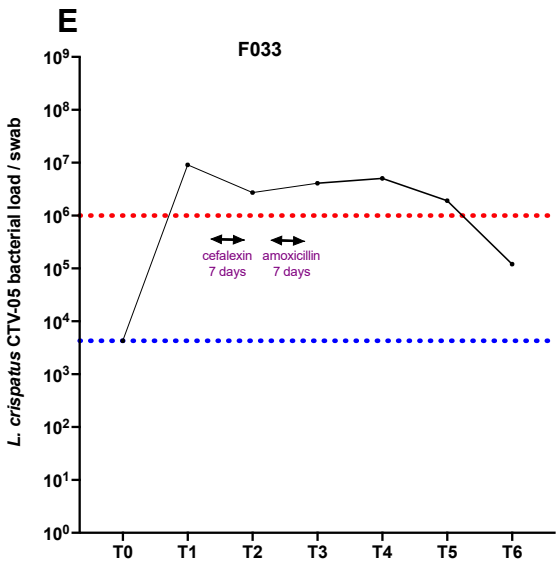


Figures 4.18A-M - *L. crispatus* CTV-05 load/swab in 13 participants using antibiotics during the study. Participant study ID numbers are included (e.g. F005) as is the class, duration and timing of administered antibiotics. All antibiotics were oral, unless specified otherwise. Trends in *L. crispatus* CTV-05 bacterial load are recorded at each timepoint. If results were unavailable, the value from the preceding timepoint was included for visual purposes (green plots). The red line indicates a *L. crispatus* CTV-05 load/swab of 10^6/swab. The blue line indicates a *L. crispatus* CTV-05 load of 4277/swab.

Figure 4.18 continued

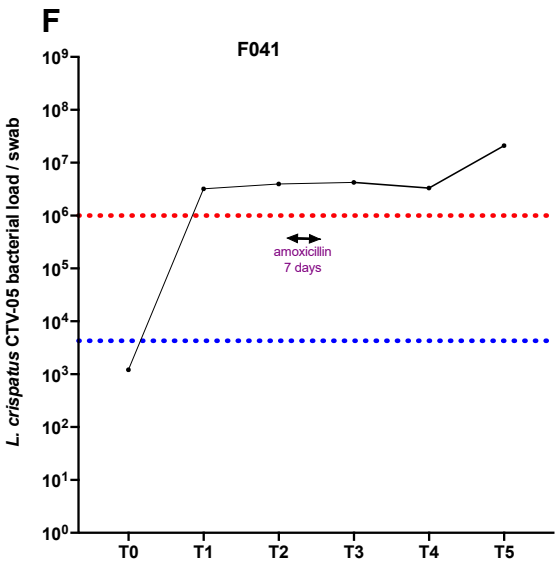


UTI



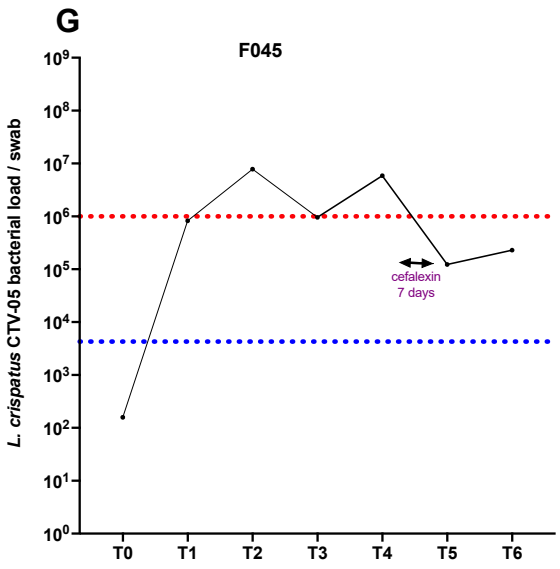
Cefalexin for UTI

Amoxicillin for chest infection

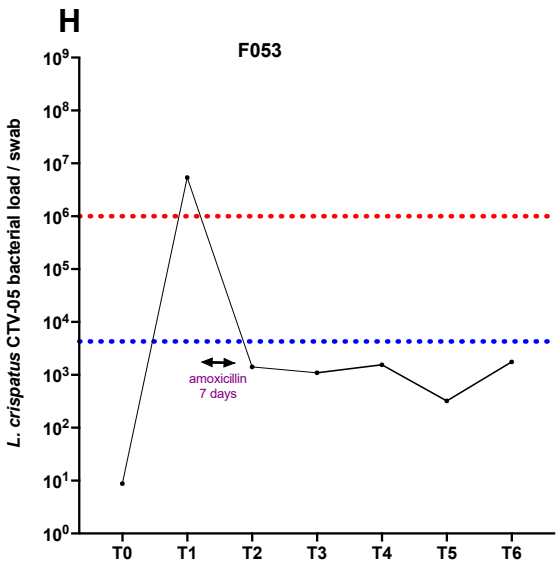


Chest infection

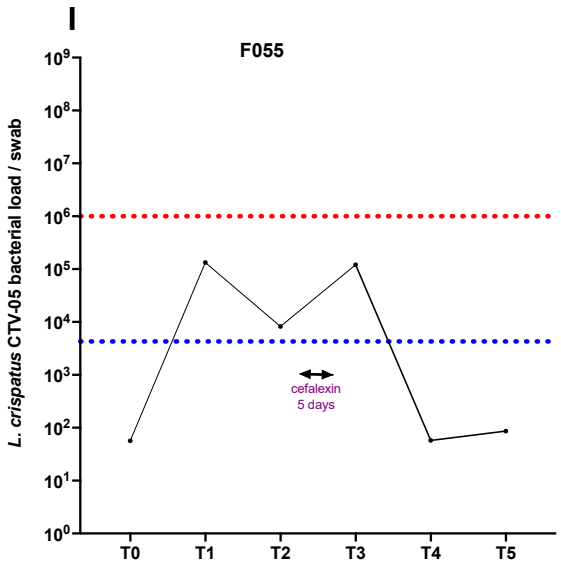
Figure 4.18 continued



UTI

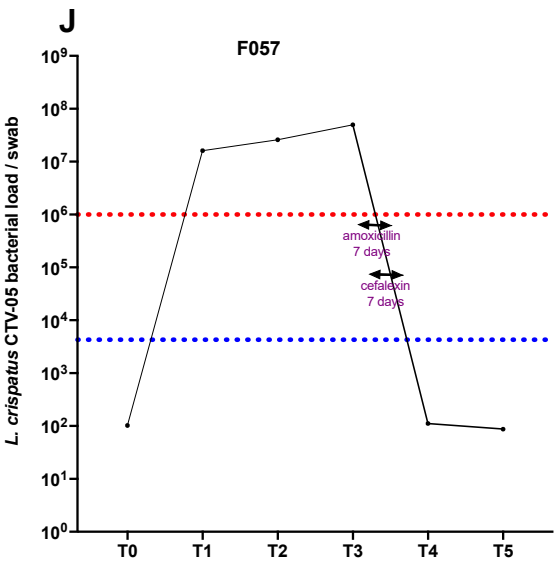


Ear infection



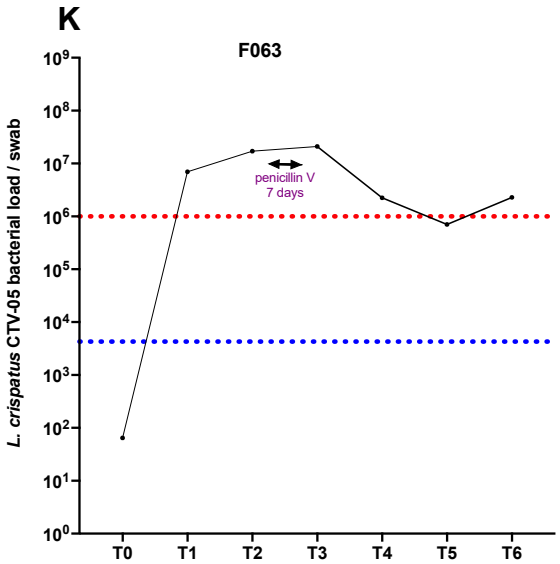
UTI

Figure 4.18 continued

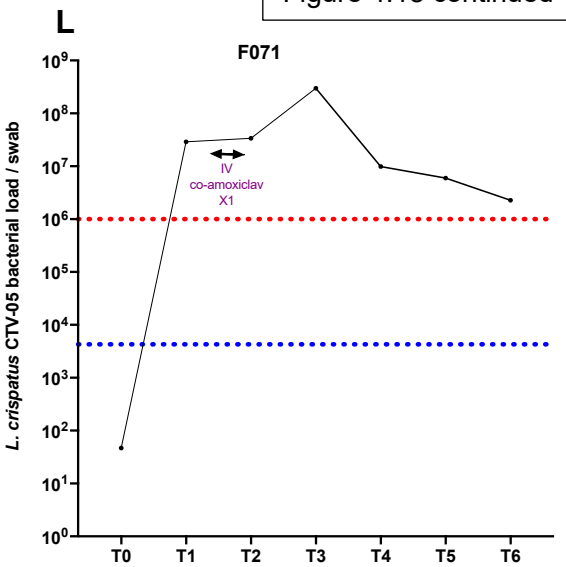


Amoxicillin for tooth infection

Cefalexin for UTI

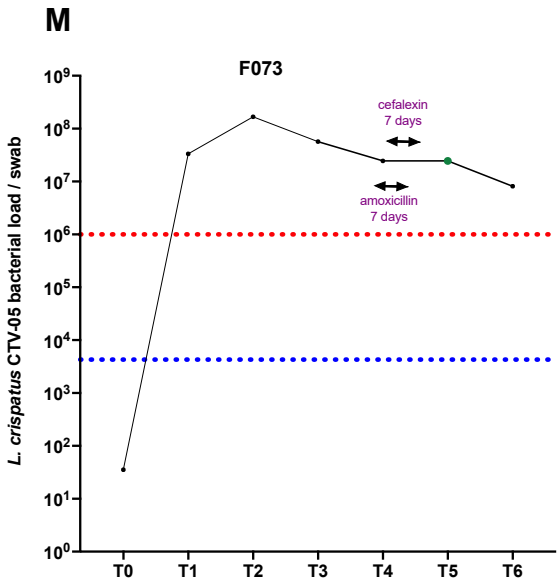


Vaginal group C/G streptococcus



During cerclage (1x intravenous dose)

Figure 4.18 continued



Amoxicillin for UTI

Cefalexin for UTI

4.3.8 LACTIN-V response in women delivering preterm

In total, nine women experienced preterm delivery in the FLIP-1 cohort, including one MTL at 19^{+4} weeks, two PTBs <34 weeks and six PTBs <37 weeks (Figure 4.19).

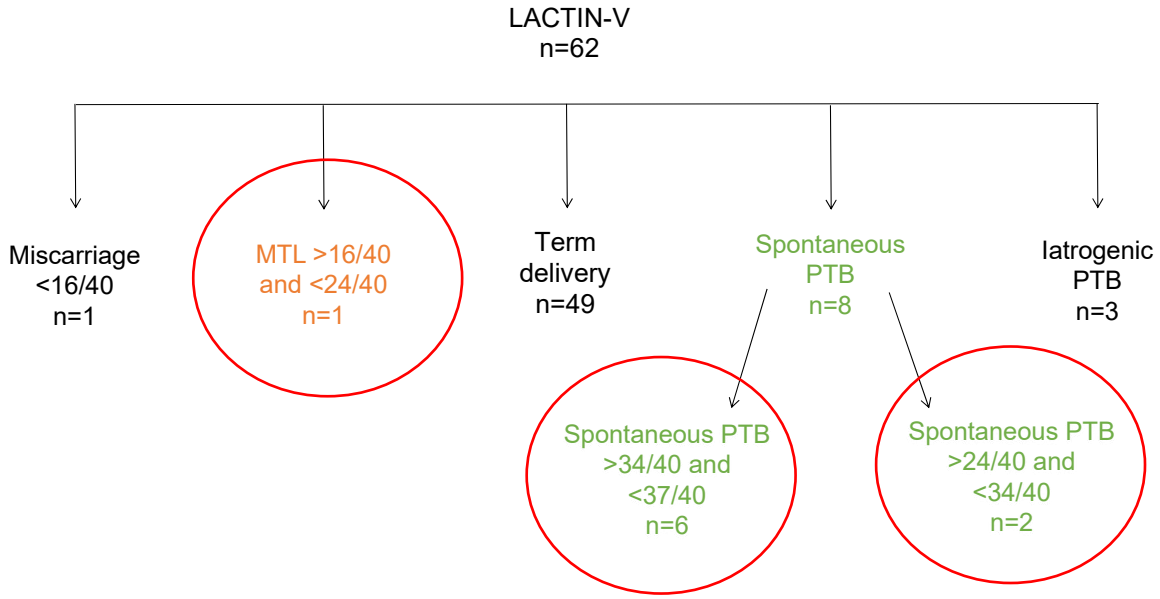
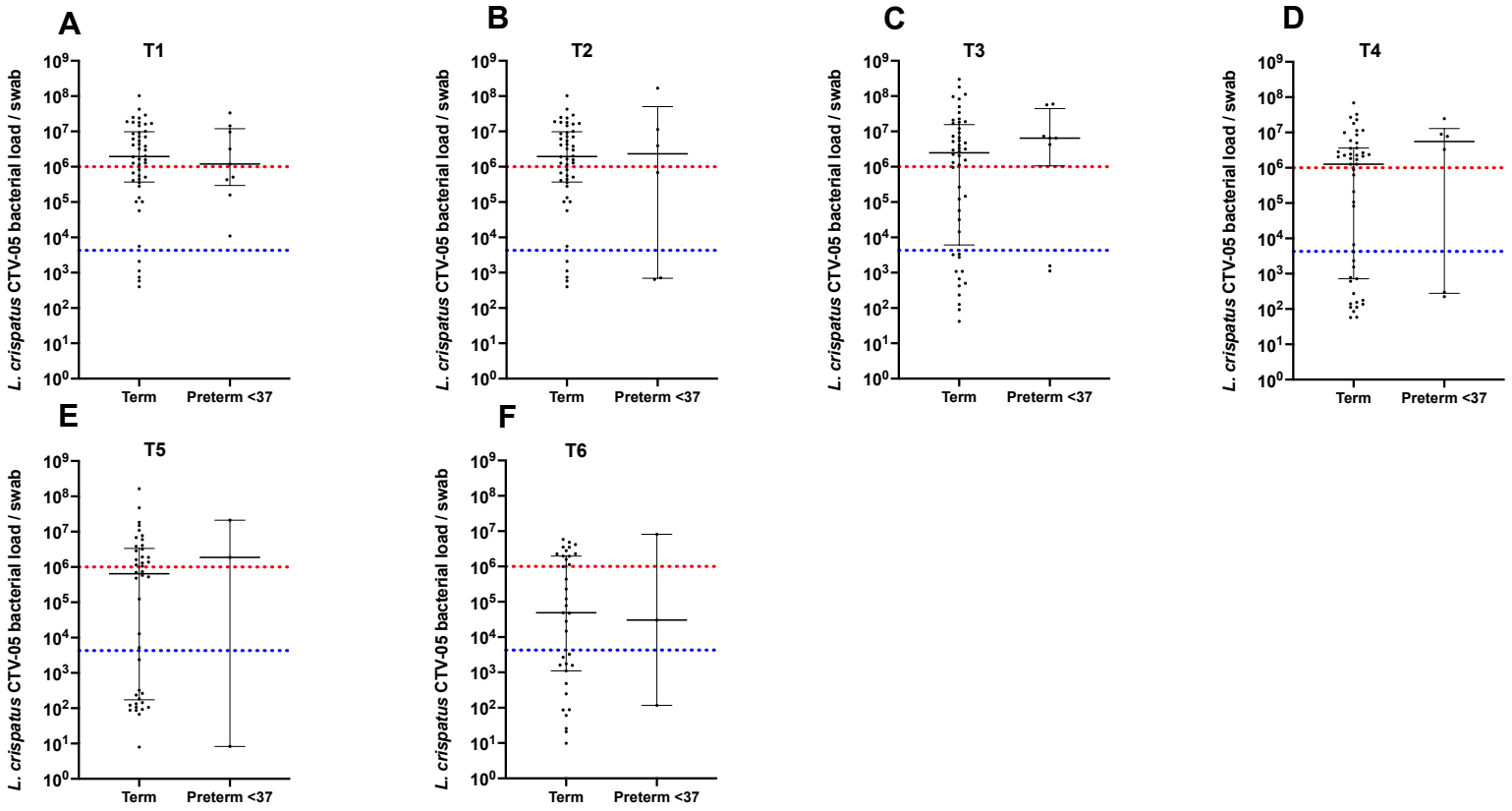


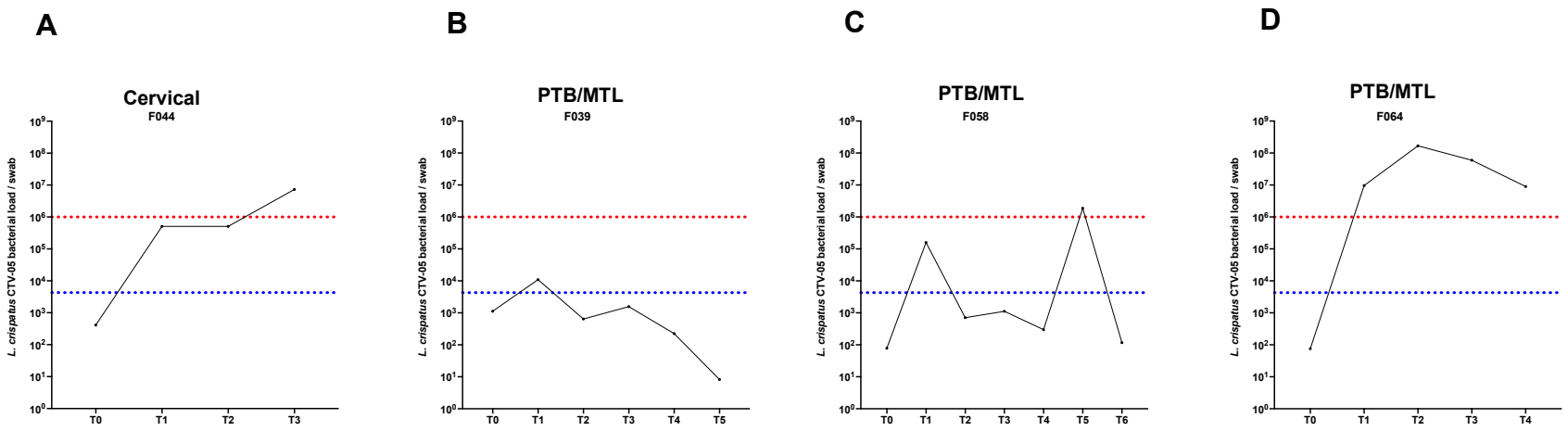
Figure 4.19 - Clinical outcomes for 62 women using LACTIN-V. Analysis of women delivering preterm are circled and included one woman experiencing a MTL and eight women with a sPTB between $<37^{+0}$ weeks. This included two women delivering prior to 34^{+0} weeks of pregnancy.

Firstly, the *L. crispatus* CTV-05 load in women delivering term versus preterm was assessed (Figure 4.20). No difference in *L. crispatus* CTV-05 load was observed between women delivering term versus preterm at any of the timepoints ($p>0.05$).

Secondly, *L. crispatus* CTV-05 load was examined longitudinally across the study period for individual participants (Figure 4.21A-I). Despite delivering preterm, most women maintained high levels of *L. crispatus* CTV-05 across the study period with seven women attaining HBLC, as represented by surpassing the red line. Only two participants (F039 and F058) show a poor response to LACTIN-V with a low *L. crispatus* CTV-05 load measured throughout the study.

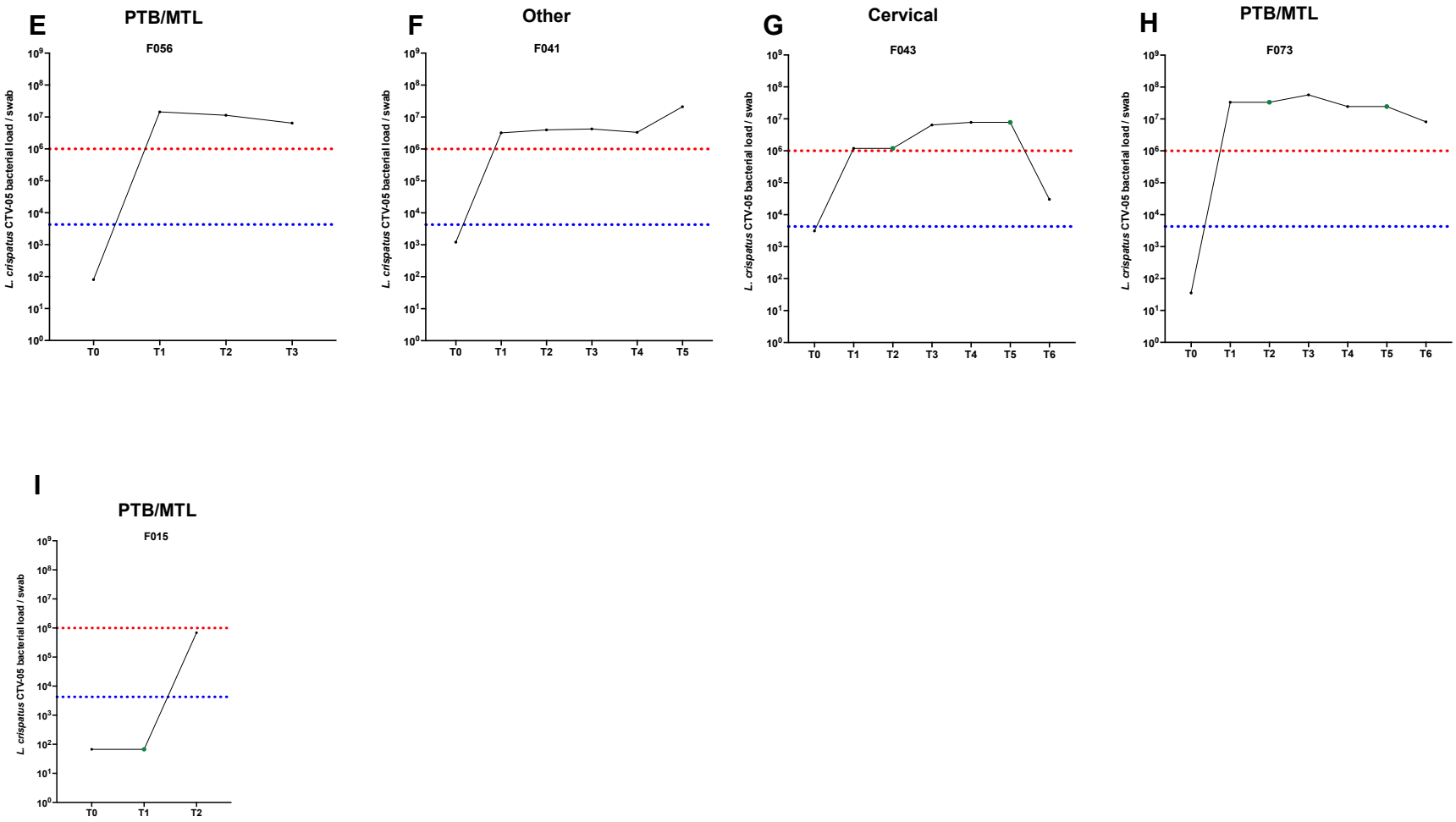


Figures 4.20A-F – *L. crispatus* CTV-05 bacterial load in women delivering term versus preterm (<37 weeks) at T1-T6. A) Median load at T1 in term (1,949,250 load/swab) versus preterm (1,189,645 load/swab) (p=0.8573). **B)** Median load at T2 in term (1,949,250 load/swab) versus preterm (2,317,603 load/swab) (p=0.7988). **C)** Median load at T3 in term (2,475,343 load/swab) versus preterm (6,428,287 load/swab) (p=0.3759). **D)** Median load at T4 in term (1,266,318 load/swab) versus preterm (5,530,600 load/swab) (p=0.3708). **E)** Median load at T5 in term (640,686 load/swab) versus preterm (1,871,223 load/swab) (p=0.7804). **F)** Median load at T6 in term (48,927 load/swab) versus preterm (30,247 load/swab) (p=0.8385). *L. crispatus* CTV-05 load did not differ between women delivering term or preterm. Statistics was performed using one-tailed Mann-Whitney U testing. Values are presented as median values with IQR. The red line indicates a *L. crispatus* CTV-05 load/swab of 10^6/swab. The blue line indicates a *L. crispatus* CTV-05 load of 4277/swab.



Figures 4.21A-I - *L. crispatus* CTV-05 load from T0-T6 in women delivering preterm. The participant study ID numbers are included (e.g. F005) as is the risk factor for sPTB. Most participants display high levels of *L. crispatus* CTV-05 throughout including **(A)** F044, **(D)** F064, **(E)** F056, **(F)** F041, **(G)** F043, **(H)** F073, **(I)** F015. Only two participants had a low *L. crispatus* CTV-05 load; **(B)** F039 and **(C)** F058. If results were unavailable, the value from the preceding timepoint was included for visual purposes (green plots). The red line indicates a *L. crispatus* CTV-05 load of 10^6/swab. The blue line indicates a *L. crispatus* CTV-05 load of 4277/swab.

Figure 4.21 continued



Of the nine women delivering preterm, six switched to a *L. crispatus* dominant state after commencement of LACTIN-V (Figure 4.22). Three women had CST I at baseline. By T3, six women switched to CST I. By the end of the study, only two women displayed a non-*L. crispatus* dominant state.

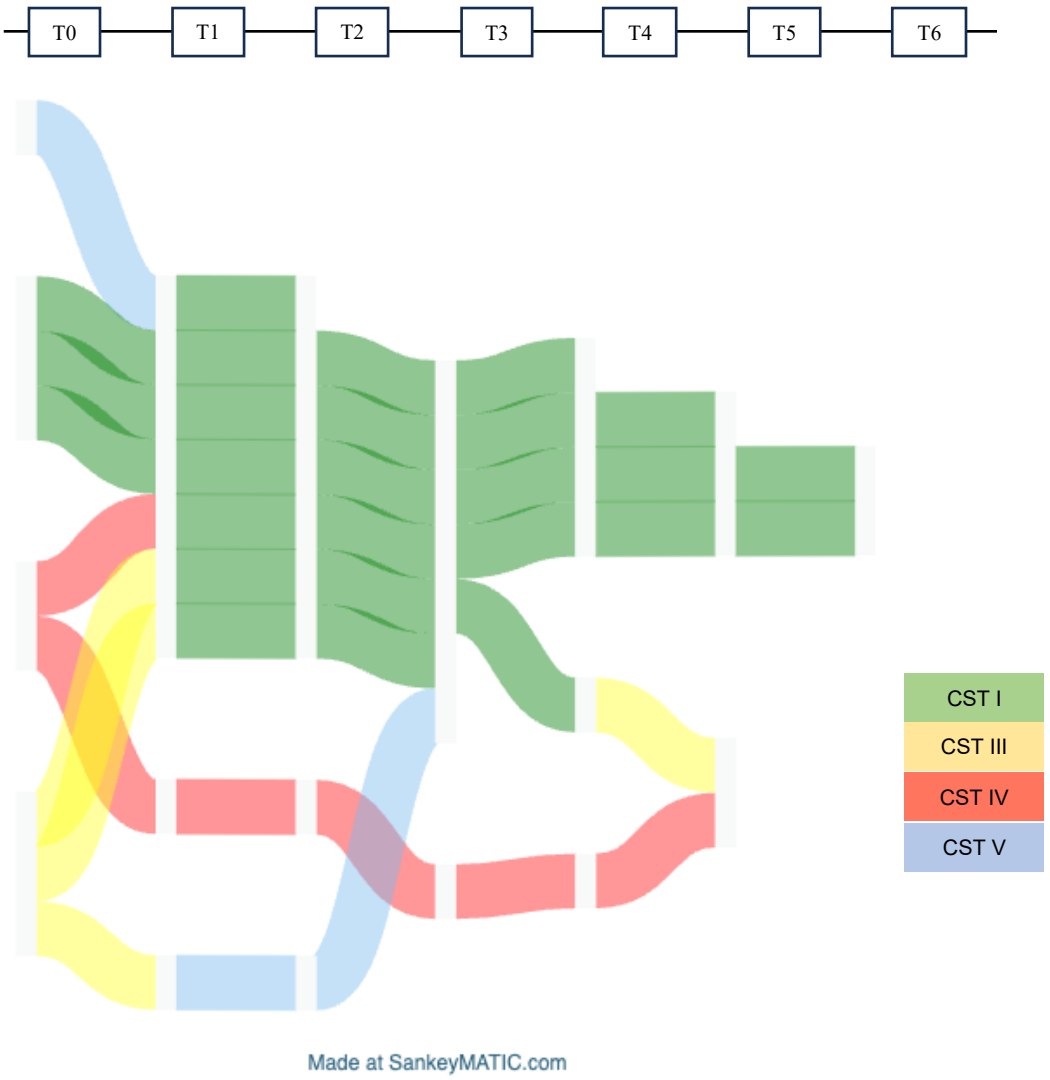


Figure 4.22 – Sankey diagram showing CSTs in nine women delivering preterm. CST II is not included as no participants were of CST II. At T2 results for 3 participants were missing and at T5 one result was missing. In these cases, CSTs from the preceding timepoint were used to enable visualisation. At T0 three women had CST I observed. A switch to CST I occurred for most women, by the end of the study only two women did not switch to CST I (made using SankeyMATIC.com).

The alpha diversity at T4 in women delivering preterm was also explored. Figure 4.23 shows similar alpha diversity in women delivering term and spontaneously preterm, with no statistical difference seen (p=0.2374).

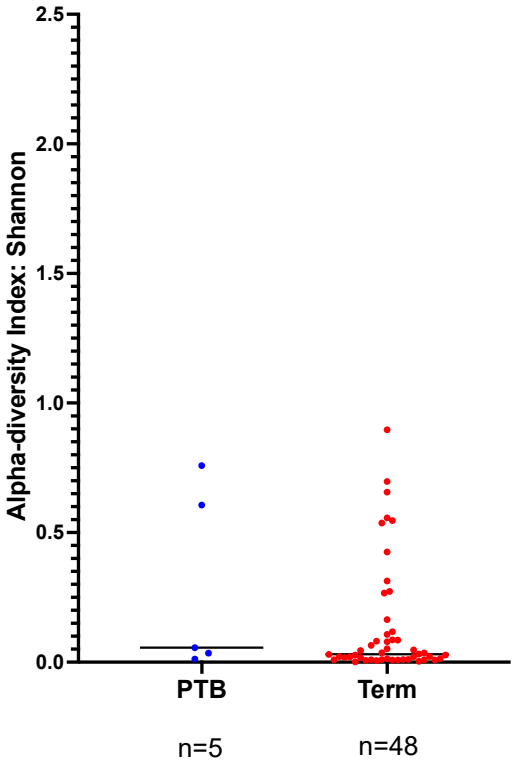


Fig 4.23 – Assessment of Alpha Diversity (Shannon Index) in women delivering spontaneously preterm and term. The Shannon Index at T4 (28^{+0} weeks) did not differ in women delivering spontaneously preterm and term, p=0.2374. Statistics: Mann-Whitney U test.

4.3.9 Assessment of potential *L. crispatus* CTV-05 colonisation of placenta and fetal membranes

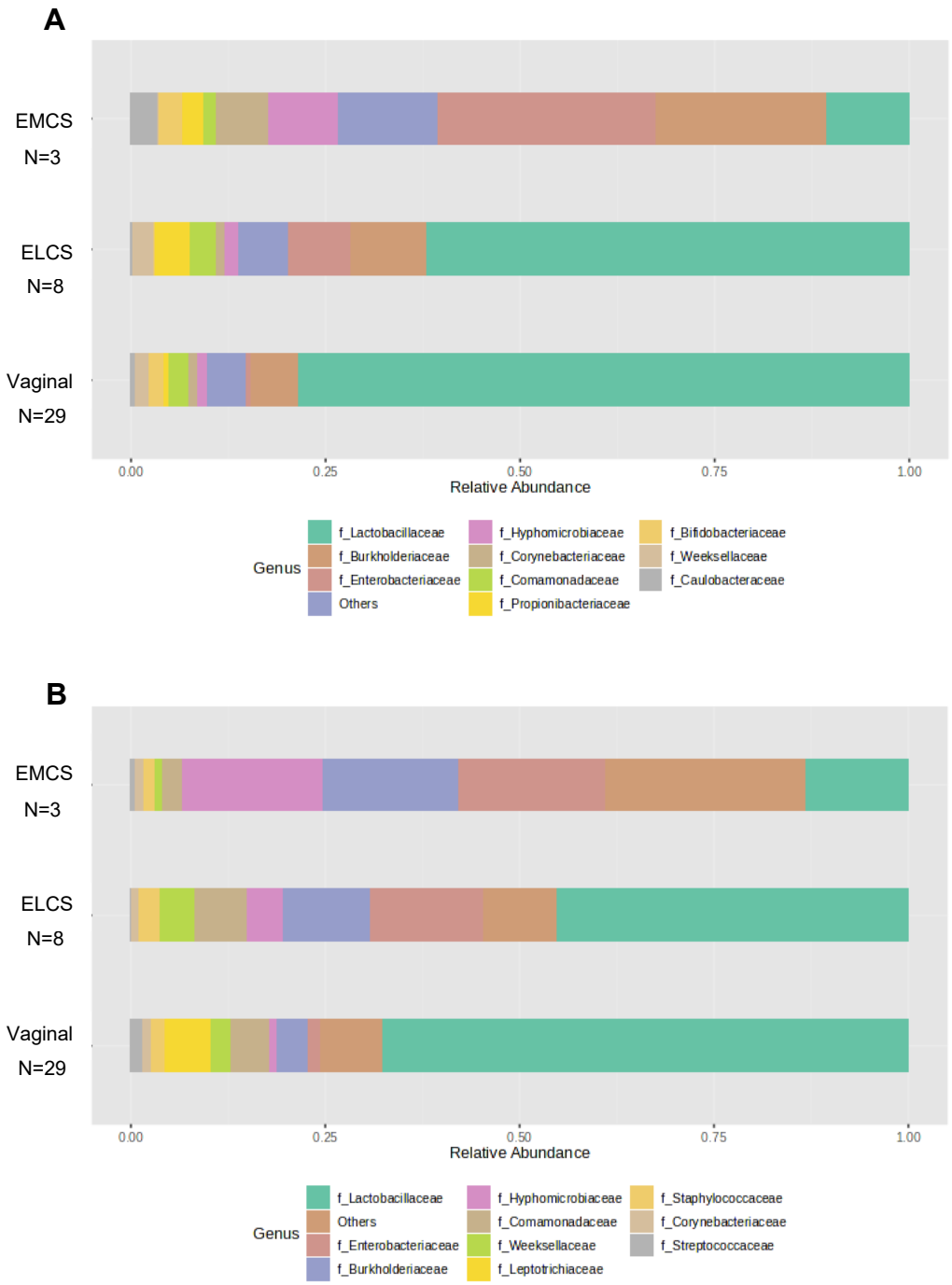
Delivery samples were collected from 40 women and included both placental and fetal membrane sampling. Twenty-nine women (73%) delivered vaginally, eight (20%) had an elective caesarean-section (ELCS) and 3 women (7%) had an emergency caesarean-section (EMCS). Extraction of bacterial DNA was confirmed by performing PCR amplification using 16S rRNA universal primers in all placenta and fetal membrane samples. Negative controls included PCR reagents.

Placenta

Metataxonomic profiling of placental samples generated a total of 661,765 reads. The average read count was 16,544 per sample ranging from 546 to 35,624. A total of 164 ASVs were detected more than twice. The top ten genera identified in placental tissue are summarised in Figure 4.24A. The most abundant genera in placenta obtained after vaginal delivery and ELCS was *Lacticaseibacillae*.

Fetal membranes

Fetal membranes obtained at delivery (n=40) were also analysed generating a total of 592,061 reads. The average read count was 14,801 per sample ranging from 3,656 to 41,876. 187 ASVs were detected more than twice. The top ten genera identified in fetal membranes are summarised in Figure 4.24B. Similarly to analysis of placenta, the most abundant genera following vaginal delivery was *Lacticaseibacillae,* which was also the most abundant genus in fetal membranes obtained at ELCS.



Figures 4.24 A-B - The top ten most abundant genera in delivery samples. Results are presented for 40 women according to mode of delivery including EMCS (n=3), ELCS (n=8) and vaginal delivery (n=29). **A)** The top ten most abundant genera in placenta. The most abundant genus in placenta was *Lacticaseibacillae* in women delivering both vaginally and by ELCS. **B)** The top ten most abundant genera in fetal membranes. The most abundant genus in both placenta and fetal membrane samples was *Lacticaseibacillae* in women delivering vaginally or by ELCS.

L. crispatus CTV-05 detection in delivery samples was defined as either having an amplicon identified using targeted PCR or the presence of ASV-2. Results are described according to mode of delivery.

Vaginal delivery (n=29)

Of the 29 women delivering vaginally, 12 women (41%) had *L. crispatus* CTV-05 detected in placenta and/or fetal membranes. *L. crispatus* CTV-05 was detected in the placentas of 9 women (31%) and the fetal membranes of 11 women (38%). Eight participants (28%) had *L. crispatus* CTV-05 detected in both placenta and fetal membranes.

ELCS (n=8)

Only one woman delivering by ELCS had *L. crispatus* CTV-05 detected placenta. Although this participant demonstrated an amplicon with targeted PCR, ASV-2 was not detected. *L. crispatus* CTV-05 was not detected in the fetal membranes in any women delivering by ELCS.

EMCS (n=3)

In women requiring an intra-partum EMCS, *L. crispatus* CTV-05 was detected in the fetal membranes of three women. Two of these women also had *L. crispatus* CTV-05 detected in placenta.

4.3.10 Assessment of the neonatal gut microbiome

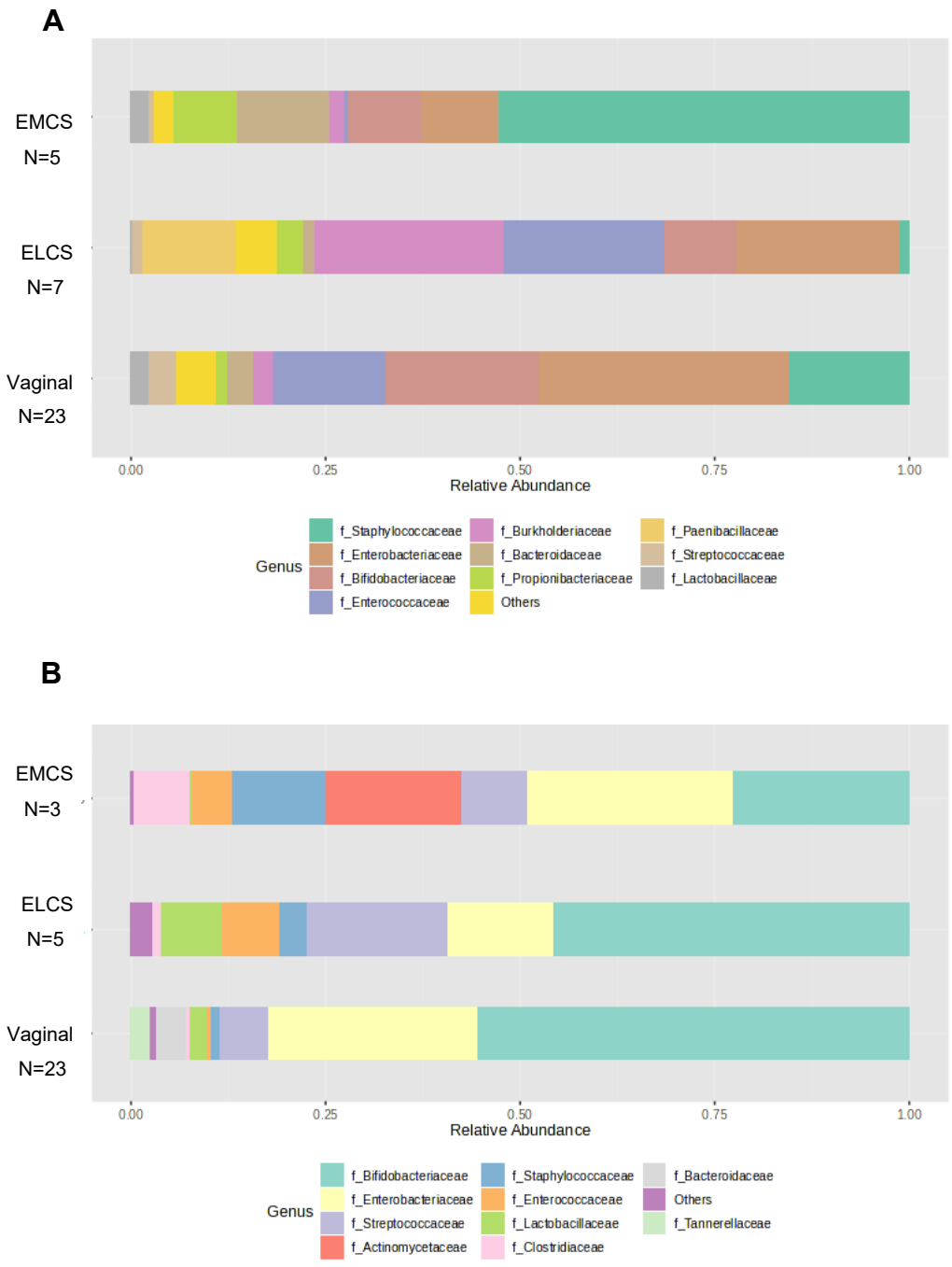
First-pass meconium was obtained from 35 participants and day 14 neonatal stool was obtained from 31 new-borns. Matched meconium and neonatal stool samples were available for 29 participants.

Meconium

Metataxonomic analysis of meconium samples (n=35) generated a total of 814,174 reads. The average read count was 23,262 per sample ranging from 7,260 to 40,664. 184 ASVs were detected more than twice. The top ten genera identified in meconium are summarised in Figure 4.25A. Meconium was obtained from 23 women delivering vaginally, 7 by ELCS and 5 by EMCS. For women delivering vaginally, the most abundant genus in meconium included *Enterobacteriaceae*, followed by *Bifidobacteriaceae*. The most abundant genera identified in meconium following ELCS included *Burkholderiaceae*, *Enterococcaceae* and *Enterobacteriaceae*. The most abundant genera identified following EMCS included *Staphylococcaceae*.

Neonatal stool

Analysis of neonatal stool (n=31) generated a total of 617,169 reads. The average read count was 19,908 per sample ranging from 1,157 to 35,435. 143 ASVs were detected more than twice. The top ten genera are shown in Figure 4.25B. Neonatal stool was obtained from 23 women delivering vaginally, 5 by ELCS and 3 by EMCS. Following vaginal delivery, the most abundant genera in neonatal stool included *Bifidobacteriaceae*, followed by *Enterobacteriaceae*. After ELCS delivery, the most abundant species included *Bifidobacteriaceae,* followed by *Streptococcaceae*. Neonatal stool collected from those born by EMCS showed *Enterobacteriaceae* to be the most abundant genus.



Figures 4.25A-B - The top ten most abundant genera in neonatal gut samples. A) The top ten most abundance genera in meconium (n=35) obtained following vaginal delivery (n=23), ELCS (n=7) and EMCS (n=5). With vaginal delivery the most abundant genera included *Enterobacteriaceae* and *Bifidobacteriaceae*. With ELCS *Burkholderiaceae, Enterococcaceae* and *Enterobacteriaceae* were most abundant. Following EMCS *Staphylococcaceae* was the most abundant genus. **B)** The top ten most abundance genera in day 14 neonatal stool (n=31) obtained following vaginal delivery (n=23), ELCS (n=5) and EMCS (n=3). The most abundant genera following vaginal delivery included *Bifidobacteriaceae* and *Enterobacteriaceae*. With ELCS, *Bifidobacteriaceae* and *Streptococcaceae* were most abundant.

Detection of *L. crispatus* CTV-05 was determined using targeted PCR and ASV-2 presence (Table 4.6). *L. crispatus* CTV-05 was detected in the meconium of three participants (9%) including detection by PCR for two women and detection by ASV-2 in one woman. For two of these women, *L. crispatus* CTV-05 detection was transient as was not identified in subsequent neonatal stool sampling. The third participant did not provide a neonatal stool sample.

An amplicon was detected in only one neonatal stool sample, but not in the matched meconium sample. There was no ASV-2 detection of *L. crispatus* CTV-05 in any of the provided neonatal stool samples.

All four women in Table 4.6 were classified as colonisers, displayed HBLC and delivered vaginally at term.

Table 4.6 - *L. crispatus* CTV-05 detection in meconium (n=35) and neonatal stool (n=31). *L. crispatus* CTV-05 was detected by either PCR or ASV-2 in 3 meconium samples. One participant displayed *L. crispatus* CTV-05 detection in neonatal stool, but not in preceding meconium sampling.

Participant ID	Meconium PCR	Meconium ASV-2	Neonatal stool PCR	Neonatal stool ASV-2
F012	No	Yes	No	No
F029	Yes	No	No	No
F034	Yes	No	Not provided	Not provided
F048	No	No	Yes	No

4.4 Discussion

Comparison of *L. crispatus* CTV-05 detection methods

Three different techniques were used for the detection of *L. crispatus* CTV-05 in vaginal samples. In the first technique, PCR primers specific to *L. crispatus* CTV-05 were used. Targeted PCR detected *L. crispatus* CTV-05 in 24% of participants at T0. In Northern American non-pregnant populations, the incidence of naturally occurring *L. crispatus* CTV-05 is 2-6% (554). It is possible, that targeted PCR over-detected *L. crispatus* CTV-05. Although testing of primers against 24 *L. crispatus* isolates produced no amplicons, it is conceivable that *L. crispatus* CTV-05 primers detect genetically similar strains of *L. crispatus,* explaining the over-detection seen with targeted PCR. Consistent with the background prevalence of naturally occurring *L. crispatus* CTV-05, ASV-2 and RT-qPCR (with a threshold of *L. crispatus* CTV-05 load ≥4277/swab) detected *L. crispatus* CTV-05 in 3% of women at T0. Importantly, metataxonomic profiling by amplicon sequencing is restricted in its ability to identify *L. crispatus* to strain level (656, 657).

LACTIN-V use leads to persistent *L. crispatus* CTV-05 colonisation in pregnancy

Persistent colonisation of *L. crispatus* CTV-05 was observed in 65% and 58% of women at T4 and T5, representing 8 and 16 weeks since LACTIN-V treatment, respectively. HBLC of *L. crispatus* ($>10^6$ copies/swab) has been reported to cause pathogens displacement (548). HBLC was seen in 52% and 44% of women at T4 and T5 respectively ($p<0.0001$). These results were consistent with *in-vitro* assays showing *L. crispatus* CTV-05 to have high adherence to vaginal epithelial cells obtained from non-pregnant women of reproductive age (658).

Moreover, in this chapter, *L. crispatus* CTV-05 viability at 28^{+0} weeks was confirmed. High rates of viable *L. crispatus* CTV-05 colonisation at 28^{+0} weeks gestation is clinically relevant in the context of PTB. Our group has previously shown that babies born prematurely after PPROM who develop neonatal sepsis are significantly more likely to be born through a vaginal canal depleted of *L. crispatus* and enriched with potentially pathogenic bacteria (371).

Hemmerling et al (2010) randomised women to receive LACTIN-V (2 x 10^9 CFU/dose, pre-filled vaginal applicator) (n=18) or placebo (n=6) following metronidazole treatment of BV (553). The dosing regimen included five daily doses, followed by two additionally weekly doses. Women were sampled at 10 and 28 days after commencing LACTIN-V and were not followed up beyond day 28. Using rep-PCR analysis, colonisation with *L. crispatus* CTV-05 was seen in 50% and 44% at day 10 and 28. Results presented in this chapter show higher colonisation rates following the loading dose. After the loading dose Hemmerling et al (2010) report colonisation in 50% of women, whereas in the FLIP-1 cohort colonisation occurred in 77% of women at T1. After ten days without LACTIN-V use, Hemmerling et al (2010) report colonisation in 44% of women. In the FLIP-1 cohort, 65% of women to displayed persistent colonisation eight weeks after cessation of LACTIN-V. Although the dosing regimen, detection methodology and sampling frequency differed, results suggest higher colonisation rates among pregnant cohorts following the loading dose.

In a subsequent RCT, Cohen et al (2020) randomised women to receive LACTIN-V (2 x 10^9 CFU/dose, pre-filled vaginal applicator, 7 day loading dose followed by a twice weekly maintenance dose for 10 weeks) or placebo. Vaginal sampling was performed at 4, 8, 12, 24 weeks after commencing LACTIN-V (554). Again, the LACTIN-V dosing regimen differed, and *L. crispatus* CTV-05 detection was based on qPCR, whereby a positive result was deemed to be above the lower limit of detection. Four weeks into LACTIN-V use, Cohen et al (2020) demonstrated *L. crispatus* CTV-05 colonisation in 84% of women. This would correspond to T2 in the FLIP-1 study, where colonisation was seen in a similar proportion of women, 72%. However, Cohen et al (2020) show at 13 weeks after last LACTIN-V use, colonisation dropped to 44%. Although colonisation in the FLIP-1 cohort gradually declined from T1 to T6, higher rates of persistent colonisation were seen following completion of LACTIN-V; with 65% and 58% displaying colonisation 8 and 16 weeks later.

Stapleton et al (2011) explored the impact of LACTIN-V on UTI recurrence following antibiotic treatment. Women were randomised to receive LACTIN-V (n=48) (gelatin capsules, 10^8 CFU/dose, taken daily for five days and then once weekly for ten weeks) or placebo (n=48) (551). *L. crispatus* qPCR was performed ten weeks after commencing LACTIN-V and 93% of women were classified as having HBLC (>10^6

rRNA gene copies/swab). Of note, authors did not perform qPCR specifically for *L. crispatus* CTV-05, which was restricted to the *L. crispatus* species. Interestingly, the placebo group also displayed HBLC in 68% of women. This highlights the need to refine qPCR to capture *L. crispatus* CTV-05, which Stapleton et al (2011) omitted. Comparisons between results from Stapleton et al (2011) and the FLIP-1 study are also limited in that gelatin capsules were used, which dissolve poorly, limiting *L. crispatus* CTV-05 colonisation efficacy (551, 553). Furthermore, Stapleton et al (2011) did not sample women after LACTIN-V use as the dosing regimen lasted for 11 weeks and final sampling was performed at the tenth week.

LACTIN-V use in pregnancy leads to more persistent *L. crispatus* CTV-05 colonisation when compared to use in non-pregnancy, reflective of the microbial and endocrine stability seen in pregnancy. Prior to menarche, a state of diversity exists characterised by anaerobic and enteric bacteria (337, 603). Towards menarche, oestrogen levels increase and promote proliferation of vaginal epithelial cells and glycogen deposition. Glycogen is metabolised by human α-amylase to substrates including maltose, maltotriose and α-dextrines, which are subsequently metabolised preferentially by *Lactobacillus* spp., producing lactic acid (365). This acidic environment promotes *Lactobacillus* spp. growth and is tolerated poorly by other microbes (338, 340).

In women of reproductive age, the menstrual cycle and physiological fluctuations of oestrogen influence the vaginal ecosystem (365). Research exploring how the menstrual cycle impacts the vaginal microbiome require caution, as some studies include women using hormonal contraception or with BV, obscuring results (659, 660). For most women, microbial stability is maintained during the menstrual cycle (346). For some, the fall in oestrogen and endometrial shedding causes depletion of *Lactobacillus* species (346, 347). The effects of the menstrual cycle on the vaginal microbiome are not limited to hormonal fluctuations. The presence of menstrual blood is also relevant. Menstrual blood increases the vaginal pH, contributing to a surge in anaerobic microbes (661). An iron rich micro-environment during menses also provides a nutrition source for anaerobes (662). Such factors may explain why cessation of LACTIN-V in non-pregnancy associates with reduced in colonisation.

In contrast, pregnancy represents a state of endocrine stability. Placental oestrogen production creates an environment favouring *Lactobacillus* spp. dominance. Healthy pregnancies associate with *Lactobacillus* spp. dominance, which is maintained throughout pregnancy (654). High levels of oestrogen promote conversion of glycogen to lactic acid by vaginal epithelial cells, increasing the glycogen availability for *Lactobacillus* spp. (347). Overall, the oestrogen surge seen in pregnancy, enhances *L. crispatus* CTV-05 colonisation. The post-partum drop in circulating oestrogen and passage of lochia, increases vaginal diversity and *Lactobacillus* depletion (349, 654). Although FLIP-1 participants were not assessed postnatally, a post-partum reduction in *L. crispatus* CTV-05 colonisation is likely.

LACTIN-V alters the vaginal microbial composition

Although LACTIN-V did not increase total vaginal bacterial load, a significant increase in *L. crispatus* relative abundance was observed. In most women, this was at the expense of *G. vaginalis* and *L. iners*, which are both implicated in BV. BV was detected in 10 (16%) women at least once during the study, which spontaneously resolved for 8 of these women. Yeasts were identified on at least one occasion in 23 women (37%). The UK prevalence of BV and *C. albicans* in low-risk antenatal cohorts is 3.5% and 12.5% respectively (663). The higher rates observed in the FLIP-1 cohort may relate to the frequent sampling of women. A higher prevalence of BV is unsurprising in women at high-risk of PTB. A high *L. crispatus* CTV-05 load associated with statistically significant protection from BV. Median *L. crispatus* CTV-05 loads were also lower in women yeasts detected when compared to women without yeasts.

Many pathways have been suggested for the displacement of pathogens by *L. crispatus*. The key role of *Lactobacillus* as a regulator of the vaginal ecosystem lies with its ability to produce H_2O_2. Acidic environments inhibit pathogens, including *E. Coli* (507, 664). The anti-microbial effects of H_2O_2 are enhanced by substances such as chloride anion and myeloperoxidase, which are present in the vagina (543). *In-vitro* and animal studies show that *Lactobacillus* spp. compete with pathogens to adhere to epithelial cells (664, 665). *Lactobacilli* also produce anti-microbial substances, including bacteriocins (665). *Lactobacilli* can antagonise and destroy opportunistic pathogens, by supressing the formation of biofilms (666). Additionally, by inhibiting

bacterial transcytosis, a cellular process allowing vesicles to be transported in and out of cells, lactobacilli prevent the dissemination of toxins (667). Results from this chapter are in keeping with *in-vitro* studies showing *L. crispatus* inhibition of pathogens including *G. vaginalis* and *Prevotella,* within just four hours of co-culture, making *L. crispatus* CTV-05 an ideal strain to study in disease prevention (668, 669). Ngugi et al (2011) trialled LACTIN-V, reporting higher concentrations of BV-associated bacteria, including *Atopobium* spp. and *G. vaginalis* in women without *L. crispatus* CTV-05 colonisation, when compared to women that colonise (655). Similarly, Cohen et al (2020) demonstrated reduced BV recurrence in 30% of women using LACTIN-V versus 45% of receiving placebo (p=0.001) (554).

Candida albicans, is a commensal with pathogenic properties. When causing symptoms *C. albicans* causes vulvovaginal candidiasis. The vaginal microbiota and host immune system provide balance between the commensal and pathogenic functioning of *C. albicans*. *Lactobacilli* inhibit pathogenic activity of *C. albicans* through the production of lactic acid, H_2O_2 and biosurfactants (670). *In-vitro*, *L. crispatus* lactic acid production causes pH-dependent restriction of *C. albicans* growth (671). 16S rRNA analysis has shown *L. iners* dominance to relate to *Candida* with *L. crispatus* providing protection (671). The protective role of lactobacilli against vulvovaginal candidiasis has however been challenged. 16S rRNA analysis has not always demonstrated an association between *Lactobacillus* and candidiasis in women experiencing recurrent vulvovaginal candidiasis (672). Such results may be due to the study of women with a history of recurrent vulvovaginal candidiasis rather than active symptoms. Lactobacillus and yeast co-culture, show *Lactobacillus* spp. inhibition of the yeast-hyphae switch in *C. albicans*, prohibiting proliferation rather than colonisation of *C. albicans* (673, 674).

Although yeasts presence was assessed in FLIP-1, symptomology was not recorded so as not to distort the assessment of adverse events associated with LACTIN-V use (Chapter 3). Moreover, some women received clotrimazole treatment, interfering with the interpretation results.

The FLIP-1 trial also assessed vaginal GBS colonisation, identifying 12 (19%) women to have GBS on at least one occasion. By T5/T6, GBS detection reduced to just four women (6%). Women with GBS displayed lower *L. crispatus* CTV-05 loads. Although

statistical significance was not reached, a larger sample size should explore this trend. GBS assessment was restricted to vaginal sampling. Swabbing both the vagina and rectum, increases culture yield when compared to vaginal sampling alone (675). The FLIP-1 GBS prevalence (19%) was lower than the GBS prevalence among antenatal populations in North West London (29%) and reflects the omission of rectal swabbing in FLIP-1 participants (518).

GBS colonisation from 34^{+0} weeks' gestation predisposes to neonatal sepsis. If proof-of-concept demonstrates *L. crispatus* CTV-05 to reduce GBS colonisation, clinical implementation may necessitate lengthening the LACTIN-V course to maintain *L. crispatus* CTV-05 colonisation until delivery.

In-vitro work has demonstrated *L. crispatus* to eradicate GBS within just 60 minutes (676). Mechanisms exploring how Lactobacilli prohibit GBS adherence to vaginal epithelial cells have been assessed and described as blockage by exclusion, competition and displacement (677). Oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have not shown significant changes in GBS detection (678, 679).Others have shown oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 to significantly lower GBS incidence when compared to no intervention (528). Daily oral *L. salivarius* from 26^{+0} to 38^{+0} weeks' gestation in GBS positive women showed 72% and 68% of participants to be GBS-negative in rectal and vaginal samples respectively, by 38^{+0} weeks (523). Oral iNatal® (*Enterococcus faecium* L3 LMG P-27496, *Bifidobacterium animalis ssp. lactis* BB12 DSM 15954, *Lactobacillus casei* R025 CNCM I-3429 and *Lactococcus lactis ssp. lactis* SP 38 DSM 26868) was given to 127 pregnant women and when compared to a control group 279 women, showed a 6% decrease in GBS vaginal-rectal colonisation and a 30% lowered incidence of PPROM (680). Although the largest trial assessing probiotics and GBS to date, authors failed to report on compliance. Importantly, most clinical trials have taken a multi-strain approach, challenging the identification of a superior strain. Sample sizes have been small, compliance is often omitted from published data and all studies investigate oral probitoics. Whether vaginal probiotics have potential to reduce GBS colonisation warrants further work in well-designed clinical trials.

Starting CST impacts colonisation

Pre-existing *L. crispatus* dominance associates with non-colonisation. Meanwhile, women with CST III/IV and T0 are more likely to colonise. Similarly, Antonio and Hillier (2003) demonstrate L. crispatus CTV-05 colonisation to be less effective in women with pre-existing *L. crispatus* dominance and in these cases, LACTIN-V did not displace endogenous lactobacilli (542). Ngugi et al (2011) also demonstrated reduced *L. crispatus* CTV-05 colonisation in women with a pre-existing abundance of *L. crispatus* (655). Hemmerling et al (2010) did not find baseline lactobacillus levels to influence colonisation (553). Interestingly, demographics including ethnicity were not reported. It is therefore difficult to determine whether other factors influenced colonisation.

Czaja et al (2007) conducted a phase 1 randomised trial in women with recurrent UTIs. Although there was no significant increase in women with *L. crispatus* CTV-05 detection by rep-PCR following LACTIN-V use, 28/30 participants displayed *L. crispatus* dominance at the outset. Targeting women with sub-optimal vaginal microbial communities, could increase colonisation efficiency but larger clinical trials with good representation from women with CST III/IV is required.

In a micro-environment already dominated by *L. crispatus*, endogenous may *L. crispatus* has advantageous survival properties. For example, release of anti-microbial components by endogenous *L. crispatus* may prohibit *L. crispatus* CTV-05 adherence and growth. Alternatively, endogenous *L. crispatus* may out-compete exogenous *L. crispatus* for energy sources.

Factors influencing colonisation

Not all non-colonisers displayed *L. crispatus* dominance prior to LACTIN-V use. Multiple factors can influence *L. crispatus* CTV-05 colonisation including shedding, biofilms and non-compliance.

The cervicovaginal epithelium provides defence against pathogens through production of mucous and trapping of pathogens, pathogen recognition through TLRs and the

release of anti-microbial peptides. The balance between cervicovaginal epithelial cell proliferation and maturation, known as shedding, also provides protection from infection (681). Accelerated shedding of the vaginal epithelium associates with increased susceptibility to STIs and GBS colonisation (682, 683). Shedding is accelerated in women with high diversity and *Lactobacillus* spp. depletion, when compared to women with a *Lactobacillus* spp. dominant microbiota (684). Increased cell shedding, may interfere with attachment and adherence of exogenous *L. crispatus* CTV-05, contributing to reduced colonisation.

Colonisation resistance may occur due to biofilms. Biofilms are communities of microorganisms, consisting primarily of *G. vaginalis* clusters that are strongly adherent to the vaginal epithelium (685). Some women may be resistant to *L. crispatus* CTV-05 colonisation due to the presence of biofilms.

Another important consideration for non-colonisers, is compliance. Compliance to LACTIN-V was self-reported with 93% of women being fully compliant (Chapter 3) Trypan blue staining detects apoptosed cells and can therefore confirm whether applicators have been used intra-vaginally (686). The return of used applicators and staining with trypan blue could therefore verify compliance. Hemmerling et al (2010) sub-analysed women with positive and negative staining of LACTIN-V applicators (553). Colonisation rates were higher (78%) in women with positive trypan blue staining when compared to participants with inconsistent positive staining (61%). Identifying non-compliant women through trypan blue staining could enhance analysis of results.

Spermicidal products containing nonoxynol-9 can disrupt the vaginal microbiome by depleting lactobacilli (687). Nonoxynol-9 has a direct toxic effect on lactobacilli (688). *In-vitro* assays testing *L. crispatus* CTV-05 adherence to vaginal epithelial cells show spermicides do not impact *L. crispatus* CTV-05 vaginal adherence (658). It is unlikely women in FLIP-1 were utilising spermicide, a contraceptive agent, however impact of exogenous substances on colonisation requires consideration.

Sexual intercourse, including number of sexual partners, frequency of intercourse and receptive oral sex, can transiently alter the vaginal microbiome (689). The frequency of sexual intercourse has been associated with loss of H_2O_2-producing lactobacilli

(690). Although study participants were asked to abstain from sexual intercourse, sexual intercourse was not recorded. Vaginal intercourse impair *L. crispatus* CTV-05 colonisation when trialled in non-pregnant women with BV (655). Cohen et al (2020) showed no impact with sexual intercourse or condom use (554). Larger clinical trials are required to explore the impact of sexual intercourse and condom use on LACTIN-V effect in pregnancy.

Smoking has also been identified to influence the vaginal microbiome. Smokers show a lower proportion of *Lactobacillus* spp. within the vaginal microbiome when compared to non-smokers (691, 692). Although only two FLIP-1 participants were smokers, exploration of LACTIN-V response in pregnant smokers is required. Another consideration is the use of e-cigarettes. E-cigarettes can modify the oral microbiome, but their impact on the vaginal microbiome remains unknown (693).

Secretor status, the presence or absence of ABO blood group antigens within the CVF, have been implicated in sPTB aetiology. A lack of maternal H-antigen is an independent risk factor for sPTB (694). Secretor status influences susceptibility to bacterial pathogens (695). Non-secretors, with *Lactobacillus* depletion, are predisposed to PTB (696). In women with cervical treatment as a PTB risk factor, blood group B heightens sPTB risk (697). In contrast, women with previous PTB or MTL with blood group O are further predisposed to PTB (697). Further work should explore whether blood group influences LACTIN-V response. Interestingly, women with non-secretor blood group antigens, display increased adherence of *L. crispatus* CTV-05 to vaginal epithelial cells when compared with secretors (658).

Antibiotics

Antenatally antibiotics were used by 13 (21%) women, including cefalexin, amoxicillin, nitrofurantoin, amoxicillin and penicillin V. Nitrofurantoin, a nitrofuran antibiotic, is effective against gram-negative and gram-positive bacteria, and inhibits DNA, RNA and protein synthesis (698). Amoxicillin and penicillin V (penicillin antibiotics), target a range of gram-positive and some gram-negative bacteria (699). Co-amoxiclav contains amoxicillin and clavulanic acid and is effective against beta-lactamase producing bacteria. Cefalexin, a cephalosporin, is also effective against beta-lactamase

producing gram-negative and gram-positive bacteria. Gram-positive *Lactobacilli* spp. are sensitive to penicillin but resistant to metronidazole, sulphonamides, trimethoprim and cephalosporins (700).

In some instances, a reduction in *L. crispatus* CTV-05 load was seen following antibiotic use. This was seen following cefalexin (F024, F045, F057) and amoxicillin use (F053 and F057). Penicillin-based antibiotics (e.g. amoxicillin) produce direct anti-microbial effects on *Lactobacilli* spp. Although *Lactobacilli* spp. are resistant to cephalosporins, a decline in *L. crispatus* CTV-05 load was seen following cefalexin. In these cases, antibiotic-related disruption of the vaginal microbiota may contribute to colonisation resistance. Antibiotic depletion of other vaginal microorganisms can impair microbiota-dependent pathways that support *L. crispatus* CTV-05 growth, leading to *L. crispatus* CTV-05 depletion. If antibiotics target microorganisms that produce metabolites essential for the growth of *L. crispatus* CTV-05, such as short-chain fatty acids, substrate depletion can result in reduced *L. crispatus* CTV-05 load. This effect termed 'colonisation resistance' has been described as an important modulator of microbial communities (701). Mice and human studies demonstrate microbial communities to be influenced by availability of carbon energy substrates (702, 703).

In addition to antibiotic class, the timing of antibiotic use in the LACTIN-V dosing regimen is also important. Persistent *L. crispatus* CTV-05 colonisation with HBLC occurred in some women, despite antibiotic use. This was the case for participants F033 (cefalexin and amoxicillin use during maintenance dose), F041 (amoxicillin), F063 (penicillin V), F071 (co-amoxiclav) and F073 (amoxicillin and cefalexin). All except for one (F073) received antibiotics during the LACTIN-V maintenance dose. Antibiotic administration during concomitant LACTIN-V use can permit persistent and HBLC.

Further work is needed to explore antibiotic sensitivity and resistance towards *L. crispatus* CTV-05. Different lactobacilli strains show different susceptibilities to nitrofurantoin (700). Culture-based species level identification of lactobacillus from vaginal sampling of non-pregnant women show antibiotics cause a loss of H_2O_2-producing lactobacilli, (690). Clinical trials should investigate *L. crispatus* CTV-05 susceptibility to antibiotics, particularly as antibiotics account for 80% of prescribed

medications during pregnancy (704). Based on results in this chapter, a 'rescue course' of LACTIN-V may be necessary following antibiotics.

Interestingly, a reduction in *L. crispatus* CTV-05 load was seen prior to the onset of a UTI in one participant (F005), accompanied by a preceding drop *in L. crispatus* CTV-05 load. It is plausible that an alternative insult compromising the *L. crispatus* CTV-05 load can subsequently predispose women to urogenital pathogens culminating in a UTI. An interesting sub-focus includes whether LACTIN-V can reduce UTI recurrence during pregnancy, as is seen in non-pregnant women (509, 551).

Ethnicity

Due to a small sample size of 62 women, the representation of different ethnic groups was limited. Most women were of White (60%) ethnicity. Black, Asian and mixed/other ethnicities were represented by 13%, 18% and 9% respectively. Ethnicity is a well described influencer on the vaginal microbiome (339). At T0, the proportion of Black women starting with CST I was lower than other ethnic groups, in-keeping with previously published data (369). When CST distributions at T4 were explored all ethnic groups showed an increase towards *L. crispatus* dominance. This increase was statistically significant for White women, but not for Black or Asian women likely reflecting small numbers of non-White women. Ethnicity did not influence colonisation likelihood. Although ethnicity did not influence LACTIN-V response, evaluation in a larger cohort is required.

LACTIN-V response in women delivering preterm

Women delivering <37 weeks (n=9) were assessed to identify whether an altered response to LACTIN-V occurred. The *L. crispatus* CTV-05 load did not differ in women delivering term versus preterm. The *L. crispatus* CTV-05 load was tracked in each woman delivering preterm, highlighted most women (7/9) to display HBLC. Two participants (F039 and F058) displayed low and fluctuating *L. crispatus* CTV-05 loads throughout. Only these two women delivering preterm, were classified as non-colonisers. Furthermore, alpha diversity at T4 did not differ in women delivering term or preterm.

After confirming that most women delivering preterm displayed HBLC, the microbial composition was assessed. Most women delivering preterm displayed a shift towards a state of *L. crispatus* dominance.

Despite *L. crispatus* CTV-05 colonisation and optimisation of the microbial community, preterm can occur secondary to alternative, non-microbial causes of sPTB. Three patients had pre-eclampsia or gestational diabetes, contributing to sPTB risk. Non-colonisers that delivered preterm may have been non-compliant to LACTIN-V. Interestingly, F039 maintained high diversity at all assessed timepoints. In contrast, F058 maintained *a L. crispatus* dominant state throughout. On review of clinical notes, F058 had gestational diabetes, which may have contributed to PTB risk.

Another consideration includes predispositions to PTB that could not be accounted for, including genetic predisposition. For example, gene polymorphisms associated with immune and inflammatory pathways predispose to PTB (705).

Finally, whether women that delivered preterm would have delivered even earlier had LACTIN-V not been used remains unknown.

PPROM

As only four cases of PPROM occurred, the assessment of LACTIN-V response in those with PPROM was limited. An interesting future focus includes whether LACTIN-V can prolong gestation whilst improving neonatal following PPROM. A study protocol describing the randomisation of women to receive oral *L. fermentum* or placebo following PPROM has been published (537). Oral probiotics do not alter the vaginal microbiota in pregnancy (530). Although the published protocol involving oral *L. fermentum* outlines aim to assess clinical outcomes, authors do not describe plans to assess the vaginal microbiota.

Randomisation of women with PPROM to receive antibiotics and a vaginal probiotic (*L. crispatus, L. rhamnosus, L. gasseri* and *L. jensenni)* or placebo reported no difference in median time to delivery (538). Vaginal sampling was not performed and

impact on the vaginal microbiota was not explored. Others demonstrate daily vaginal *L. rhamnosus* to prolong gestation by 26.5 days when compared to 11.6 days in placebo groups (539). Again, vaginal microbiota assessment was precluded. In a third study, outcomes of women with PPROM were compared in those receiving vaginal *L. casei rhamnosus* and ampicillin versus women receiving ampicillin alone. The mean time to delivery was 41.4 days with *L. casei rhamnosus* use and 12.3 days in those without. Significantly improved neonatal birth condition (APGAR scores), birth weights, lower neutrophil counts and lower CRP levels were also seen with vaginal *L. casei rhamnosus* use (540). A fourth trial randomised women with PPROM to receive vaginal probiotics (*L. rhamnosus* (DSM 14870) and *L. gasseri* (DSM 14869) (1×10^8 CFU)) plus standard antibiotics, or antibiotics alone. The mean gestational age at birth was higher in women receiving vaginal probiotics (35.49 weeks) when compared to the antibiotic group (32.53 weeks). The latency period and mean birth weights were significantly higher with probiotics. Although promising results, this trial could be strengthened by involving a placebo arm and assessing the vaginal microbiota (706).

Although live vaginal biotherapeutics have been explored in PPROM cohorts, assessment of the microbial composition has been neglected. Moreover, *L. rhamnosus,* an uncommon coloniser of the vagina, remains a commonly trialled probiotic strain (707-709). Future trials require careful strain selection, involving an in-depth assessment of the vaginal microbial response.

Vaginally live biotherapeutics are superior to oral live biotherapeutics

Oral probiotics have been explored in their ability to prevent sPTB. Surveys assessing ingestion of milk-based lactobacilli suggest that regular dairy-based lactobacilli intake reduces sPTB risk, particularly when consumed in early pregnancy (524, 619). Although over 18,000 women were surveyed, women were invited to participate, creating a selection bias of women motivated by health. Moreover, impact on the vaginal microbiome following dairy-based lactobacilli ingestion was not performed.

Women $\geq 35^{+0}$ weeks' gestation were randomised to receive orally ingested fermented milk (10^9 CFU/mL *L. johnsonii* La1, for two weeks) or a placebo milk (527). Following vaginal sampling, *lactobacilli* were cultured on MRS agar plates and counted. Probiotic

milk intake correlated with a significantly increased number of counted vaginal *lactobacilli*. This study was limited in its culture-based methodology and failure to determine strain specific colonisation. Authors did not comment on PTB rates. As probiotic use commenced at ≥35^{+0} weeks' gestation, targeting women close to term by default circumvented women at high-risk of sPTB.

The multi-strain approach including *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 has been commonly studied. Operational taxonomic unit analysis of this multi-strain oral preparation in pregnancy, has shown no significant change in *lactobacillus* spp. (528). Others have shown oral *L. rhamnosus GR-1* and *L. fermentum RC-14* to significantly increase vaginal *lactobacilli* abundance when compared to a placebo (529). Authors hypothesise that following oral ingestion, intestinal passage occurs leading to eventual alterations in the vaginal microbiome. Suggested mechanisms include direct transfer from the closely located rectum and altered mucosal immunity at the gut and vagina. Importantly, *lactobacilli* detection was performed using ribotyping, a technique that cannot differentiate between different strains of the same subspecies, thus vaginal colonisation with ingested strains was not confirmed.

PCR and restriction endonuclease analysis of recto-vaginal samples collected following oral *L. plantarum* and *L. crispatus* use detected *L. plantarum* in both vaginal and faecal samples whilst *L. crispatus* was only detected in faecal samples (532). The absence of vaginal *L. crispatus* isolation may reflect its susceptibility to oxygen and environmental stress factors within the gastrointestinal tract (532). Whilst migration of *L. plantarum* from the gastrointestinal tract to the vagina was shown, the same was not seen for *L. crispatus.*

In contrast to orally administered live biotherapeutics, results from this chapter demonstrate that vaginal administration of *L. crispatus* CTV-05 leads to persistent colonisation and HBLC causing alterations in the vaginal microbial community. In addition to route of administration, probiotic strain choice is a key determinant of colonisation efficacy. The combination of *L. rhamnosus* GR-1 and *L. reuteri* is commonly studied due to its safety status granted by the European Food and Safety Authority (710). However, *L. rhamnosus* is not a common coloniser of the vagina, (707-

709). It is unsurprising that 16S rRNA analysis shows that oral *L. rhamnosus* GR-1 and *L. reuteri* in pregnancy do not cause colonisation or compositional changes (530, 531).

L. crispatus CTV-05 colonisation is localised to the cervicovaginal niche

Placenta and fetal membranes were assessed for the presence of *L. crispatus* CTV-05 to determine the extent of *L. crispatus* CTV-05 colonisation. Delivery samples obtained following vaginal delivery or EMCS are likely to be contaminated by vaginal *L. crispatus* CTV-05 during vaginal passage, explaining why *L. crispatus* CTV-05 was detected in either the placenta or fetal membranes of 12/29 and 3/3 women delivering vaginally or by ELCS. Consistent with this, these women displaying *L. crispatus* CTV-05 were colonisers with HBLC throughout the study.

Assessment of delivery samples obtained at ELCS are therefore more insightful. Delivery samples were available for nine women delivering by ELCS. Only one participant had *L. crispatus* CTV-05 detected in the placenta by targeted PCR. For this participant, ASV-2 was not detected in the placenta and fetal membranes did not show evidence of *L. crispatus* CTV-05 (by PCR or ASV-2). Vaginal transfer of *L. crispatus* CTV-05 to placental tissue at ELCS is less likely but remains possible due to intra-operative handling of tissue. Overall, analysis of placental and fetal membrane samples obtained at ELCS support localisation of *L. crispatus* CTV-05 colonisation to the cervicovaginal niche. Absent intra-uterine ascension of *L. crispatus* CTV-05 contributes to the safety profile of LACTIN-V.

The placental and fetal membrane microbiome

Although the FLIP-1 study did not aim to characterise the placental microbiome some interesting observations can be made. In keeping with other published work, placental samples obtained at ELCS show the placenta to constitute a low-abundance microbiome, unlikely to contribute metabolic function (382, 383). Others have identified microorganisms that reside in the oral cavity within the placenta of women delivering preterm, supporting a role for haematogenous spread of microbes and placental colonisation in sPTB aetiology. Oral microbes were not identified in placenta or membranes collected irrespective of mode of delivery. *Sneathia* spp. was identified

and although is a common oral microbe, is also found in the reproductive tract (339, 653, 711). Differences in the placental microbiome in term and preterm deliveries have been disputed (712). In the FLIP-1 cohort, only one participant that provided delivery samples experienced a sPTB restricting further analysis.

Analyses of delivery samples were limited in that sampling was taken from only one location of the placenta and fetal membranes. Open-air room swabs were not collected for the analysis of environmental contaminants. Other authors show that placental tissue obtained under sterile conditions at ELCS from multiple placental locations, with the collection of environmental controls showed no evidence of bacteria presence when assessed with immunohistochemistry, electron microscopy, qPCR and 16S rRNA gene amplification (713). Organisms identified from ELCS delivery samples, including *Paraburkholderia, Diaphorobacter, Cloacibacterium* and *Hyphomicrobium* are found in root plants, soil and wastewater and are likely to represent processing contamination. Contaminants present in extraction reagents have also been described and importantly can distort the analysis of low biomass samples (714, 715).

L. crispatus CTV-05 is not detected within the neonatal gut microbiome

Analysis of neonatal gut samples aimed to investigate whether vaginal LACTIN-V use led to *L. crispatus* CTV-05 detection within the neonatal gut microbiome. *L. crispatus* CTV-05 was detected in 3 meconium samples. For two newborns, detection was transient. As the third participant did not supply a neonatal stool sample, persistence was undetermined.

L. crispatus CTV-05 was detected by PCR, but not with ASV-2, in only one neonate. Interpretation of targeted PCR results in the absence of ASV-2 require caution, as the *L. crispatus* CTV-05 primers may cross-react with genetically similar *L. crispatus* strains. Targeted PCR showed *L. crispatus* CTV-05 to be absent in 95% of analysed neonatal gut samples. ASV-2 analysis demonstrated *L. crispatus* CTV-05 to be absent in 98% of analysed neonatal gut samples. Overall, these results can reassure women considering LACTIN-V use in pregnancy wishing to avoid products that transfer to neonates. Due to its ability to detect as little as one copy of target DNA, qPCR

assessment of neonatal gut samples should be performed to assess *L. crispatus* CTV-05 presence and quantity (716).

The neonatal gut microbiome

Many studies show that meconium is not sterile (717, 718). The neonatal gut microbiome develops *in-utero.* 16S rRNA and qPCR analysis of 15 mother-infant pairs illustrated shared features between the placental microbiota with infant meconium, suggesting that microbial transfer occurs at the feto-maternal unit (719). *Lactobacillus* spp. isolated from the vagina of pregnant women demonstrated using PCR, that 23% of newborns were colonised by the predominant lactobacilli present in the vagina of their mothers, suggesting that mother-to-child vertical transmission of *Lactobacillus* occurs (720). If this is the case, then a high abundance of *L. crispatus* would be expected in analysed neonatal gut samples from the FLIP-1 cohort. Although vaginal *L.* crispatus CTV-05 colonisation is shown with LACTIN-V use, *Lactobacilli* were not abundantly present in meconium or neonatal stool samples.

Mode of delivery impacts the neonatal gut microbiome (721). Published work suggests that vaginal delivery leads to a gut microbiome reflective of the maternal vaginal microbiome (*Lactobacillus* and *Prevotella* species), whereas delivery by caesarean-section shows neonatal gut colonisation with epidermal (*Clostridium, Staphylococcus, Propionobacterium* and *Corynebacterium*) rather than vaginal species (722, 723). FLIP-1 study was not designed to assess impact of mode of delivery on neonatal gut microbiome and analyses of results is limited by the small sample size.

Reportedly, vaginal delivery associates with higher levels of *Bacteriodes* and *Bifidobacterium* species in the neonatal gut microbiome, especially *B. fragilis, B. longum and B. catenulatum* (724-726). The early establishment of a *Bacteroides* population within the neonatal gut microbiome relates to improved mucosal immunity and reduced risk of cardiovascular disease (727). Results in this chapter, show a high relative abundance of *Bifidobacterium* in neonatal gut samples acquired after vaginal delivery but the significance of this from a low biomass sample is unknown.

Factors influencing the neonatal gut microbiome

Exposure to antibiotics around the time of birth can reduce neonatal gut microbial diversity, predisposing infants to autoimmune conditions, inflammatory bowel disease and obesity (728). Neonatal exposure to antibiotics is associated with an increased risk of LOS (729). Murine models show that *in-utero*, fetal exposure to orally ingested antibiotics leads to reduced maternal IL-17 and G-CSF production and increased susceptibility to sepsis in neonatal mice (730). It is therefore important to consider the impact of maternal antibiotic usage on the neonatal gut microbiome. Future work assessing the neonatal gut microbiome should incorporate data on antenatal, intrapartum and postnatal antibiotics use.

The collection of neonatal gut samples was limited in its collection technique. Efforts to reduce contamination included the use of sterile spatulas, sterile collection tubes and removal of the outer layer of stool in contact with the diaper. However, use of non-sterile diapers meant contamination could not be eliminated. To reduce contamination, rectal sampling could be confirmed. This technique was however not used in view of its invasive nature, possibly leading to a reduction in consent.

Breastfeeding also influences the neonatal gut microbiome. Breastfeeding associates with higher levels of *Bifidobacterium* within the neonatal gut microbiome (724). In the analysis of neonatal gut samples, breast feeding was not accounted for. Other factors impacting the neonatal gut microbiome, that were not accounted for in this study include skin-to-skin and the maternal gut microbiome (385).

Strengths

This chapter presents results from the first in-pregnancy clinical trial of LACTIN-V. Whilst most in-pregnancy trials focus on oral probiotis, LACTIN-V was administered vaginally. Women at high-risk of sPTB were targeted, to capture women in need of microbial modulation. The regular sampling of women at seven different timepoints facilitated close monitoring of LACTIN-V response. Despite challenges faced by COVID-19 and restrictions on clinical sampling, huge efforts were made to collect a near-complete collection of samples.

Limitations

The greatest limitation of this study was our small sample size. Future work should incorporate a larger cohort to facilitate statistical analysis of outcomes. A placebo arm is crucial for understanding LACTIN-V impact. The use of Trypan blue staining to confirm compliance would strengthen analysis. Although Trypan blue staining was not performed, self-reported compliance and Trypan blue staining produce comparable results; self-reported compliance and positive trypan blue staining was 81% and 77% respectively in a trial of LACTIN-V (554).

The risk of contamination during sample collection and processing requires consideration. Although sampling was performed by trained research staff using gloves, the risk of contamination remains. Contamination from bacterial DNA existing on equipment in clinical and laboratory environments and reagents was also possible (731). Although experiments were conducted with care, including UV-treatment of consumables and the incorporation of negative controls, the risk of contamination cannot be fully eliminated. Neonatal gut samples were particularly at risk of contamination, as samples were in contact with the infant's skin and diapers. The use of an open-air control was not included for delivery samples as each woman delivered in a different room. Future utilisation of open-air swabs to account for environmental contaminants should be included.

Delivery samples were challenging to obtain, as collection depended on spontaneous obstetric events and swift arrival of research staff. Due to the unpredictable timing of delivery, vaginal samples were not collected at consistent times during delivery. Sampling was only performed if vaginal cleaning or lubricant jelly had not been used. However, the presence of blood, vaginal examinations and utilisation of vaginal induction agents were not accounted for.

The impact of PTB interventions should not be over-looked. Women were offered prevention interventions when indicated. Progesterone was used by 27% of women. Vaginal progesterone use in women with a short cervix does not alter *L. iners* or *L. crispatus* abundance and is therefore unlikely to have influenced results (353). 23% of women required a cervical cerclage. The initial study protocol aimed for homogenous cerclage placement, involving consistent surgeons, technique and the use of monofilament sutures. However, the COVID-19 pandemic challenged the

implementation of this and three participants received a braided cerclage. Whilst monofilament sutures do not alter the vaginal microbiome, a shift towards dysbiosis and reduced *Lactobacillus* spp. is seen with braided suture use (269).

Next generation sequencing is limited in that different primer sets will vary in coverage of microbial taxa. In this study, V1-V2 variable regions were selected for their capacity to distinguish *Lactobacillus* spp. Targeting of a different variable region would provide improved coverage of other relevant vaginal pathogens, albeit at the detriment of studying *Lactobacillus* species. The incorporation of other sequencing methods, such as shot gun sequencing would enable study of fungal and viral vaginal communities, which also influence the vaginal host-microbe milieu (732, 733).

Future work

Study design should be optimised to include a placebo-control randomised trial involving a larger sample size to elucidate whether observed outcomes are attributed to LACTIN-V. Incorporation of trypan blue staining would permit a more in-depth assessment of colonisation and non-colonisation.

Future work should report on sexual intercourse, use of lubricants, smoking and secretor status. Although ethnic distributions were reflective of the community served, efforts to include under-represented ethnic groups should be made. This could be achieved through a multi-centre trial.

Although costly, future work should incorporate whole genome sequencing for more detailed analysis (734). Shotgun sequencing provides a more detailed assessment of bacterial genomes, facilitating bacterial assessment at strain level.

A differential expression of metabolites in relation to CST associates with sPTB (735). The incorporation of metabolic profiling methods, such as desorption electrospray ionization mass spectroscopy (DESI-MS) will provide insight into LACTIN-V influence on vaginal metabolomics (736).

4.5 Conclusion

Two key features of the vaginal microbiome determine PTB risk; *Lactobacillus* abundance and the specific dominating *Lactobacillus* species. In this chapter, high rates of persistent *L. crispatus* CTV-05 colonisation is demonstrated with LACTIN-V use in pregnancy, leading to a shift towards a state of *L. crispatus* dominance. Data in this chapter supports the concept that vaginal administration of live vaginal biotherapeutics modulates the vaginal microbiome towards a more protective state. The vaginal microbiome does not work in isolation and works in synchrony with the host immune response. In the next chapter, LACTIN-V impact on inflammation is explored to further elucidate the protective effects of LACTIN-V.

5 LACTIN-V and the Cervicovaginal Immune Milieu

5.1 Chapter overview

Having shown that LACTIN-V use in pregnancy leads to *L. crispatus* CTV-05 colonisation and a shift towards *L. crispatus* dominance, I sought to determine the impact of LACTIN-V on the host immune response. It was hypothesised that LACTIN-V use would modulate the maternal immune response, by reducing pro-inflammatory cytokine concentrations within the cervicovaginal milieu.

The aims of the research conducted in this chapter were to:

- Describe changes in cervicovaginal fluid (CVF) cytokine concentration in women taking LACTIN-V.
- Determine whether the local immune response to LACTIN-V differs according to starting CST.
- Determine whether host immune response to LACTIN-V varies in colonisers versus non-colonisers.
- Assess whether the immune response differs in women delivering term or preterm.

Key Results

LACTIN-V use in pregnancy reduces pro-inflammatory cytokine concentrations at the cervicovaginal interface. By 28^{+0} weeks median IL-1ß concentrations were significantly reduced (p=0.0002). Median IL-8 concentrations were reduced by 28^{+0} weeks (T4), although statistical significance was not met (p=0.2384). Detectability of IL-6 was significantly reduced at 28^{+0} weeks (p=0.0003). When analysing matched longitudinal samples, a significant reduction in IL-1ß (p=0.0002), IL-8 (p=0.0191) and IL-6 (p=0.0055) concentrations were seen by 28^{+0} weeks.

Starting CST influenced the immune response to LACTIN-V. Women with a high-risk microbial community at baseline (CST III/IV) showed a more profound reduction in IL-1ß and IL-8 concentrations as well as IL-6 detectability, when compared to women with a low-risk microbial community at baseline (CST I/II/V). In women with a high-risk

microbial community at baseline, the reduction in pro-inflammatory cytokines was accompanied by a transition to *L. crispatus* dominance (CST I).

Conclusion

This study demonstrates that LACTIN-V causes a favourable immunomodulatory effect. The reduction in pro-inflammatory cytokines associated with LACTIN-V use among pregnant women at high-risk of sPTB provides a scientific rationale for its success as a preventative therapy. If results are replicated in larger cohorts, LACTIN-V has potential to modulate the immune response in women at high-risk of sPTB.

5.2 Introduction

Pregnancy is a highly regulated physiological phenomenon, whereby the maternal immune system plays a key role in healthy term birth. During pregnancy, the maternal immune system must adapt to allow growth and development of a semi-allogeneic fetus whilst balancing protection against pathogens. Cytokines are small signalling proteins involved in cell-to-cell communication and are key contributors to both physiological and pathological processes that occur in pregnancy (737).

Cytokines play an important role from conception through to implantation, pregnancy and labour. Parturition is a tightly controlled process involving synchronised activation of the cervix, myometrium and chorioamniotic membranes. Inflammatory changes during labour have been reported in the myometrium, cervix, fetal membranes and peripheral blood (469). An exaggerated inflammatory response has been suggested to contribute to the onset of sPTB (738, 739). Four widely studied signalling proteins in the context of preterm labour include IL-1ß, IL-8, IL-6 and IL-10 (740).

Of particular interest is the inflammatory response within the CVF. Studying the CVF is advantageous as it is less invasive than other techniques such as amniotic fluid sampling. CVF cytokines are also more likely to reflect biochemical processes at the cervicovaginal interface relevant to cervical remodelling, compared to other sample types like plasma and serum.

IL-1 is well-known for its pro-inflammatory role and exists in two forms; IL-1α and IL-1ß. On binding to its plasma membrane receptor, IL-1ß triggers systemic and local immune responses by altering transcription of pro-inflammatory genes. IL-1ß up-regulates prostaglandin production, contributing to the onset of uterine contractions (741, 742). IL-1ß increases the expression of IL-8 in decidual cells, thus contributing to neutrophil infiltration (741). During parturition, IL-1ß concentrations increase in the cervix, myometrium and amnion (465, 578). Histological analysis show this to associate with cervical infiltration of neutrophils and macrophages (465). In animal models, intra-amniotic injection of IL-1ß causes infiltration of neutrophils and increases pro-inflammatory cytokines as well as subsequent sPTB (579, 580). Human studies indicate that high levels of IL-1ß associate with sPTB (484, 581). CVF IL-1ß are

elevated in women who deliver preterm when compared to those who deliver term (743).

IL-8 is a pro-inflammatory chemokine, produced by monocytes, lymphocytes, neutrophils and endothelial cells (471, 744). On binding to its G protein-coupled receptor, inflammatory pathways such as Akt/protein kinase B, mitogen-activated protein kinase and protein kinase C are activated (745, 746). Subsequently, IL-8 attracts further immune cells like neutrophils to sites of inflammation, triggers monocyte and macrophage cell differentiation, angiogenesis, endothelial cell proliferation and MMP production (407, 747). IL-8 also plays a role in cervical re-modelling (582, 583). CVF IL-8 concentrations are higher in women delivering preterm when compared to those delivering term (584, 585).

IL-6 is a pleiotropic cytokine, involved in multiple physiological processes such as inflammation, metabolic function, acute-phase responses and haematopoiesis (748). Upon binding to its receptor, IL-6 activates the Janus kinase-signal transducer (JAK/STAT) pathway, activating downstream transcription factors (749). The pro-inflammatory actions of IL-6 contribute to cervical re-modelling (582, 583). Elevated CVF IL-6 levels relate to sPTB (494, 586). A systematic review of 17 studies involving 6,270 participants showed that CVF IL-6 measurements strongly associate with sPTB (494).

IL-10 is considered an anti-inflammatory cytokine and functions to regulate a pro-inflammatory immune response. Immune cells such as macrophages, Treg and NK cells release IL-10 (750, 751). IL-10 is expressed in the uterus and placenta and is also produced by non-immune cells including epithelial cells (410, 752). IL-10 inhibits monocyte and macrophage production of pro-inflammatory cytokines such as IL-1ß, IL-8, IL-6 and TNF-α (587-589, 752, 753). Low levels of IL-10 from 22^{+0} weeks' gestation are associated with an increased risk of sPTB (754).

Analysis of CVF cytokine concentrations across pregnancy have shown IL-6 and IL-8 to fall at mid-pregnancy, normalising to pre-pregnancy levels at term (461). Others have shown CVF IL-8 and TNF-α to be significantly lower in the second trimester, with no changes in IL-1α, IL-1β, IL-6, IL-10 or MMP-8 concentrations across pregnancy

(462). Although CVF IL-1α and IL-1β concentrations remain stable between the second and third trimester, a rise occurs 4-14 days prior to labour (463).

The host immune response does not work in isolation. A complex relationship between the host immune response and vaginal microbiome exists. The cervicovaginal immune response to the vaginal microbiome in pregnancy has been studied. Increased bacterial diversity relates to elevated pro-inflammatory CVF cytokines (498). Elevated CVF eotaxin, IL-1β, IL-6 and MIP-1β negatively correlates with *L. crispatus* abundance, associating with increased sPTB risk (370).

The cervicovaginal epithelium acts as a first line of defence and contributes to innate immune-mediated protection. Protection is conferred through tight junctions, mucus production and anti-microbial peptides such as lactoferrin and lysozymes (755). Epithelial cells recognise microbial pathogens through TLRs, triggering innate response pathways that confer protection (756). Cervicovaginal epithelial cells have been shown to release cytokines (757). *In-vitro* stimulation of human vaginal and cervical epithelial cells, leads to the release of pro-inflammatory cytokines including IL-1ß and IL-8 (758). Lipoproteins derived from *N. gonorrhoea* induce the release of IL-8 and IL-6 from human endocervical epithelial cells *in-vitro* following NF-κB activation (759).

The influence of the vaginal microbiota on cervical and vaginal epithelial cell function has been studied. Cytokine assays show that co-culture of ectocervical and endocervical cells with *L. iners* or *G. vaginalis* increased pro-inflammatory cytokine release when compared to *L. crispatus* (757). This suggests that the cervicovaginal microbiota influences host immune response at the cervical interface. Similarly, culture of human vaginal epithelial cells with BV-related bacteria, such as *Atopobium vaginae*, induced a pro-inflammatory immune response characterised by elevated IL-1β, IL8, IL-6 and TNF-α (760). Targeting the cervicovaginal microbiota may improve the cervicovaginal immune milieu, and so reduce sPTB.

Lactobacillus spp. containing oral probiotics show no impact on the cervicovaginal immune milieu. Oral *L. rhamnosus* GR-1 and *L. reuteri* RC-14 in pregnant women at low-risk of sPTB, showed no alterations in CVF IL-4 and IL-10 concentrations in both

placebo and intervention groups (531). Post-menopausal women given vaginal *L. rhamnosus* GR-1 and *L. reuteri* RC-14 also showed no significant difference in CVF IL-1ß, IL-6, TNF-α or GM-CSF concentrations when compared to a placebo group (761).

Studies assessing the impact of LACTIN-V on the local host immune response are limited in that most trials focus on safety, acceptability or impact on the vaginal microbiome. A randomised placebo-controlled phase 2b trial of LACTIN-V showed a significant reduction in BV recurrence with LACTIN-V use (554). A sub-analysis of this cohort investigated whether LACTIN-V altered the CVF immune profile. An association between LACTIN-V and lowered CVF IL-1α concentrations was reported (762).

This chapter presents novel data assessing LACTIN-V impact on the local host immune response in pregnant women at high-risk of sPTB.

5.3 Results

5.3.1 Study population characteristics

Sixty-two women completed the full course of LACTIN-V. The study population characteristics and clinical outcomes are summarised in Tables 4.1 and 4.2 (Chapter 4). From 62 participants, a total of 237 swabs were obtained for CVF cytokine analysis. At T3 and T4, six and five samples were absent respectively. Nine of these samples were missing due to restrictions related to the COVID-19 pandemic. Two samples were missing from two women that delivered prior to T4.

5.3.2 Standardisation for multiple Luminex® assay

Cytokine concentrations were calculated by the xPONENT4.3 software. This was based on the amount of emitted fluorescence, which represented the amount of bound analyte in a directly proportionate trend. Standard concentrations were supplied by the manufacturer and the measured analyte concentrations were compared to the standard ranges.

Due to the high number of samples being analysed by Luminex® assays, 96 well plates were used. CVF cytokine concentrations were determined for a total of 237 samples obtained from 62 women, including T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57). Samples were run in duplicates and the mean was used for analysis. IL-1ß, IL-6 and IL-10 were run neat on the sample multiplex plate and a separate single plex plate was used for IL-8 since the sample needed a 1:10 dilution. Six multiplex and six single plex plates were required to complete the analyses on the total cohort. A pooled calibrator and blank were used on all plates.

To account for inter-assay variation, the co-efficient of variation was calculated for standards and the internal pool calibrator (Table 5.1). A co-efficient of variation of <10% was regarded as an appropriate level of variation between plates. The level of variation between the plates was low at <10% for all analytes (Table 5.1). For IL-1ß, IL-8 and IL-6, all standard curves generated a R^2 of >0.99 (Table 5.2). The R^2 or Coefficient of Multiple Determination is a statistical measure of the proportion of variation on the data points explained by a regression model. An R^2 of 0.99 means that 99% of the variation is explained by the regression model and that 99% of the variation is explained by the variation in the response. An R^2 of 0.0 means that the regression model does not describe the data any further than a horizontal line passing through the average data. An R^2 value of 1.0 means that the curve passes through every standard data point. Overall, an R^2 value of >0.99 indicates excellent assay linearity (577).

Table 5.1 – Coefficient of variation calculated for each analyte. Six standards (S) were made by performing serial dilutions with S1 serving as the highest standard. The internal pool calibrator aimed to assess plate-to-plate variation.

	IL-1ß	IL-8	IL-6	IL-10
S1	6.31	0.64	2.77	2.68
S2	7.09	1.51	4.67	4.01
S3	7.59	0.99	5.20	3.05
S4	7.01	1.30	2.75	4.51
S5	6.28	1.07	3.72	4.71
S6	6.71	0.27	3.63	7.35
Pool	6.93	6.36	5.40	5.88

Table 5.2 – R^2 values. R^2 values for IL-1ß, IL-8, IL-6 and IL-10 on each plate are tabulated.

Plate	IL-1ß	IL-8	IL-6	IL-10
1	0.99641861	0.998928494	0.997912657	0.970983719
2	0.996347845	0.998352868	0.997556272	0.977427014
3	0.997786996	0.999245242	0.999090202	0.987423960
4	0.996485483	0.999492137	0.995019847	0.994860942
5	0.998775275	0.99953077	0.996104711	0.991481604
6	0.998844183	0.99898426	0.998913512	0.984723042

A standard curve was generated for each analyte. If results fell either above or below the standard curve range but within the sensitivity range of the assay, the computer software generated extrapolated values using a five-parameter logistic curve fit (577).

Samples were considered as detectable if the concentration fell within the standard curve, or an extrapolated value was determined by the software. Samples were considered out of range (OOR) if values were outside the limit of detection (sensitivity) of the assay. As summarised in Table 5.3, the most detectable cytokine was IL-8; as all samples were either within the standard curve or extrapolated. For IL-8, 74% were

within the standard range, 26% were extrapolated and no results were OOR. The next most detectable cytokine was IL-1ß with 79% of samples within the standard curve, 15% extrapolated and 6% of results OOR below the lower limit of detection. For IL-6, 24% of samples were measured to be within the standard curve, 40% of results were extrapolated and 36% were OOR below the lower limit of detection. For IL-10, 153 samples (65.5%) were OOR, all of which were below the lower limit of detection. Only 40 (17%) samples were within the standard curve and 44 (18.5%) were extrapolated. Merged plotting of standard curves across all six plates is illustrated in Appendix 5.1 for IL-1ß, IL-8 and IL-6. If analytes were not detectable (OOR), concentrations were assigned to be half the value of the sensitivity level for the assay to avoid introducing a biased exclusion of results.

Table 5.3 – Detection ranges for CVF analytes. Standard curve ranges, sensitivity of detection and assigned OOR values described. Proportions of results within the standard curve, extrapolated and OOR listed. All OOR results were below the lower limit of detection.

	Standard curve range (pg/mL)	Sensitivity (pg/mL)	Assigned OOR value (pg/mL)	Within standard curve (%)	Extrapolated (%)	OOR (%)
IL-1ß	17.7 - 4300	0.8	0.4	186 (79)	36 (15)	15 (6)
IL-8	4.12 - 1000	1.8	0.9	176 (74)	61 (26)	0 (0)
IL-6	4.53 - 1100	1.7	0.85	57 (24)	95 (40)	85 (36)
IL-10	1.89 - 460	1.6	0.8	40 (17)	44 (18.5)	153 (65.5)

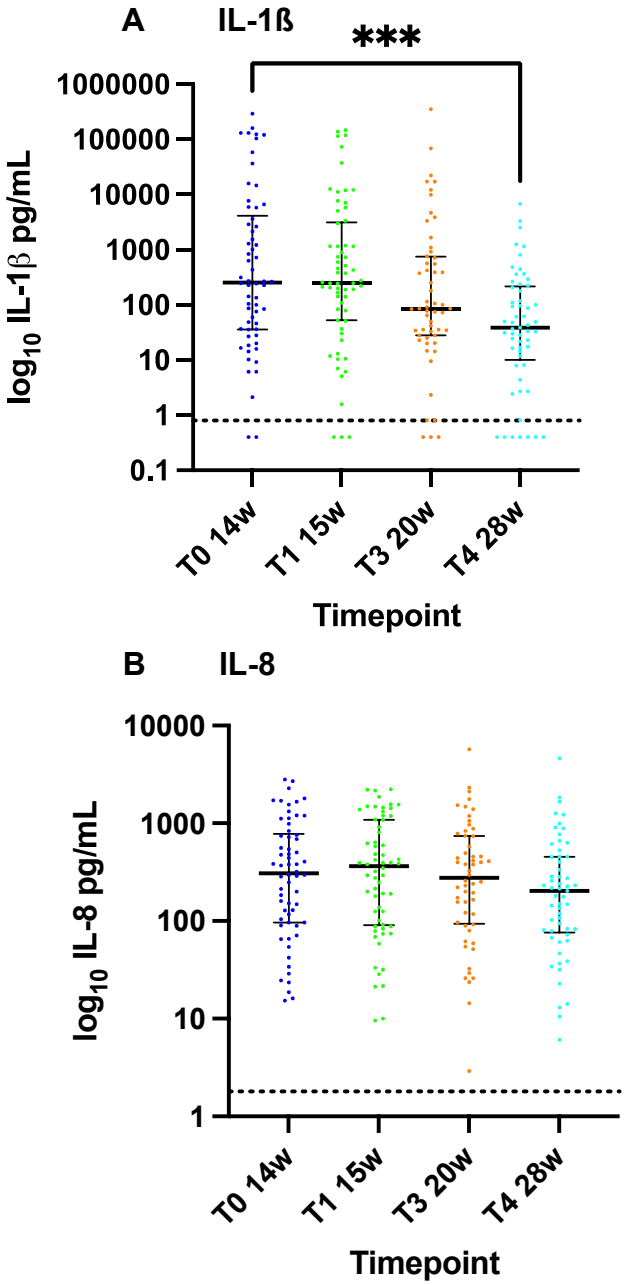
Previous work from within the research team has utilised the Bradford protein assay to measure total cellular protein in each sample (pg/mL) (763). As part of this, the concentration of protein in each sample was calculated per analyte. The concentration of protein per sample was calculated by multiplying the concentration of protein (Bradford assay) with the volume of sample used on the assay. After, the concentration of analyte in pg/mL/µg was determined by dividing raw observed concentrations (Luminex) by the concentration of protein (Bradford Assay). As work from within our research team has previously shown there to be no differences between raw and normalised concentrations for each of the studied analytes, in this chapter raw concentrations are presented. Additionally, cytokine concentrations are reported as pg/mL rather than pg/swab to reduce swab-to-swab variation, allowing for reliable comparison of analyte concentrations between swabs.

5.3.3 CVF cytokine response to LACTIN-V

To assess whether LACTIN-V influences CVF cytokine production, concentrations at T1, T3 and T4 were compared to concentrations prior to LACTIN-V use, at T0. Figures 5.1, 5.2 and 5.3 show cytokine concentrations across the study timepoints. Data presented in Figures 5.1A-B, 5.2A and 5.3A are presented as median values with interquartile ranges (IQR) and statistical analyses comparing concentrations at T0 with concentrations at subsequent timepoints were performed using a one-way ANOVA Kruskal-Wallis Test, with Dunn's correction. The dotted lines in Figures 5.1A-B, 5.2A and 5.3A represent the sensitivity of detection for respective cytokines. One-way Chi-squared analysis was used to compare detectability of IL-6 and IL-10 in Figures 5.2B-C and 5.3B-C.

IL-1ß concentrations are illustrated in Figure 5.1A. The median IL-1ß concentrations were 254.4, 251.4, 85.6 and 38.8 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. IL-1ß concentrations at T1, T3 and T4 were compared to baseline concentrations at T0. Between T0 and T1, median IL-1ß concentrations were stable and there was no significant difference in IL-1ß concentrations at T0 versus T1 ($p>0.9999$). From T3, the median IL-1ß concentration reduced. There was no significant difference in IL-1ß concentrations at T0 versus T3 ($p>0.3276$). By T4 the reduction in IL-1ß concentrations reached statistical significance ($p=0.0002$).

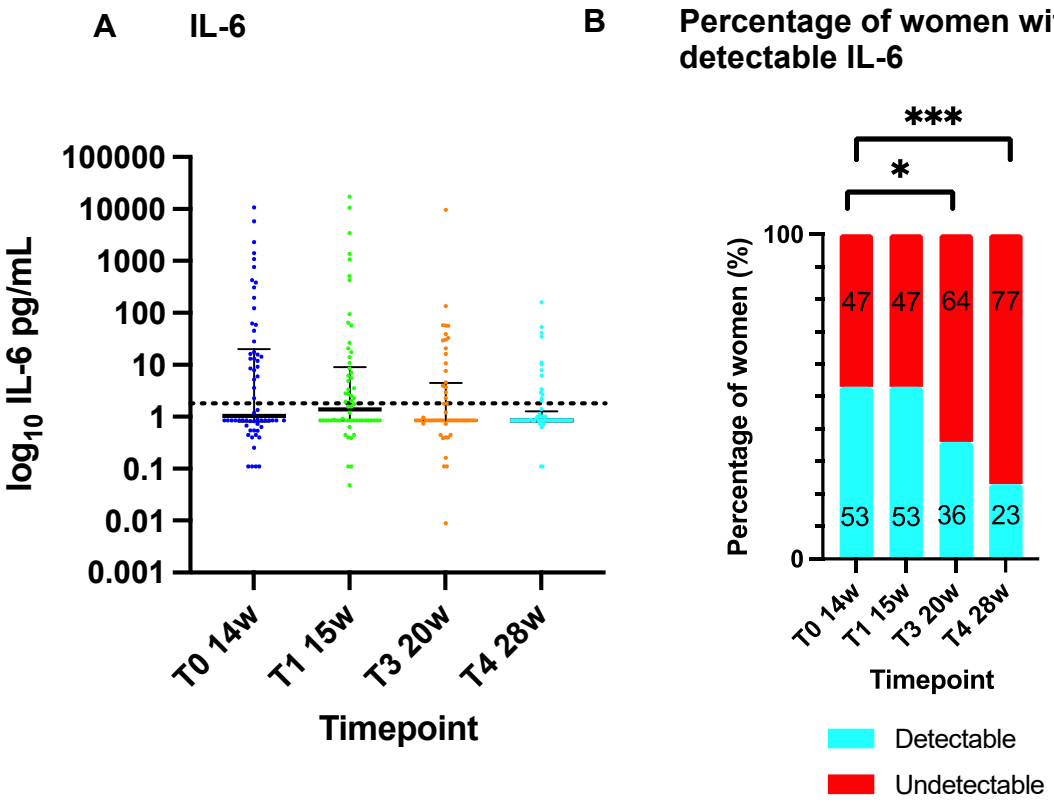
IL-8 concentrations across the study period are shown in Figure 5.1B. The median IL-8 concentrations were 307.4, 364.3, 277.0 and 204.1 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. The median IL-8 concentration increased at T1, although there was no significant increase in IL-8 concentrations at T1 when compared to T0 ($p>0.9999$). From T3, the median IL-8 concentration reduced. The median IL-8 concentration reduced further at T4. When compared to T0 the reductions in IL-8 concentrations were not significant at T3 ($p>0.9999$) or T4 ($p=0.2384$).



Figures 5.1A-B – IL-1ß and IL-8 response to LACTIN-V. (A) IL-1ß concentrations across timepoints. The median IL-1ß concentrations were 254.4, 251.4, 85.6 and 38.8 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. There was no significant difference between IL-1ß concentrations at T0 versus T1 (p>0.9999) or T3 (p>0.3276). There was a significant difference between T0 and T4 (p=0.0002). **(B)** IL-8 concentrations across timepoints. The median IL-8 concentrations were 307.4, 364.3, 277.0 and 204.1 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. There was no significant difference in IL-8 concentrations at T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.2384) when compared to T0. Data is presented as median values with IQR. Dotted lines represent the sensitivity of detection level. Statistics: one-way ANOVA Kruskal-Wallis Test, with Dunn's correction. ***p<0.001. (w=weeks gestation)

Figures 5.2A-C demonstrate the IL-6 response to LACTIN-V. The median IL-6 concentrations were 1.034, 1.383, 0.85 and 0.85 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively (Figure 5.2A). The median IL-6 concentration increased at T1, after which there was a reduction at T3 that was maintained at T4. There was no significant difference between IL-6 concentrations at T1 ($p>0.9999$), T3 ($p=0.7201$) or T4 ($p=0.4243$) when compared to baseline.

As a high proportion of IL-6 results were below the sensitivity of detection, as represented by the dotted line in Figure 5.2A, IL-6 was analysed based on detectability (Figure 5.2B-C). Detectability was defined as concentrations above the sensitivity of detection. The proportion of women with detectable IL-6 was unchanged from T0 to T1, with 33 women (53%) having detectable IL-6 at both T0 and T1 ($p=0.5000$) (Figure 5.2B-C). The proportion of women with detectable IL-6 reduced at T3 and T4 where 20 women (36%) and 13 women (23%) had detectable IL-6 respectively. When compared with T0, the decline in detectable IL-6 reached statistical significance at T3 ($p=0.0281$) and T4 ($p=0.0003$). Raw numbers, percentages and p values are summarised in Figure 5.2C.

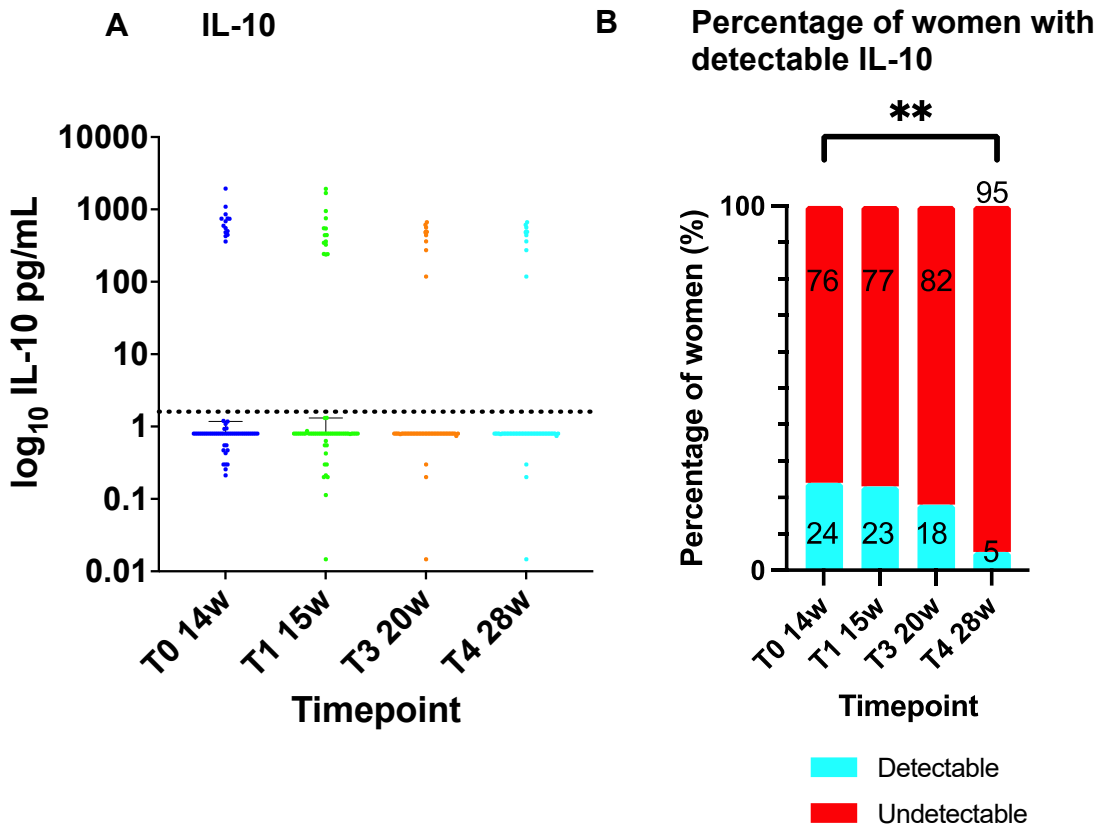


C Number of women with detectable IL-6

Timepoint	Detectable (%)	Undetectable (%)	Total	P value
T0	33 (53)	29 (47)	62	
T1	33 (53)	29 (47)	62	0.5000
T3	20 (36)	36 (64)	56	0.0281*
T4	13 (23)	44 (77)	57	0.0003***

Figures 5.2A-C – IL-6 response to LACTIN-V. (A) IL-6 concentrations across timepoints. IL-6 median concentrations were 1.034, 1.383, 0.85 and 0.85 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. There was no significant difference between IL-6 concentration at T0, versus T1 (p>0.9999), T3 (p=0.7201) or T4 (p=0.4243). Data is presented as median values with IQR. The dotted line represents the sensitivity of detection level. Statistics: one-way ANOVA Kruskal-Wallis Test, with Dunn's correction. **(B)** Percentage of women with detectable IL-6. There was no significant difference in the number of women with detectable IL-6 at T0 versus T1 (p=0.5000). Significantly less women had detectable IL-6 at T3 (p=0.0281) and T4 (p=0.0003) when compared with T0. Statistics: one-way Chi-squared **(C)** Tabulated data describing the number of women with detectable IL-6 from T0 to T4. *p<0.05; ***p<0.001. (w=weeks gestation)

Figures 5.3A-C demonstrate the IL-10 response to LACTIN-V. The median IL-10 concentration was 0.8 pg/mL at all timepoints. Analysis was performed for n=62, n=62, n=56 and n=57 at T0, T1, T3 and T4 respectively. There were no significant differences in IL-10 concentrations at T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p>0.9999$) when compared to T0. As a high proportion of IL-10 results were below the sensitivity of detection, similarly to IL-6, the IL-10 was analysed according to detectability (Figure 5.3B-C). At T0 15 women (24%) had detectable IL-10. Similarly, at T1 14 women (23%) had detectable IL-10. T0 and T1 IL-10 detectability did not significantly differ ($p=0.4160$). By T3, the proportion of women with detectable IL-10 reduced to 10 women (18%) ($p=0.2001$). At T4 a further decrease in IL-10 detectability was seen, with IL-10 detectability in 3 women (5%). When compared with T0, the decrease in IL-10 detectability at T4 was significant ($p=0.0020$). Raw numbers, percentages and p values are summarised in Figure 5.3C. As most IL-10 results (153, 65.5%) were OOR and assigned a value of 0.8 pg/mL, no further IL-10 analysis was performed.



C Number of women with detectable IL-10

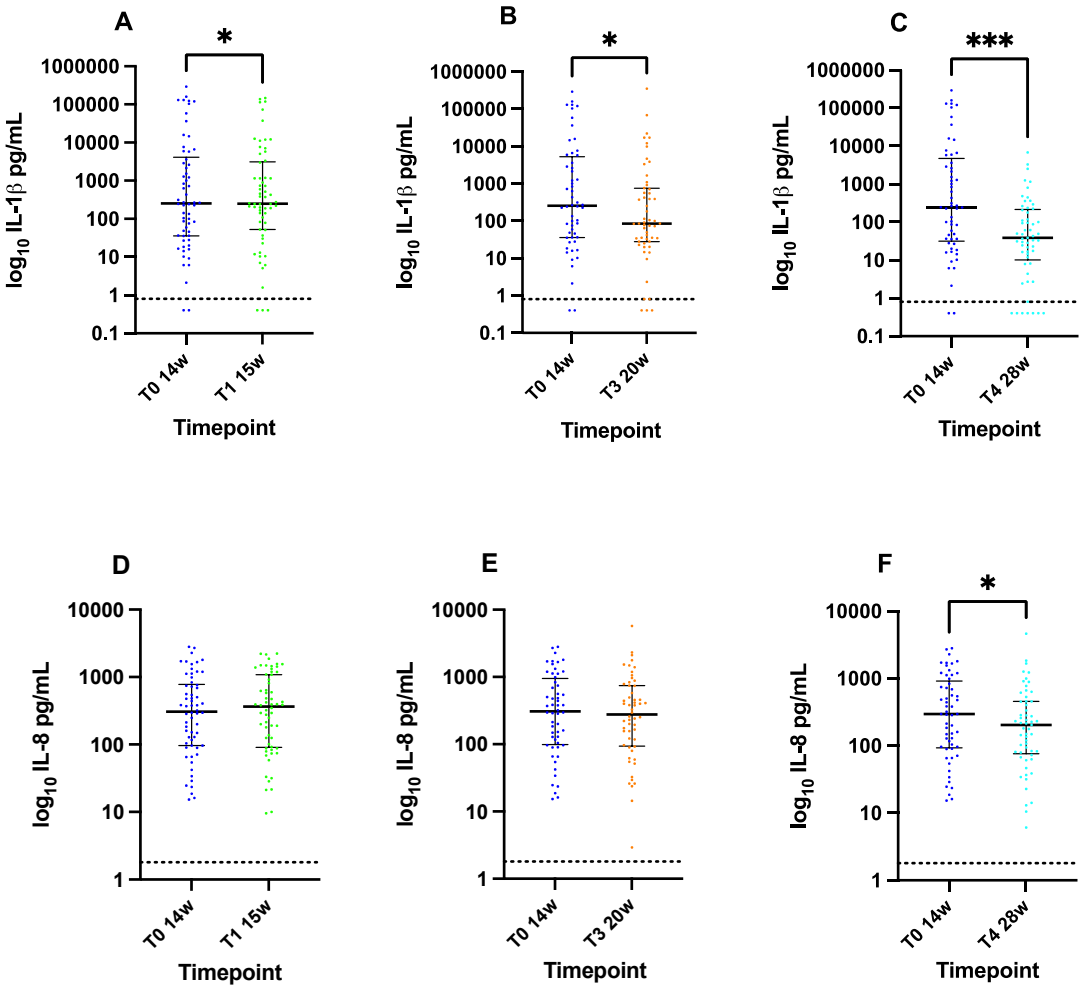
Timepoint	Detectable (%)	Undetectable (%)	Total	P value
T0	15 (24)	47 (76)	62	
T1	14 (23)	48 (77)	62	0.4160
T3	10 (18)	46 (82)	56	0.2001
T4	3 (5)	54 (95)	57	0.0020

Figures 5.3A-C – IL-10 response to LACTIN-V. (A) IL-10 concentrations across timepoints. IL-10 median concentrations were 0.8, 0.8, 0.8 and 0.8 pg/mL at T0 (n=62), T1 (n=62), T3 (n=56) and T4 (n=57) respectively. There were no significant differences between IL-10 at T0 versus T1 (p>0.9999), T3 (p>0.9999) or T4 (p>0.9999). Data is presented as median values with IQR. The dotted line represents the sensitivity of detection level. Statistics: one-way ANOVA Kruskal-Wallis Test, with Dunn's correction. **(B)** Percentage of women with detectable IL-10. A one-way Chi-squared analysis was used for analysing IL-10 detectability. There was no significant difference in the number of women with detectable IL-10 at T0 versus T1 (p>0.4160) or versus T3 (p=0.2001). At T4 IL-10 detectability decreased when compared to T0 (p<0.0020). Statistics: one-way Chi-squared **(C)** Tabulated data describing the number of women with detectable IL-10 from T0 to T4. **p<0.01. (w=weeks gestation)

Due to 11 missing samples (six missing samples from T3 and five missing samples at T4), matched analysis could not be performed for the whole cohort. Nine of these missing samples were due to COVID-19 sample attrition. Two T4 samples were absent from two participants that delivered before T4 (MTL at 19^{+6} weeks' gestation and sPTB at 27^{+5} weeks' gestation). A matched analysis comparing cytokine concentrations at T1 (n=62), T3 (n=56) and T4 (n=57) with concentrations at T0 was performed (Figure 5.4A-F, 5.5A-F). To retain matched data and avoid the exclusion of women delivering preterm, data at each timepoint is presented in separate graphs. To assess differences between matched samples, a Wilcoxon signed rank test was used. Matched analysis was performed at T0 versus T1 (n=62), T0 versus T3 (n=56), and T0 versus T4 (n=57).

Matched analysis of IL-1ß is shown in Figures 5.4A-C. Although the median concentrations at T0 (254.4 pg/mL) compared to T1 (251.4 pg/mL) were similar, a significant reduction was seen from T0 to T1 (p=0.0363) (Figure 5.4A). Comparison of results at T0 (median 256.1 pg/mL) and T3 (median 85.6 pg/mL) also showed a significant reduction at T3 (p=0.0254) (Figure 5.4B). Matched data at T0 (median 276.4 pg/mL) and T4 (median 38.8 pg/mL) were compared and showed a significant reduction by T4 (p=0.0002) (Figure 5.4C).

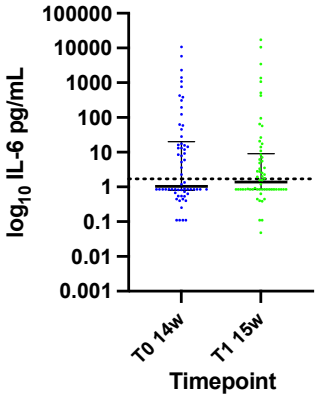
Matched analysis of IL-8 is shown in Figures 5.4D-F. The median IL-8 concentration at T0 (307.4 pg/mL) increased slightly at T1 (364.3 pg/mL). Between T0 and T1 there were no significant changes in IL-8 concentrations (p=0.3129) (Figure 5.4D). Comparison of matched IL-8 concentrations at T0 and T3 showed median IL-8 to decrease at T3 from 307.4 pg/mL at T0 to 277.0 pg/mL (p=0.2494) (Figure 5.4E). The median IL-8 concentration reduced from 295.1 pg/mL at T0 to 204.1 pg/mL at T4. This reduction was significantly lower (p=0.0191) (Figure 5.4F).



Figures 5.4A-F – Matched analysis of IL-1ß and IL-8 concentrations. (A) IL-1ß concentrations at T0 versus T1 (n=62). The median IL-1ß concentrations at T0 and T1 were 254.4 pg/mL and 251.4 pg/mL respectively **(B)** IL-1ß concentrations at T0 versus T3 (n=56). The median IL-1ß concentrations at T0 and T3 were 256.1 pg/mL and 85.6 pg/mL respectively. **(C)** IL-1ß concentrations at T0 versus T4 (n=57). The median IL-1ß concentrations at T0 and T4 were 276.4 pg/mL and 38.8 pg/mL respectively. IL-1ß concentrations were statistically lower at T1 (p=0.0363), T3 (p=0.0254) and T4 (p=0.0002) when compared with T0. **(D)** IL-8 concentrations at T0 versus T1 (n=62). The median IL-8 concentrations at T0 and T1 were 307.4 pg/mL and 364.3 pg/mL respectively. **(E)** IL-8 concentrations at T0 versus T3 (n=56). The median IL-8 concentrations at T0 and T3 were 307.4 pg/mL and 277.0 pg/mL respectively. **(F)** IL-8 concentrations at T0 versus T4 (n=57). The median IL-8 concentrations at T0 and T4 were 295.1 pg/mL and 204.1 pg/mL respectively. No significant difference in IL-8 at T0 versus T1 (p=0.3129) or T3 (p=0.2494) occurred. When compared with T0, IL-8 at T4 was significantly lower (p=0.0191). Dotted lines represent the sensitivity of detection level. Data is presented as median values with IQR. Statistics: Wilcoxon signed rank testing. *p<0.05; ***p<0.001. (w=weeks gestation)

Matched analysis of IL-6 concentrations (Figures 5.5A-F) was also performed. T0 and T1 IL-6 concentrations (Figure 5.5A) and detectability (Figure 5.5B) were compared. Median IL-6 concentrations at T0 and T1 were 1.0 pg/mL and 1.4 pg/mL respectively (p=0.4318). There was also no significant difference in IL-6 detectability between T0 and T1 (p=0.4287). Comparison at T0 and T3 showed that the median IL-6 concentration was significantly lower at T3 (0.85 pg/mL) when compared to T0 (1.3 pg/mL) (p=0.0139) (Figure 5.5C). IL-6 detectability did not differ significantly between T0 and T3 (p=0.0901) (Figure 5.5D). The median IL-6 concentration was 0.85 pg/mL at both T0 and T4, but matched analysis showed a significant reduction by T4 (p=0.0055) (Figure 5.5E). At T4, significantly less women had detectable IL-6 when compared to T0 (p=0.0355) (Figure 5.5F).

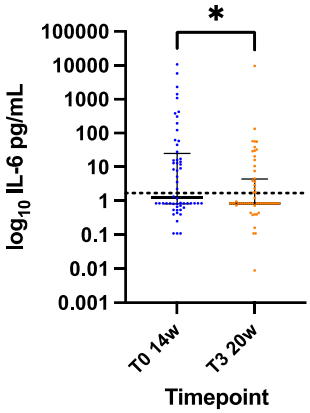
A



B

Timepoint (n=62)	Detectable (%)	Undetectable (%)	P value
T0	31 (50)	31 (50)	
T1	32 (52)	30 (48)	0.4287

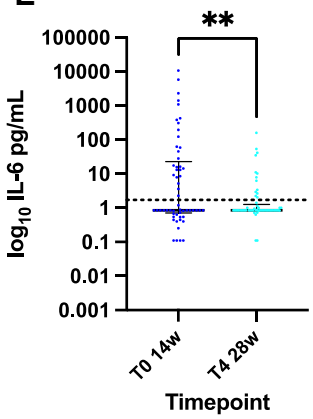
C



D

Timepoint (n=56)	Detectable (%)	Undetectable (%)	P value
T0	27 (48)	29 (52)	
T3	20 (36)	36 (64)	0.0901

E



F

Timepoint (n=57)	Detectable (%)	Undetectable (%)	P value
T0	26 (46)	31 (54)	
T4	13 (23)	44 (77)	0.0355*

Figures 5.5A-F – Matched analysis of IL-6 concentrations and detectability. (A) IL-6 concentrations at T0 (median 1.0 pg/mL) and T1 (median 1.4 pg/mL) (n=62) (p=0.4318). **(B)** Tabulated data for IL-6 detectability at T0 and T1. At T0, 31 women (50%) had detectable IL-6 and at T1, 32 women (52%) had detectable IL-6 (p=0.4287). **(C)** IL-6 concentrations at T0 (median 1.3 pg/mL) and T3 (median 0.85 pg/mL) (n=56) (p=0.0139). **(D)** Tabulated data for IL-6 detectability at T0 and T3. At T0 27 women (48%) had IL-6 detectable, decreasing to 20 women (36%) at T3 (p=0.0901). **(E)** IL-6 concentrations at T0 (median 0.85 pg/mL) and T4 (median 0.85 pg/mL) (n=57) (p=0.0055). **(F)** Tabulated data for IL-6

detectability at T0 and T4. At T0 26 women (46%) had detectable IL-6, decreasing to 13 women (23%) at T4 (p=0.0355). Dotted lines represent the sensitivity of detection level. Data is presented as median values with IQR. Statistics: one-tailed Wilcoxon signed rank test (matched analysis). Differences in IL-6 detectability were analysed using a one-way Chi-squared analysis. *p<0.05; **p<0.01. (w=weeks gestation)

5.3.4 Cytokine response according to starting CST

Cytokine response according to starting CST was explored. It was hypothesised that women starting with CST III or IV would demonstrate a greater cytokine response to LACTIN-V.

IL-1ß

The plot colours represent the CST of individual participants at corresponding timepoints. Dark green, red and light green plots represent CST I, III/IV and II/V respectively. Figure 5.6A demonstrates that women with CST I at T0, mostly maintain their CST with LACTIN-V use.

At T0, CST I was observed in 27 women (44%). In this group of women, an increase in IL-1ß concentrations was observed from T0 (n=27, median 35.6 pg/mL) to T1 (n=27, median 222.3 pg/mL) (Figure 5.6A). At T3 (n=26, median 85.6 pg/mL) and T4 (n=27, median 29.9 pg/mL), the IL-1ß concentrations reduced. The median IL-1ß concentration at T4 was comparable to that at T0. Overall, there was no significant difference in IL-1ß concentrations when T1 (p=0.9353), T3 (p>0.999) and T4 (p=0.5684) were compared to T0.

At T3, only one woman (F017) changed to CST III, which persisted for this participant at T4. By T4 a second participant (F023) displayed CST III. As illustrated in Figure 5.6A, these two participants that changed to CST III displayed high IL-1ß concentrations that were above the median. Participant F017 had an IL-1ß concentration of 381.8 and 809.2 pg/mL at T3 and T4 respectively. Participant F023 had an IL-1ß concentration of 236.0 pg/mL at T4. The change to CST III was not clinically significant for these two participants who delivered at term.

At T0, CST III was observed in 15 women (Figure 5.6B). The median IL-1ß concentrations at T0 (n=15), T1 (n=15), T3 (n=15) and T4 (n=14) were 718.9, 383.4, 84.6 and 38.4 pg/mL respectively. Women starting with CST III, had a higher starting median IL-1ß concentration, when compared to women starting with CST I (35.6 pg/mL). Figure 5.6B demonstrates a decline in IL-1ß median concentrations with LACTIN-V use. Although there was no significant difference in IL-1ß concentrations between T0 and T1 (p>0.9999) or T3 (p=0.1552), at T4 the IL-1ß concentrations were significantly lower (p=0.0014).

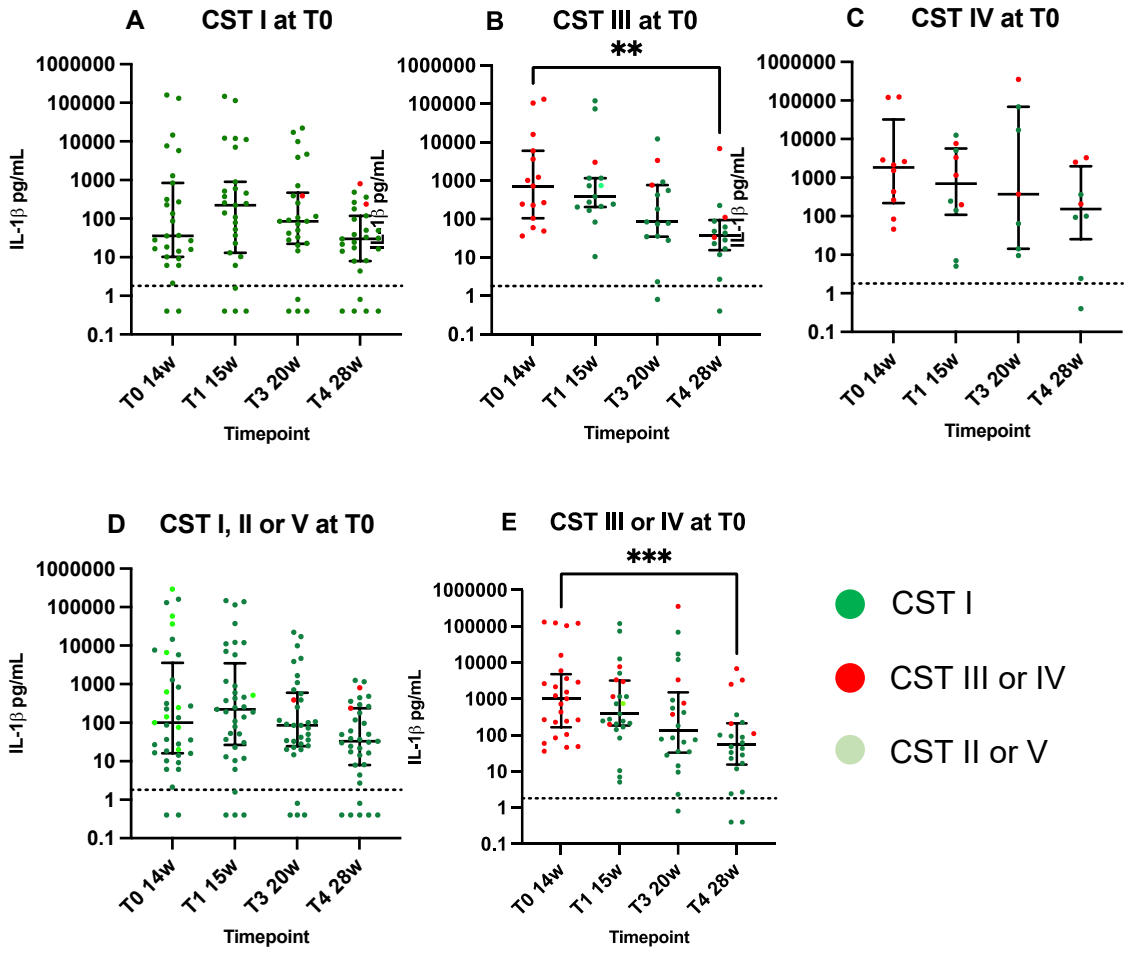
Figure 5.6B also demonstrates a trend for women to switch to CST I with LACTIN-V use, as shown by the increase in dark green plots over time. In total, 13 (87%) women switched to CST I at T1. At T1 one participant (F044) switched to CST V (741.7 pg/mL) and one participant (F021) maintained CST III (3019.7 pg/mL). At T3, F021 continued to maintain CST III (761.5 pg/mL) but by T4 converted to CST I (2.7 pg/mL). A second participant reverted to CST III at T3 (F026, 3335.9 pg/mL), which persisted at T4 (6775.4 pg/mL). At T4, two additional participants switched to CST III (F057, 110.5 pg/mL and F043, 33.3 pg/mL). Interestingly, except for participant F043, women retaining CST III at subsequent timepoints displayed high IL-1ß concentrations, above the median.

Figure 5.6C summarises IL-1ß concentrations in women starting with CST IV. The median IL-1ß concentrations at T0 (n=10), T1 (n=10), T3 (n=7) and T4 (n=8) were 1842.0, 705.5, 374.1 and 154.0 pg/mL respectively. In this group of women, the median IL-1ß concentrations were also higher at T0 when compared to the T0 median women with a starting CST I (median 35.6 pg/mL). A trend for IL-1ß concentrations to drop across the study period was seen, although significance at T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.2994) was not reached.

Figure 5.6C also shows that by T4, five women switched to CST I. At T1 four women failed to switch their CSTs (F039, F035, F049 and F054). Interestingly, by T4 three of these women retained their starting CST (F039, F035 and F049), one of which had a spontaneous PTB at 35^{+5} (F039). The other two had iatrogenic PTBs secondary to obstetric cholestasis and pre-eclampsia.

In Figure 5.6D, sub-group analysis of women starting with CST I, II or V (n=37) was performed to categorise women with a low-risk vaginal microbial composition. At T0, T1, T3 and T4 analysis included n=37, n=37, n=34 and n=35 women respectively. The median IL-1ß concentrations were 100.2, 222.3, 85.6 and 33.0 pg/mL at T0, T1, T3 and T4 respectively. An increase in IL-1ß concentrations was observed at T1, with a gradual return to baseline levels at T3 and a trend to reduce further at T4. There was no significant difference in IL-1ß concentrations at T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p=0.1257$) when compared to T0. Figure 5.6D shows that by T4, all except for two women had CST I observed.

The IL-1ß response in women starting with CST III or IV was performed to categorise women with a high-risk vaginal microbial composition (Figure 5.6E). Results are shown for n=25 at T0-T1 and n=22 at T3-T4. Median IL-1ß concentrations From T0-T4 were 1010.0, 383.4, 132.5 and 54.9 pg/mL respectively. Overall, a reduction in median IL-1ß concentrations was seen with LACTIN-V use. Although there was no significant difference between IL-1ß concentrations at T0 and T1 ($p>0.9999$) or T3 ($p=0.1224$), statistical significance was reached by T4 ($p=0.0006$). By T4, most women 17/22, (77%) switched to CST I. As shown in Figure 5.6E, women that did not switch to CST I by T4 displayed higher IL-1ß concentrations.



Figures 5.6A-E IL-1ß response according to starting CST (A) CST I at T0. Data is reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. Median IL-1ß concentrations were 35.6, 222.3, 85.6 and 29.9 pg/mL at T0-T4 respectively. No significant difference was seen when T0 was compared with T1 (p=0.9353), T3 (p>0.999) or T4 (p=0.5684). **(B)** CST III at T0. Data is reported for n=15 at T0, T1 and T3 and n=14 at T4. The median IL-1ß concentrations at T0, T1, T3 and T4 were 718.9, 383.4, 84.6 and 38.4 pg/mL respectively. No significant difference was seen when T0 was compared with T1 (p>0.9999) and T3 (p=0.1552). Statistical significance was reached at T4 (p=0.0014). **(C)** CST IV at T0. Data is reported for n=10 at T0 and T1, n=7 at T3 and n=8 at T4. The median IL-1ß concentrations were 1842.0, 705.5, 374.1 and 154.0 pg/mL at T0-T4 respectively. No significant difference was seen when T0 was compared with T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.2994). **(D)** CST I/II/V at T0. Data is reported for n=37 at T0 and T1, n=34 at T3 and n=35 at T4. The median IL-1ß concentrations were 100.2, 222.3, 85.6 and 33.0 pg/mL at T0, T1, T3 and T4 respectively. There was no significant difference in concentrations at T0 versus at T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.1257). **(E)** CST III/IV at T0. Data is reported for n=25 at T0-T1 and n=22 at T3-T4. Median IL-1ß concentrations were 1010.0, 383.4, 132.5 and 54.9 pg/mL at T0-T4 respectively. At T4 IL-1ß concentrations were significantly lower

(p=0.0006). At T1 and T3 a reduction in concentrations was observed, although statistical significance was not met (p>0.9999 and p=0.1224 respectively). Statistics: Kruskal-Wallis test with Dunn's correction. Dotted lines represent the sensitivity of detection for IL-1ß. Data is presented as median values with IQR. **p<0.01; ***p<0.001. (w=weeks gestation)

IL-8

The IL-8 response according to starting CST is summarised in Figures 5.7A-E.

Figure 5.7A shows the IL-8 response in women with CST I at T0. At T0 (n=27) the median IL-8 concentration was 183.4 pg/mL. The median IL-8 concentration increased slightly upon completion of the loading dose at T1 (n=27) (median 248.4 pg/mL). At T3 (n=26) the median IL-8 concentration reduced to 179.7 pg/mL and reduced further to 120.2 pg/mL by T4 (n=27). By T4, the median IL-8 concentration was comparable to baseline. There was no significant difference in the IL-8 concentrations at T0 when compared with T1 (p>0.9999), T3 (p>0.9999), or T4 (p=0.7145).

Figure 5.7A also demonstrates that where CST III was observed for one participant at T3 (F017) and two women at T4 (F017 and F023), high IL-8 concentrations above the median values were seen. The IL-8 concentration for F017 was 1060.9 and 1661.3 pg/mL at T3 and T4 respectively. For F023 the IL-8 concentration was 901.2 pg/mL at T4.

15 women started with CST III (Figure 5.7B). At T0 the median IL-8 concentration in this group of women was 370.5 pg/mL. The IL-8 concentration increased at T1 (n=15) to 582.0 pg/mL, before slightly reducing at T3 (n=15) to 455.4 pg/mL. By T4 (n=14), the median IL-8 concentration reduced to 223.4 pg/mL, lower than the median at T0. Although the median IL-8 concentrations reduced by T4, this was preceded by an initial increase in the median IL-8 concentration at T1. There was, no statistical difference in the IL-8 concentrations at T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.5088) when compared to baseline.

Like Figure 5.6B, Figure 5.7B highlights that most women (11, 79%) starting with CST III transitioned to CST I by T4. Participant F026 reverted to CST III at T3 and T4 and

displayed the highest IL-8 concentrations recorded within the cohort at T3 (5744.8 pg/mL) and at T4 (4614.0 pg/mL). Participant F021 had CST III transiently observed at T3, but by T4 demonstrated *L. crispatus* dominance. At T4, three participants showed CST III, including the participant F026, as well as participants F057 (IL-8 concentration 532.6 pg/mL) and F043 (IL-8 concentration 81.23 pg/mL).

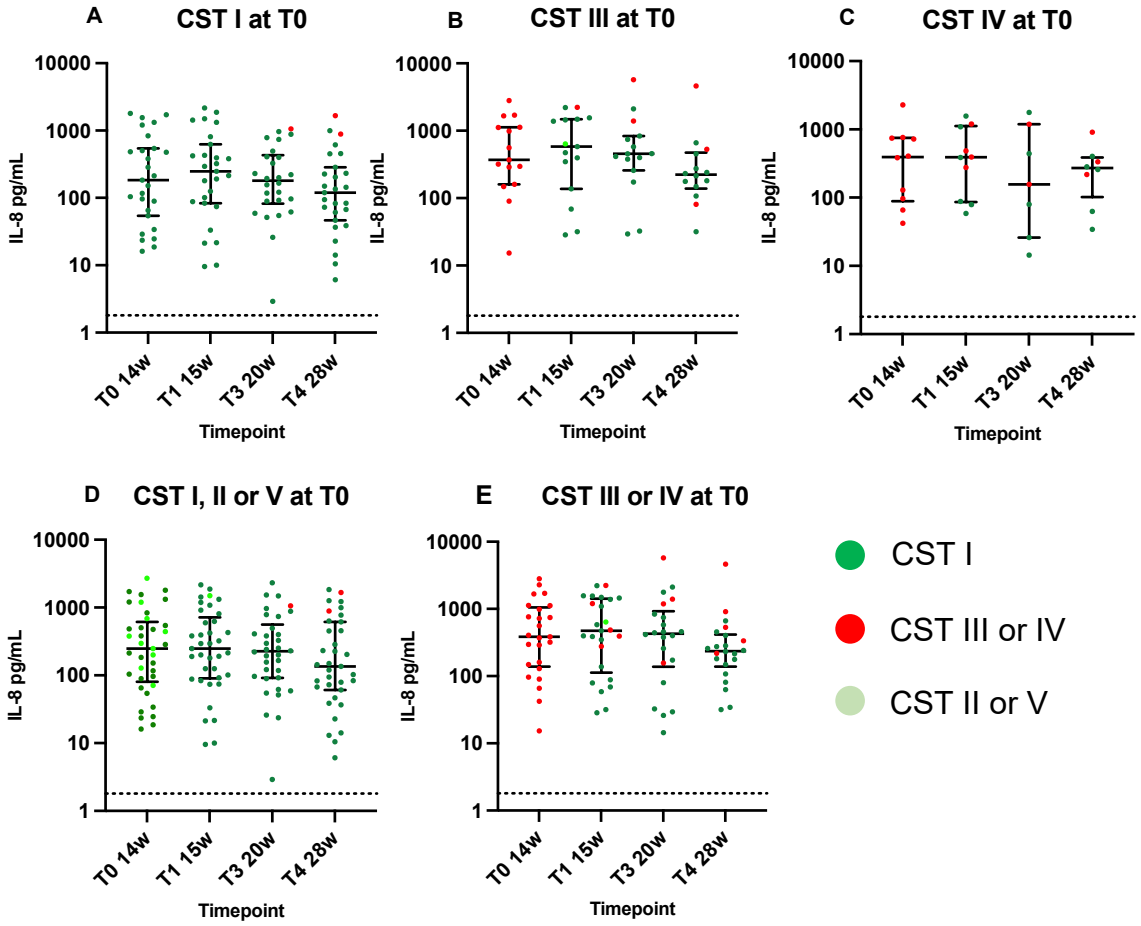
For women starting with CST IV (Figure 5.7C), the median IL-8 concentrations were 394.4, 390.2, 156.9 and 271.3 pg/mL at T0 (n=10), T1 (n=10), T3 (n=7) and T4 (n=8) respectively. The median IL-8 concentrations remained stable between T0 to T1, before reducing at T3, followed by an upward trend by T4. There was no significant difference in IL-8 concentrations at T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p>0.9999$), when compared with T0. By T4, most women displayed an optimised vaginal microbial profile to CST I (5, 63%).

In Figure 5.7D, sub-group analysis of women with a low-risk risk vaginal microbial composition at baseline (CST I, II or V) was performed. Data is reported for n=37 at T0 and T1, n=34 at T3 and n=35 at T4. Median IL-8 concentrations were 248.3, 248.4, 226.2 and 135.0 pg/mL at T0 to T4 respectively. Between T0, T1 and T3, the median IL-8 concentrations remained stable, after which, a decrease in the median IL-8 concentration occurred T4. Comparison with T0 showed no significant difference at T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p=0.6950$).

In Figure 5.7E, sub-group analysis of women with a high-risk vaginal microbial composition at baseline (CST III, IV) was performed. Data was reported for n=25 at T0-T1 and n=22 at T3-T4. The median IL-8 concentrations were 383.5, 473.8, 427.4 and 236.0 pg/mL at T0-T4 respectively. In this group of women, an increase in the median IL-8 concentration was seen at T1. At T3, the median IL-8 concentration began to fall. By T4 the median IL-8 concentration was lower than the median at T0. Statistical analysis showed no significant difference in IL-8 concentrations at T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p=0.3889$) when compared to T0.

Once again, in Figure 5.7E women starting with a sub-optimal CST are seen to shift to a more protective state. By T4 only five women displayed a sub-optimal CST. For three of these women, the IL-8 concentrations were much higher than the median IL-8 value

(F026 4615.0 pg/mL, F035 908.4 pg/mL and F057 532.6 pg/mL). However, for two women, IL-8 concentrations were close to the median value (F039 335.8 pg/mL, F049 218.2 pg/mL).



Figures 5.7A-E IL-8 response according to starting CST (A) IL-8 CST 1 at T0. Data is reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. Median concentrations were 183.4, 248.4, 179.7 and 120.2 pg/mL at T0-T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.7145). **(B)** IL-8 CST III at T0. Data is reported for n=15 at T0-T3 and n=14 at T4. Median IL-8 concentrations were 370.5, 582.0, 455.4 and 223.4 pg/mL at T0-T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p>0.0.9999) or T4 (p=0.5088). **(C)** IL-8 CST IV at T0. Data is reported for n=10 at T0-T1, n=7 at T3 and n=8 at T4. Median IL-8 concentrations were 394.4, 390.2, 156.9 and 271.3 pg/mL at T0-T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p>0.9999) or T4 (p>0.9999). **(D)** IL-8 CST I/II/IV at T0. Data was reported for n=37 at T0-T1 and n=34 at T3 and n=35 at T4. The median IL-8 concentrations were 248.3, 248.4, 226.2 and 135.0 pg/mL at T0 to T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.6950). **(E)** CST III/IV at T0. Data was reported for n=25 at T0-T1 and n=22 at T3-T4. The median IL-8 concentrations were 383.5, 473.8, 427.4 and 236.0 pg/mL at T0-T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p>0.9999) or T4 (p=0.3889). Statistics: Kruskal-Wallis test with Dunn's correction. Dotted lines represent the sensitivity of detection for IL-8. Data is presented as median values with IQR. (w=weeks gestation)

IL-6

IL-6 concentrations are demonstrated in Figure 5.8A-E according to starting CST.

In women with CST I at T0 (Figure 5.8A), the median IL-6 concentrations were stable at 0.85 pg/mL across all timepoints. Results are reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. There was no statistical difference in IL-6 concentrations at T1, T3 or T4 ($p > 0.9999$).

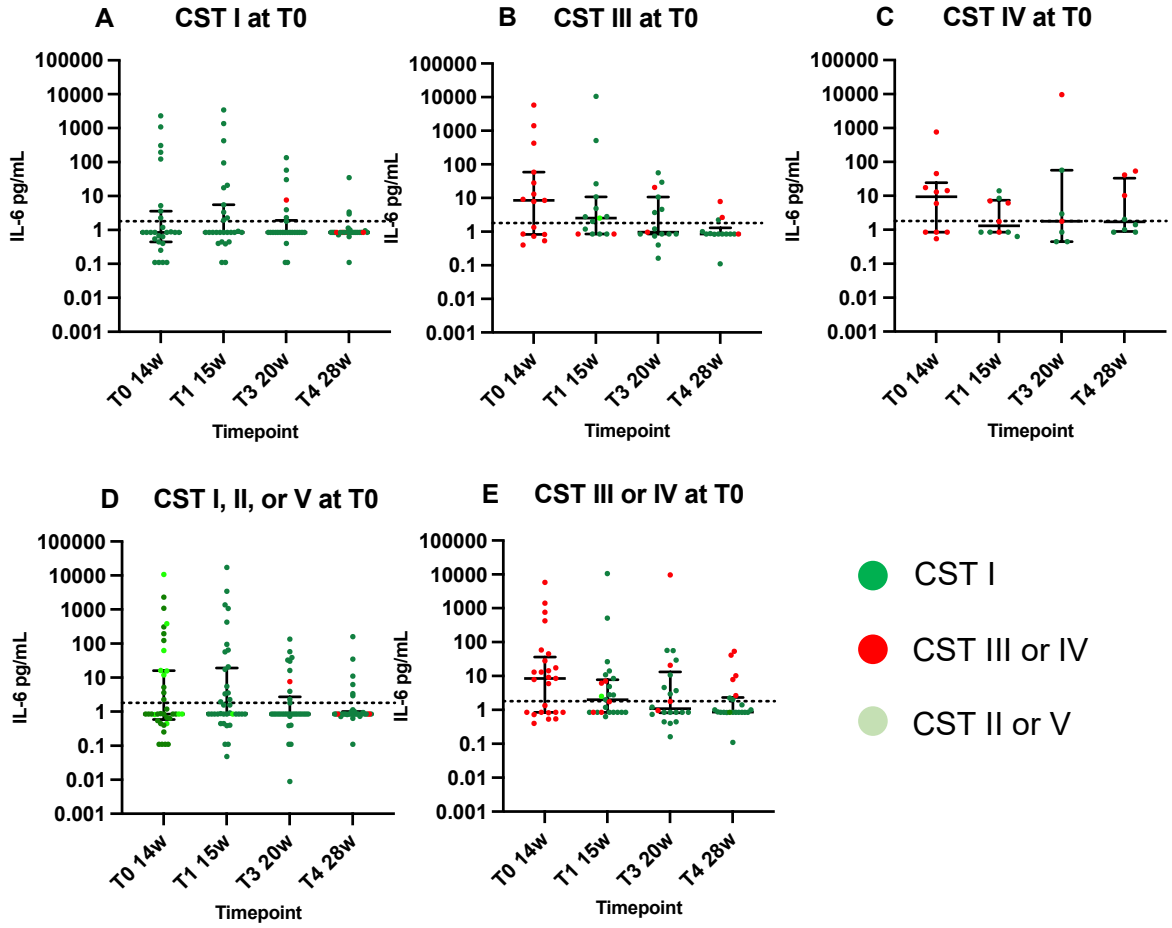
Women starting with CST III are shown in Figure 5.8B. Results are reported for n=15 at T0-T3 and n=14 at T4. The median IL-6 concentrations were 0.85, 2.5, 0.9 and 0.85 pg/mL at T0, T1, T3 and T4 respectively. Although the median IL-6 concentration increased at T1, a reduction back to baseline levels was seen by T4. There was no significant difference in IL-6 concentrations at T1 ($p>0.9999$), T3 ($p=0.7895$) or T4 ($p=0.1042$). Interestingly, two of the participants with a CST III at T4 had the highest IL-6 concentrations detected.

Women with CST IV observed at T0 are shown in Figure 5.8C. Data is reported for n=10 at T0-T1, n=7 at T3 and n=8 at T4. Median IL-6 concentrations in this group of women were 9.5, 1.3, 1.8 and 1.7 pg/mL at T0-T4 respectively. The IL-6 median concentration reduced from T0 at T1 and was maintained from T3 to T4. There was no significant difference in the IL-6 concentrations at T1 ($p=0.9452$), T3 ($p>0.9999$) or T4 ($p>0.9999$). Those that retained their baseline CST displayed the highest IL-6 concentrations.

In Figure 5.8D, sub-group analysis of women with a low-risk vaginal microbial composition at baseline (CST I, II or V) was performed. Data was reported for n=37 at T0 and T1, n=34 at T3 and n=35 at T4. Median IL-6 concentrations were 0.85, 0.87, 0.85 and 0.85 pg/mL at T0-T4 respectively. Median values remained stable across all timepoints. There was no significant difference in IL-6 concentrations at T1, T3 or T4 ($p>0.9999$).

Analysis of women with a high-risk vaginal microbial composition at baseline (CST III/IV) was performed (Figure 5.8E). Data is reported for n=25 at T0-T1 and n=22 at

T3-T4. Median IL-6 concentrations were 9.5, 2.0, 1.1 and 0.9 pg/mL at T0-T4 respectively. Following the loading dose, a reduction in the median IL-6 concentration was seen at T1, which further reduced at T3 before stabilising at T4. There was no statistical difference in IL-6 concentrations at T1 ($p>0.9999$), T3 ($p=0.5331$) or T4 ($p=0.2207$). Those failing to transition to CST I by T4 had the highest IL-6 concentrations.



Figures 5.8 A-E IL-6 response according to starting CST (A) IL-6 CST 1 at T0. Data is reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. Median IL-6 concentrations were 0.85 pg/mL at T0, T1, T2 and T3. No statistical difference was seen when T0 was compared with T1 (p>0.9999), T3 (p>0.9999) or T4 (p>0.9999). **(B)** IL-6 CST III at T0. Data is reported for n=15 at T0-T3 and n=14 at T4. The median IL-6 concentrations were 0.85, 2.5, 0.9 and 0.85 mg/mL at T0, T1, T3 and T4 respectively. No significant difference when compared T0 with T1 (p>0.9999), T3 (p=0.7895) or T4 (p=0.1042). **(C)** IL-6 CST IV at T0. Data is reported for n=10 at T0-T1, n=7 at T3 and n=8 at T4. Median IL-6 concentrations were 9.5, 1.3, 1.8 and 1.7 pg/mL at T0-T4 respectively. No significant difference occurred when T0 was compared with T1 (p=0.9452), T3 (p>0.9999) or T4 (p>0.9999). **(D)** IL-6 CST I/II/IV at T0. Data was reported for n=37 at T0-T1 and n=34 at T3 and n=35 at T4. The median IL-6 concentrations were 0.85, 0.87, 0.85 and 0.85 pg/mL at T0-T4 respectively. No significant difference was seen when T0 was compared with T1 (p>0.9999), T3 (p>0.9999) or T4 (p>0.9999). **(E)** CST III/IV at T0. Data was reported for n=25 at T0-T1 and n=22 at T3-T4. Median IL-6 concentrations were 9.5, 2.0, 1.1, 0.9 pg/mL at T0, T1, T3 and T4 respectively. No statistical difference when compared T0 with T1 (p>0.9999), T3 (p=0.5331) or T4 (p=0.2207). Statistics: Kruskal-Wallis test with Dunn's correction. Dotted lines represent the sensitivity of detection for IL-6. Data is presented as median values with IQR. (w=weeks gestation)

As a high proportion of IL-6 results were below the sensitivity of detection, starting CST impact on IL-6 detectability was also assessed (Figures 5.9A-J).

Figures 5.9A-B illustrate IL-6 detectability in women with CST I at T0. Detectability of IL-6 was reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. At T0, 8 women (30%) had detectable IL-6. This increased to 10 women (37%) at T1. At T3 a reduction in IL-6 detectability occurred, with 7 women (27%) having detectable IL-6. At T4 IL-6 detectability continued to reduce, with only 3 women (11%) having detectable IL-6. By T4 the proportion of women with detectable IL-6 was lower than that at T0. One-way Chi-squared testing showed no significant difference in IL-6 detectability at T1 (p=0.2819) or T3 (p=0.4135) when compared to T0. At T4 IL-6 detectability was significantly lower (p=0.0456).

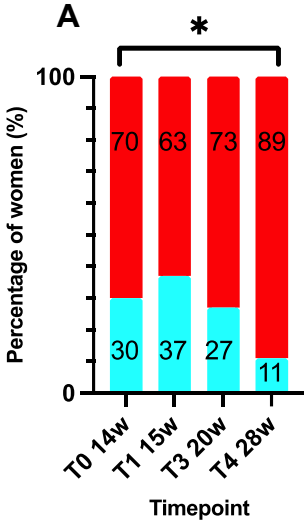
Figures 5.9C-D show IL-6 detectability in women with CST III at T0. IL-6 detectability was reported for n=15 at T0-T3 and n=14 at T4. Detectability remained stable between T0 to T1 with 9 women (60%) having detectable levels at both T0 and T1 (p=0.5000). At T3 IL-6 detectability reduced to six women (40%) (p=0.1367) and reduced significantly to just three women (21%) at T4 (p=0.0175), reaching statistical significance.

Figures 5.9E-F describes IL-6 detectability in women with CST IV at T0. Results are presented for n=10 at T0-T1 and n=8 at T3-4. Detectability remained mostly stable throughout the timepoints with 6, 5, 4 and 4 women having detectable IL-6 at T0-T4. There was no significant reduction in IL-6 detectability at T1 (p=0.3265), T3 (p=0.3357) or T4 (p=0.3357) when compared to T0.

In Figures 5.9G-H IL-6 detectability was analysed in women with a low-risk microbial community at T0. Data is presented for n=37 at T0-T1, n=34 at T3 and n=35 at T4. At T0 16 women (43%) had detectable IL-6, increasing to 19 women (51%) at T1. At T3, a reduction in IL-6 detectability occurred; 10 women (29%) had detectable IL-6 at T3 and this was maintained through to T4. The changes in IL-6 detectability were not significant at T1 (p=0.2424) but did reach significance at T3 (p=0.0041) and T4 (p=0.0032).

Figures 5.9I-J, illustrate IL-6 detectability in women with a high-risk starting microbial community. Results are shown for n=25 at T0-T1 and n=22 at T3-T4. The detectability from T0 to T4 gradually reduced across the timepoints. At T0, 16 women (64%) had detectable IL-6. This reduced at 15 women (60%) at T1 and further reduced to 12 women (55%) and 11 women (50%) at T3-T4. Statistical significance was not reached at T1 (p=0.3854), T3 (0.2549) or T4 (p=0.1664).

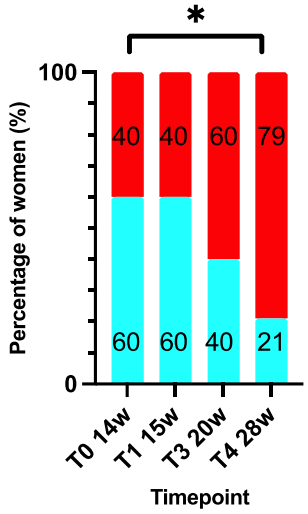
A



B

Starting CST I				
	Detectable (%)	Undetectable (%)	Total	P value
T0	8 (30)	19 (70)	27	
T1	10 (37)	17 (63)	27	0.2819
T3	7 (27)	19 (73)	26	0.4135
T4	3 (11)	24 (89)	27	0.0456*

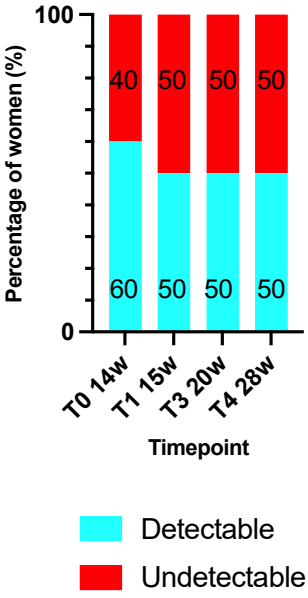
C



D

Starting CST III				
	Detectable (%)	Undetectable (%)	Total	P value
T0	9 (60)	6 (40)	15	
T1	9 (60)	6 (40)	15	0.5000
T3	6 (40)	9 (60)	15	0.1367
T4	3 (21)	11 (79)	14	0.0175*

E

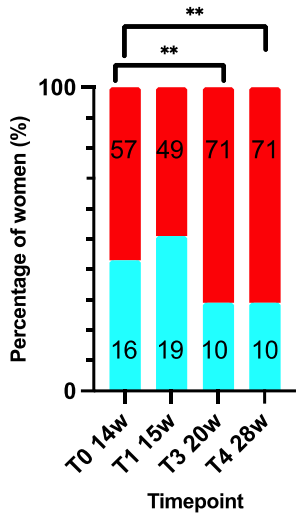


F

Starting CST IV				
	Detectable (%)	Undetectable (%)	Total	P value
T0	6 (60)	4 (40)	10	
T1	5 (50)	5 (50)	10	0.3265
T3	4 (50)	4 (50)	8	0.3357
T4	4 (50)	4 (50)	8	0.3357

Figure 5.9 continued

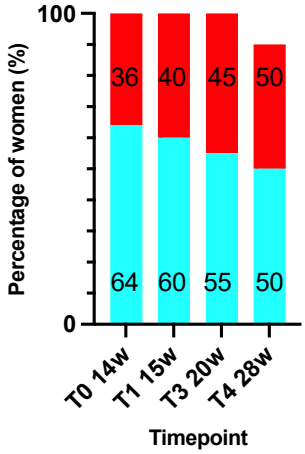
G



H

CST at T0 I, II or V				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
T0	16 (43)	21 (57)	37	
T1	19 (51)	18 (49)	37	0.2424
T3	10 (29)	24 (71)	34	0.0041**
T4	10 (29)	25 (71)	35	0.0032**

I



J

CST at T0 III, IV				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
T0	16 (64)	9 (36)	25	
T1	15 (60)	10 (40)	25	0.3854
T3	12 (55)	10 (45)	22	0.2549
T4	11 (50)	11 (50)	22	0.1664

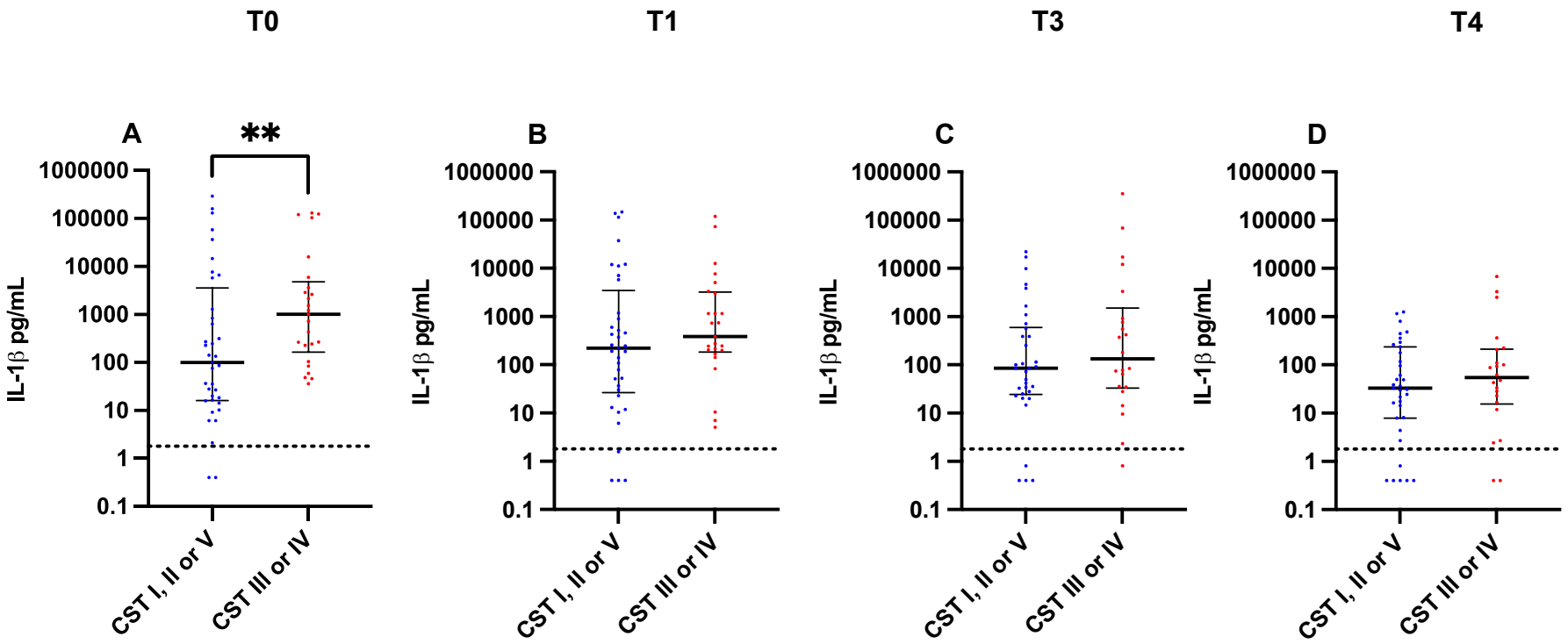
Figures 5.9 A-J – Detectability of IL-6 according to starting CST. (A) CST I at T0. Data is reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. 30%, 37%, 27% and 11% of women had detectable IL-6 from T0-T4 respectively. **(B)** Tabulated raw data for women with CST I at T0. When compared to T0 IL-6 detectability did not statistically differ at T1 (p=0.2819), T3 (p=0.4135) or T4 (p=0.0456). **(C)** CST III at T0. Data is reported for n=27, n=27, n=26 and n=27 at T0-T4 respectively. 60%, 60%, 40% and 21% of women had IL-6 detectable across T0-T4 respectively. **(D)** Tabulated raw data for women with CST III at T0. When compared to T0, IL-6 detectability at T1 (p=0.5000) and T3 (p=0.1367) did not statistically differ. At T4, IL-6 detectability significantly reduced (p=0.0175). **(E)** CST IV at T0. Data is reported for n=10 at T0-T1 and n=8 at T3-T4. IL-6 detectability was seen in 60% at T0 and 50% at T1-T4. **(F)** Tabulated raw data for women with CST IV at T0. When compared to T0 IL-6 detectability did not statistically differ at T1 (p=0.3265), T3 (p=0.3357) or T4 (p=0.3357). **(G)** CST I, II or V at T0. Data is

reported for n=37 at T0-T1, n=34 at T3 and n=35 at T4. IL-6 was detectable in 16%, 19%, 10% and 10% of women at T0-T4 respectively. **(H)** Tabulated raw data for women with CST I, II or V at T0. IL-6 detectability did not significantly change at T1 (p=0.2424). A significant reduction occurred at T3 (p=0.0041) and T4 (p=0.0032). **(I)** CST III or IV at T0. Data is reported for n=25 at T0-T1 and n=22 at T3-T4. IL-6 was detectable in 64%, 60%, 55% and 50% of women at T0-T4 respectively. **(J)** Tabulated raw data for women with CST III or IV at T0. IL-6 detectability remained stable at T1 (p=0.3854) and reduced at T3 (p=0.2549) and at T4 (p=0.1664). Statistics: One-way Chi-squared testing, *p<0.05; **p<0.01, (w=weeks gestation)

The response to LACTIN-V was compared according to whether women had a low-risk (CST I, II or V) or high-risk microbial community (CST III or IV) at enrolment (T0). Cytokine concentrations between these two groups of women were compared at each study timepoint (Figures 5.10-5.12).

Figures 5.10A-D, compares the IL-1ß response at T0-T4 according to starting CST. At T0 there was a significant difference in median IL-1ß concentrations between women a low-risk or high-risk microbial community at the study start (Figure 5.10A). The median IL-1ß concentration for women starting with CST I/II/V was 100.2 pg/mL (n=37). For women starting with CST III/IV, the median IL-1ß concentration was 1010 pg/mL (n=25) at T0 (p=0.0075) (Figure 5.10A).

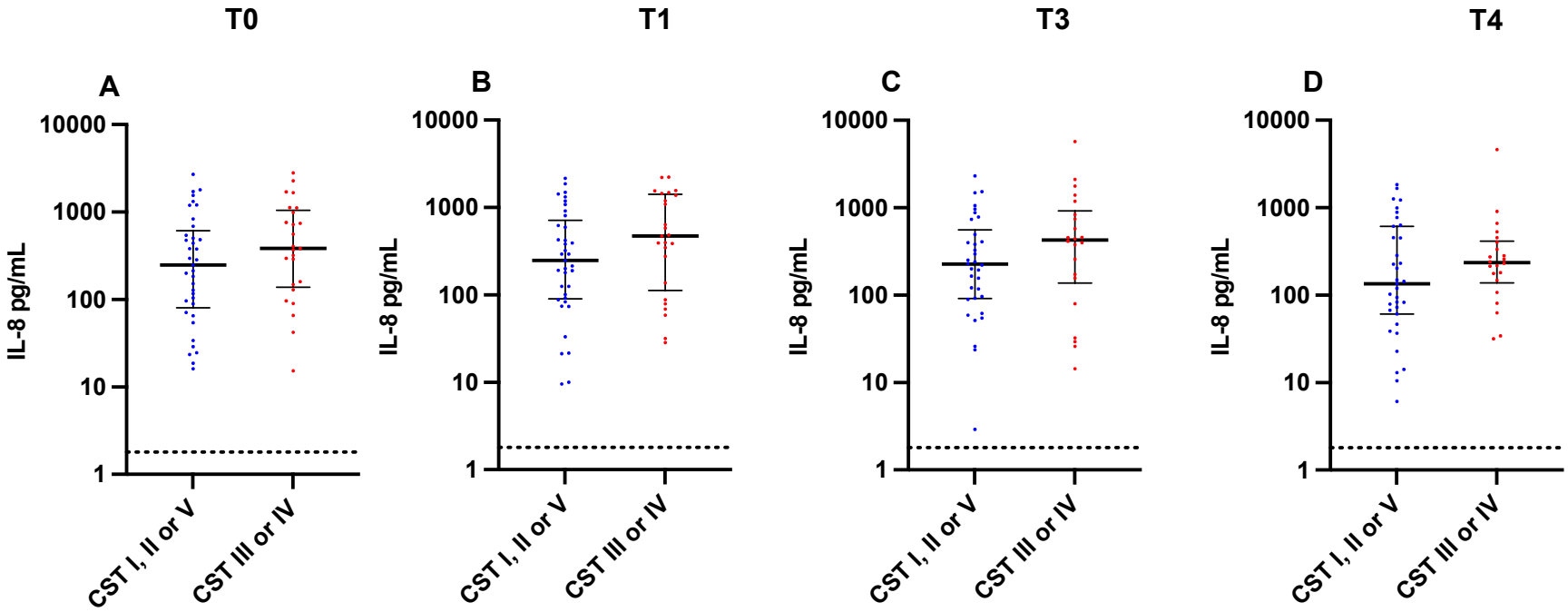
With LACTIN-V use the difference between low and high-risk microbial community groups lessens. At T1, the median IL-1ß concentration for women with CST I/II/V at baseline was 222.3 pg/mL (n=37) and 383.4 pg/mL (n=25) for women with CST III/IV at baseline (p=0.1178) (Figure 5.10B). At T3, the median IL-1ß concentration for women with CST I/II/V at baseline was 85.6 pg/mL (n=34) and 132.5 pg/mL (n=23) for women CST III/IV at baseline (p=0.2059) (Figure 5.10C). By T4, the median IL-1ß concentration for women with CST I/II/V at baseline was 33.0 pg/mL (n=35) and 54.9 pg/mL (n=22) for women CST III/IV at baseline (p=0.2304) (Figure 5.10D). Although the median IL-1ß concentration in women with a pre-existing high-risk microbial community reduces across the study period, the median IL-1ß remains higher at all timepoints when compared to women with a low-risk microbial community at basleine.



Figures 5.10 A-D – IL-1ß response to LACTIN-V according to low-risk (I/II/V) or high-risk (III/IV) starting CST. **(A)** T0 IL-1ß concentrations in women with CST I/II/V (median 100.2 pg/mL, n=37) and CST III/IV at T0 (median 1010 pg/mL, n=25), p=0.0075. **(B)** T1 IL-1ß concentrations in women with CST I/II/V (median 222.3 pg/mL, n=37) and CST III/IV at T0 (median 383.4 pg/mL, n=25), p=0.1178. **(C)** T3 IL-1ß concentrations in women with CST I/II/V (median 85.6 pg/mL, n=34) and CST III/IV at T0 (median 132.5 pg/mL, n=23), p=0.2059. **(D)** T4 IL-1ß concentrations in women with CST I/II/V (median 33.0 pg/mL, n=35) and CST III/IV at T0 (median 54.9 pg/mL, n=22), p=0.2304. Statistics: Mann-Whitney U Test. Dotted lines represent the sensitivity of detection for IL-1ß. Data is presented as median values with interquartile range. **p<0.01.

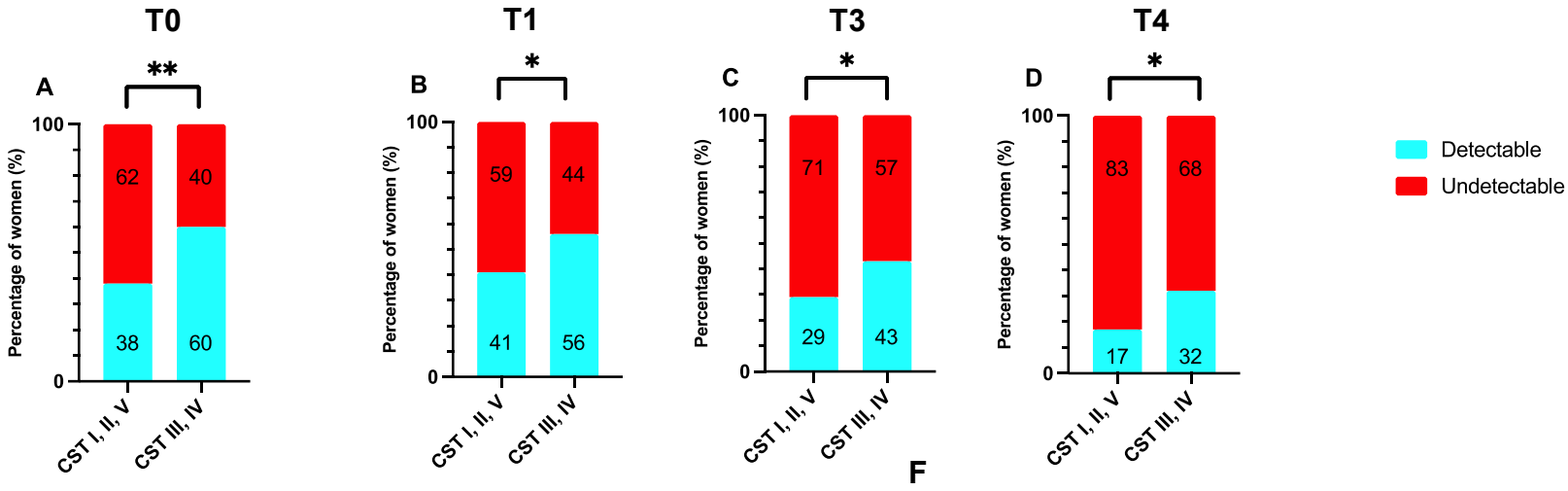
Figures 5.11A-D compares the IL-8 response at T0-T4 according to starting CST. At all timepoints, median IL-8 concentrations were higher in women with CST III/IV at T0 when compared to women with CST I/II/V at T0.

At T0, the median IL-8 concentration for women with CST I/II/V at T0 was 248.3 pg/mL (n=37) and 383.5 pg/mL (n=25) for women CST III/IV at T0 (p=0.1016) (Figure 5.11A). At T1, the median IL-8 concentration for women with CST I/II/V at T0 was 248.4 pg/mL (n=37) and in women with CST III/IV at T0, increased to 473.8 pg/mL (n=25) (p=0.0609) (Figure 5.11B). At T3, the median IL-8 concentration for women with CST I/II/V at T0 was 226.2 pg/mL (n=34) and 427.4 pg/mL (n=23) for women with CST III/IV at T0 (p=0.0976) (Figure 5.11C). At T4, the median IL-8 concentration for women with CST I/II/V at T0 reduced to 135.0 pg/mL (n=35). The median IL-8 concentration in women with CST III/IV at T0 also reduced 236.0 pg/mL (n=22) by T4 (p=0.1285) (Figure 5.11D). Unlike results for IL-1ß, LACTIN-V use does not minimise the difference I IL-8 between these two groups of women across the study period.



Figures 5.11 A-D – IL-8 response to LACTIN-V according to low-risk (I/II/V) or high-risk (III/IV) starting CST. (A) T0 IL-8 concentrations in women with CST I/II/V at T0 (median 248.3 pg/mL, n=37) and CST III/IV at T0 (median 383.5 pg/mL, n=25), p=0.1016. **(B)** T1 IL-8 concentrations in women with CST I/II/V at T0 (median 248.4 pg/mL, n=37) and CST III/IV at T0 (median 473.8 pg/mL, n=25), p=0.0609. **(C)** T3 IL-8 concentrations in women with CST I/II/V at T0 (median 226.2 pg/mL, n=34) and CST III/IV at T0 (median 427.4 pg/mL, n=23), p=0.0976. **(D)** T4 IL-8 concentrations for women with CST I/II/V at T0 (median 135.0 pg/mL, n=35) and CST III/IV at T0 (median 236.0 pg/mL, n=22), p=0.1285. Statistics: Mann-Whitney U Testing. Dotted lines represent the sensitivity of detection. Data is presented as median values with IQR.

Figures 5.12A-F, compares the IL-6 response according to starting CST. At T0, more women with CST III/IV at T0 (15/25, 60%) had IL-6 detectable when compared to women with CST I/II/V at T0 (14/37, 38%) (p=0.0019) (Figures 5.12A, E). At T1, more women with CST III/IV at T0 (14/25, 56%) had IL-6 detectable when compared to women with CST I/II/V at T0 (15/37, 41%) (p=0.0338) (Figures 5.12B, E). At T3, IL-6 detectability reduced for both groups of women. More women with CST III/IV at T0 (10/23, 43%) had IL-6 detectable when compared to women with with CST I/II/V at T0 (10/34, 29%) (p=0.0392) (Figures 5.12C, F). At T4, IL-6 detectability in women with CST III/IV at T0 (7/22, 32%) was greater than for women with with CST I/II/V at T0 (6/35, 17%), p=0.0137 (Figures 5.12D, F). Across the study period, detectability of IL-6 reduced in both groups of women. At all time points, IL-6 detectability was significantly higher in women with a baseline high-risk microbial community.



E

Timepoint 0				
CST at T0	**Detectable (%)**	**Undetectable (%)**	**Total**	**P value**
CST I, II, V	14 (38)	23 (62)	37	0.0019**
CST III, IV	15 (60)	10 (40)	25	
Timepoint 1				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
CST I, II, V	15 (41)	22 (59)	37	0.0338*
CST III, IV	14 (56)	11 (44)	25	

F

Timepoint 3				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
CST I, II, V	10 (29)	24 (71)	34	0.0392*
CST III, IV	10 (43)	13 (57)	23	
Timepoint 4				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
CST I, II, V	6 (17)	29 (83)	35	0.0137*
CST III, IV	7 (32)	15 (68)	22	

Figures 5.12 A-F– IL-6 response to LACTIN-V according to low-risk of high-risk starting CST. (A) T0 IL-6 in women starting with CST I/II/V (14/37, 38%) versus CST III/IV (15/25, 60%), p=0.0019. **(B)** T1 IL-6 detectability in women starting with CST I/II/V (15/37, 41%) versus CST III/IV (14/25, 56%), p=0.0338. **(C)** T3 IL-6 detectability in women starting with CST I/II/V (10/34, 29%) versus CST III/IV (10/23, 43%), p=0.0392. **(D)** T4 IL-6 detectability women starting with CST I/II/V (6/35, 17%) versus CST III/IV (7/22, 32%), p=0.0137. **(E)** Tabulated raw data presented for T0 and T1 (F) Tabulated raw data presented for T3 and T4. Statistics: using one-way Chi-squared testing. *p<0.05; **p<0.01.

5.3.5 Immune response in colonisers and non-colonisers

In Chapter 4, non-colonisers were defined as women without colonisation by T4. A total of 20 women were identified to be non-colonisers. The immune response in colonisers and non-colonisers was assessed. In Figures 5.13-5.15, results for colonisers are reported for n=37, n=37, n=36 and n=37 women at T0-T4 respectively. Results for non-colonisers are reported for n=20, n=20, n=18 and n=20 at T0-T4 respectively.

Figure 5.13 illustrates the IL-1ß concentrations in colonisers and non-colonisers at each timepoint. It was hypothesised that non-colonisers would display higher IL-1ß concentrations at each timepoint.

At T0, colonisers had higher IL-1ß concentrations than non-colonisers. At T0 the median IL-1ß concentration for colonisers and non-colonisers was 432.4 and 61.5 pg/mL respectively (p=0.0423). At T1, the median IL-1ß concentrations were similar between colonisers (242.0 pg/mL) and non-colonisers (313.9 pg/mL) (p>0.9999). At T3 the median IL-1ß concentration for colonisers and non-colonisers was similar at 87.7 and 85.6 pg/mL respectively (p>0.9999). At T4 the median IL-1ß concentration for colonisers and non-colonisers was 47.9 and 27.2 pg/mL respectively (p>0.9999). In colonisers, the IL-1ß concentrations significantly reduced from T0 to T4 (p=0.0007). In non-colonisers the IL-1ß concentration did not differ significantly from T0 to T4 (p>0.9999).

At T0, colonisers had higher median IL-1ß concentrations than non-colonisers. Interestingly, at T1 the median IL-1ß concentrations in non-colonisers increased. The IL-1ß concentrations gradually reduced across T0-T4 in both groups of women. This was significant for colonisers, but not for non-colonisers.

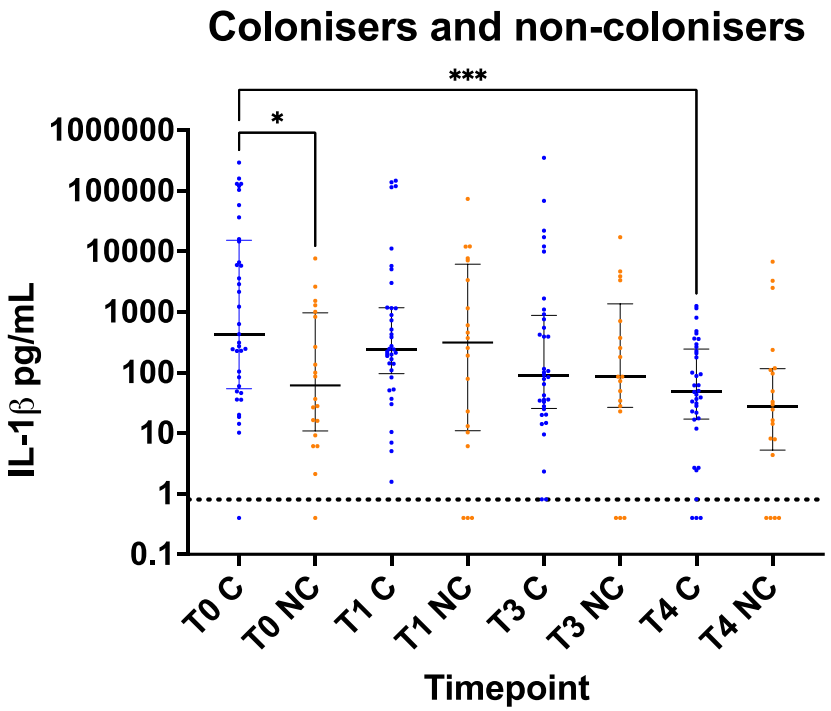


Figure 5.13 - IL-1ß concentrations in colonisers (C) and non-colonisers (NC). Results for colonisers are reported for n=37, n=37, n=36 and n=37 women at T0-T4 respectively. Results for non-colonisers are reported for n=20, n=20, n=18 and n=20 at T0-T4 respectively. The median IL-1ß concentrations for C and NC at T0 was 432.4 and 61.5 pg/mL respectively (p=0.0423). The median IL-1ß concentrations for C and NC at T1 was 242.0 and 313.9 pg/mL respectively (p>0.9999). The median IL-1ß concentrations for C and NC at T3 was 87.7 and 85.6 pg/mL respectively (p>0.9999). At T4 the median IL-1ß concentration for colonisers and non-colonisers was 47.9 and 27.2 pg/mL respectively (p>0.9999). IL-1ß concentrations in colonisers significantly reduced from the T0 (432.4 pg/mL) to T4 (47.9 pg/mL) (p=0.0007). IL-1ß concentrations at T0 (61.5 pg/mL) to T4 (27.2 pg/mL) did not significantly differ (p>0.9999). Statistics: one-way ANOVA Kruskal-Wallis Test, with Dunn's correction. Dotted lines represent the sensitivity of detection. Data is presented as median values with IQR. *p<0.05; ***p<0.001.

Figure 5.14 shows IL-8 concentrations in colonisers and non-colonisers at each timepoint. At T0, colonisers and non-colonisers show similar median IL-8 concentrations. At T0 the median IL-8 concentration for colonisers and non-colonisers was 295.8 and 248.8 pg/mL respectively ($p>0.9999$). At T1, the median IL-8 concentrations in colonisers and non-colonisers were 392.4 and 231.3 pg/mL respectively ($p>0.9999$). At T1 the median IL-8 increased in colonisers. At T3 the median IL-8 concentration for colonisers and non-colonisers was 165.7 and 312.3 pg/mL respectively ($p>0.9999$). At T4 the median IL-8 concentration for colonisers and non-colonisers was 111.5 and 218.2 pg/mL respectively ($p>0.9999$). Although the IL-8 concentration in colonisers showed a trend to reduce from T0 to T4 ($p=0.4290$), the IL-8 concentration in non-colonisers remained stable from T0-T4. Changes in IL-8 concentrations at T0 and T4 in non-colonisers showed no significant difference ($p>0.9999$).

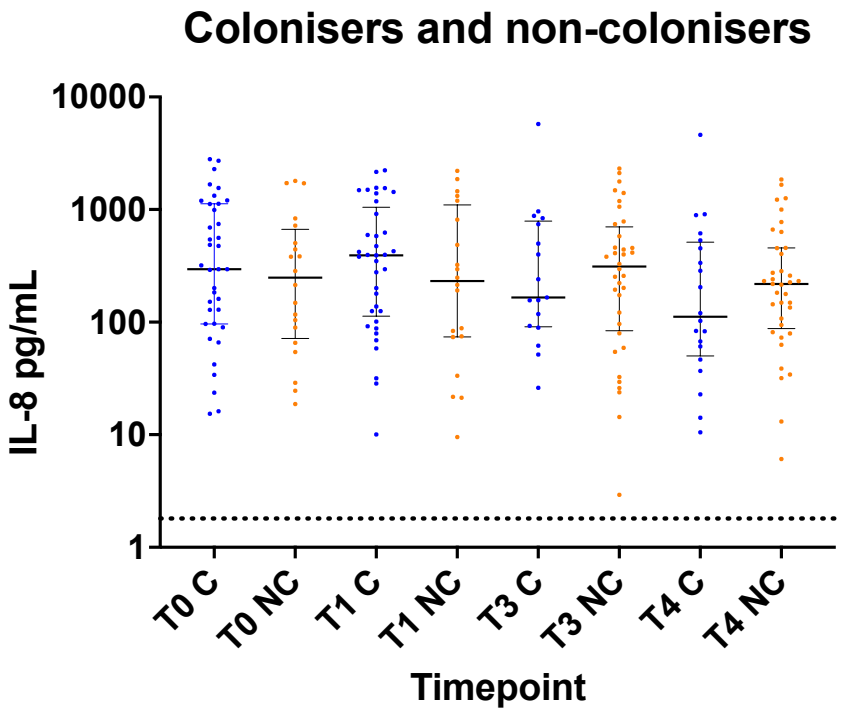
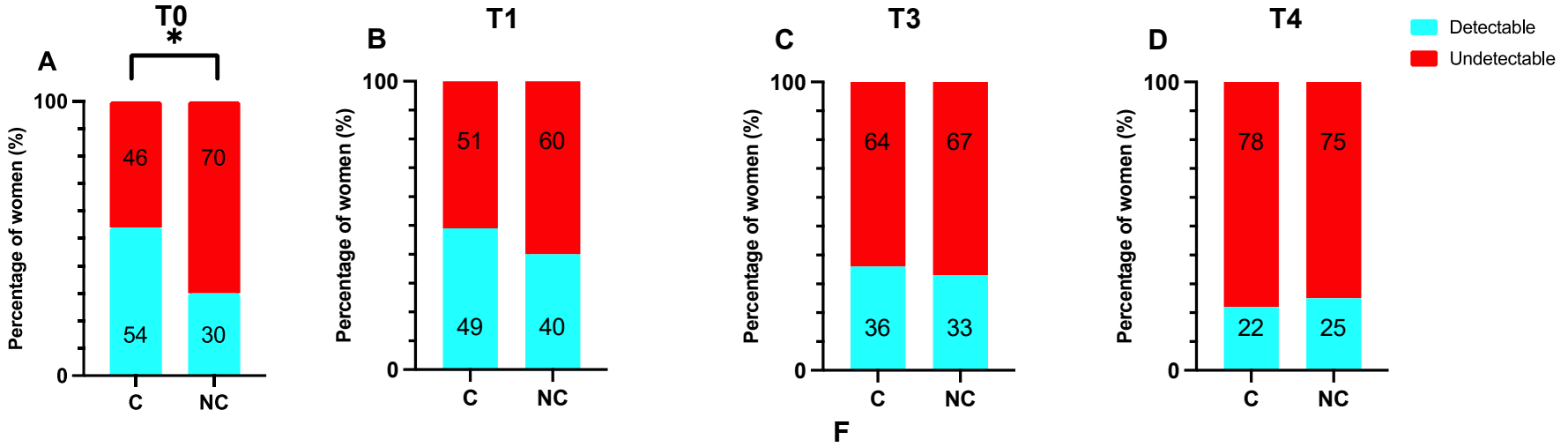


Figure 5.14 - IL-8 concentrations in colonisers (C) and non-colonisers (NC). Results for colonisers are reported for n=37, n=37, n=36 and n=37 women at T0-T4 respectively. Results for non-colonisers are reported for n=20, n=20, n=18 and n=20 at T0-T4 respectively. The median IL-8 concentrations for C and NC at T0 was 295.8 and 248.8 pg/mL respectively (p>0.9999). The median IL-8 concentrations for C and NC at T1 was 392.4 and 231.3 pg/mL respectively (p>0.9999). The median IL-8 concentrations for C and NC at T3 was 165.7 and 312.3 pg/mL respectively (p>0.9999). At T4 the median IL-8 concentration for colonisers and non-colonisers was 111.5 and 218.2 pg/mL respectively (p>0.9999). IL-8 concentrations in colonisers reduced reduce from T0 (392.4 pg/mL) to T4 (111.5 pg/mL) (p=0.4290). IL-8 concentrations in NC remained stable from T0 (248.8 pg/mL) to T4 (218.2 pg/mL) (p>0.9999). Statistics: Kruskal-Wallis Test. The dotted line represents the sensitivity of detection. Data is presented as median values with interquartile range.

IL-6 detectability was compared in colonisers and non-colonisers (Figures 5.15A-F). At T0 IL-6 detectability was significantly higher in colonisers than non-colonisers (p=0.0409) (Figures 5.15A, E). At T0 54% of colonisers had detectable IL-6 whereas 30% of non-colonisers did. From T0 to T4 the detectability in IL-6 reduced in colonisers from 54%, to 49%, 36% and 22% at T0-T4 respectively. In contrast, IL-6 detectability in non-colonisers remained stable throughout the study period at 30%, 40%, 33% and 25% from T0-T4 respectively. There was no statistical significance between IL-6 detectability between colonisers and non-colonisers at T1 (p=0.2658, Figures 5.15B, E), T3 (p=0.4202, Figures 5.15C, F) or T4 (p=0.3859, Figures 5.15D, F).



E

Timepoint 0				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
C	20 (54)	17 (46)	37	0.0409*
NC	6 (30)	14 (70)	20	
Timepoint 1				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
C	18 (49)	19 (51)	37	0.2658
NC	8 (40)	12 (60)	20	

F

Timepoint 3				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
C	13 (36)	23 (64)	36	0.4202
NC	6 (33)	12 (67)	18	
Timepoint 4				
	Detectable (%)	**Undetectable (%)**	**Total**	**P value**
C	8 (22)	29 (78)	37	0.3859
NC	5 (25)	15 (75)	20	

Figures 5.15A-F - IL-6 detectability in colonisers (C) and non-colonisers (NC). Results are reported for n=37, n=37, n=36 and n=37 colonisers at T0-T4 respectively and for n=20, n=20, n=18 and n=20 non-colonisers at T0-T4 respectively. **(A)** At T0, 20 (54%) colonisers and 6 non-colonisers (54%) had detectable IL-6 (p=0.0409). **(B)** At T1 18 colonisers (49%) and 8 non-colonisers (40%) had detectable IL-6 (p=0.2658). **(C)** At T3 13 colonisers (36%) and 6 non-colonisers (33%) had detectable IL-6 (p=0.4202). **(D)** At T4 8 colonisers (22%) 5 non-colonisers (25%) had detectable IL-6 (p=0.3859). **(E)** Tabulated raw data presented for T0 and T1 **(F)** Tabulated raw data presented for T3 and T4. Statistics: one-way Chi-squared testing, *p<0.05.

5.3.6 LACTIN-V response in women delivering preterm

The immune response to LACTIN-V in women delivering term and spontaneously preterm (sPT) was explored (Figures 5.16-5.18). Outcomes of women are described in Chapter 4. From 62 women, 49 women delivered at term and 9 delivered sPT (<37 weeks) including one MTL. Three women had iatrogenic preterm (iPT) births and are presented separately and excluded from analysis. MTL was defined as delivery between 16^{+0} and 23^{+6} weeks' gestation. Results in Figures 5.16-5.18 are reported for n=49, n=9, n=3 at T0-T1 for women delivering term, sPT and iPT, respectively. At T3 results are reported for n=46, n=8 and n=2 for women delivering at term, sPT and iPT, respectively. At T4 results are reported for n=48, n=6 and n=3 for term, sPT and iPT groups.

Figure 5.16 shows the IL-1β response at each timepoint according to clinical outcome. Although this study was not powered to detect differences according to clinical outcomes, data is presented for clarity and completeness.

At T0 median IL-1ß concentrations in women delivering term, sPT and iPT were 246.4, 718.9 and 1,527 pg/mL respectively. sPT women had higher starting median IL-1ß concentrations at T0 than term women, although significance was not reached (p>0.9999).

At T1 median IL-1ß concentrations in term, sPT and iPT groups were 246.8, 893.7 and 1,164 pg/mL respectively. Median IL-1ß concentrations remained stable between T0 (246.4 pg/mL) and T1 (246.8 pg/mL) in women delivering term. In contrast, for sPT women IL-1ß concentrations increased from T0 (718.9 pg/mL) to T1 (893.7 pg/mL). At T1 there was no statistically significant difference in IL-1ß concentrations between term and sPT groups (p>0.7677).

At T3 the median IL-1ß concentrations in term, sPT and iPT groups were 88.3, 81.8 and 175,479 pg/mL respectively. At T3 the median IL-1ß concentrations reduced for both term (88.3 pg/mL) and sPT (81.8 pg/mL) groups, and median IL-1ß concentrations were comparable. At T3 there was no significant difference in IL-1ß concentrations between term and sPT groups (>0.9999).

The median IL-1ß concentrations at T4 were 41.1, 31.5 and 208.1 pg/mL for term, sPT and iPT groups. At T4, the median IL-1ß concentration further reduced for term and sPT women. Again, the T4 IL-1ß concentrations in these two groups were comparable with no statistical difference (p>0.9999).

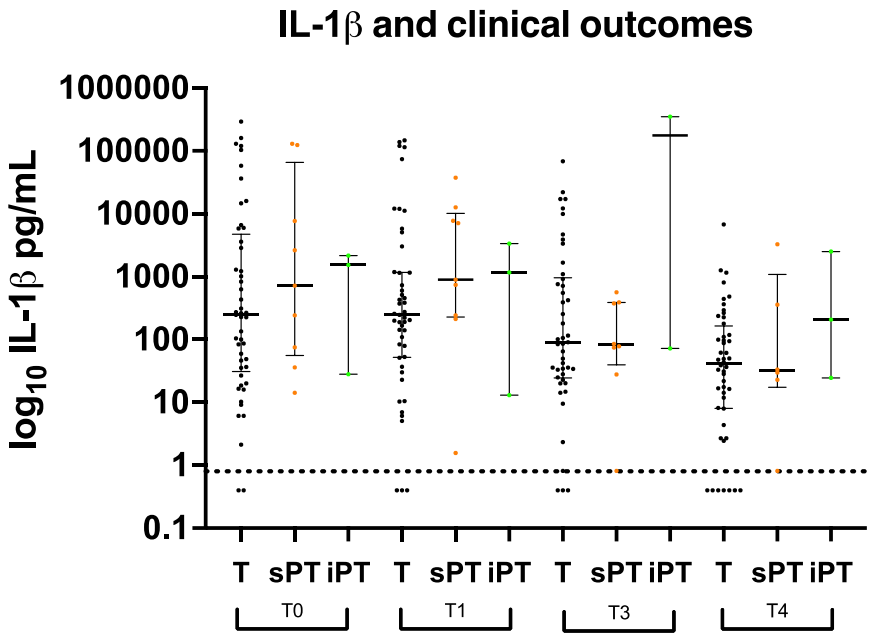


Figure 5.16 – IL-1ß concentrations according to clinical outcome. At T0 and T1 results are presented for n=49, n=9, n=3 for T, sPT and iPT groups respectively. At T3 results are reported for n=46, n=8 and n=2 for T, sPT and iPT groups respectively. At T4 results are reported for n=48, n=6 and n=3 for T, sPT and iPT groups respectively. Median T0 IL-1ß concentrations were 246.4, 718.9 and 1,527 pg/mL for T, sPT and iPT groups respectively. Median T1 IL-1ß concentrations were 246.8, 893.7 and 1,164 pg/mL respectively for T, sPT and iPT groups respectively. Median T3 IL-1ß concentrations were 88.3, 81.8 and 175,479 pg/mL for T, sPT and iPT groups respectively. At T4 median IL-1ß concentrations were 41.1, 31.5, 208.1 pg/mL for T, sPT and iPT groups respectively. IL-1ß concentrations did not differ significantly in T versus sPT groups at T0 (p>0.9999), T1 (p=0.7677), T3 (p>0.9999) or T4 (p>0.9999). Statistis: Kruskal-Wallis test with Dunn's correction. The dotted line represents the sensitivity of detection. Data is presented as median values with IQR. Abbreviations: T=term, sPT = spontaneously preterm, iPT = iatrogenic preterm.

Figure 5.17 shows the IL-8 response at each timepoint according to clinical outcome. At T0 the median IL-8 concentrations in women delivering term, sPT and iPT were 295.1, 377.8 and 719.1 pg/mL respectively. Women delivering sPT had higher starting median IL-8 concentrations at T0 than women that delivered term (p>0.9999).

At T1 the median IL-8 concentrations in term, sPT and iPT groups were 347.9, 473.8 and 277.2 pg/mL respectively. Like IL-1ß, median IL-8 concentrations remained stable between T0 (295.1 pg/mL) and T1 (347.9 pg/mL) in women delivering term. For sPT women, the median IL-8 concentration increased from T0 (377.8 pg/mL) to T1 (473.8 pg/mL). At T1 there was no significant difference in IL-8 concentrations between term and sPT groups (>0.9999).

At T3 the median IL-8 concentrations in term, sPT and iPT groups were 255.5, 253.4 and 677.7 pg/mL respectively. As seen with IL-1ß, at T3 the median IL-8 concentrations reduced for both T and sPT women, who both had comparable median IL-8 concentrations. At T3 there was no significant difference in IL-8 concentrations between term and sPT groups (>0.9999).

Median IL-8 concentrations at T4 were 209.6, 95.6, 218.2 pg/mL for term, sPT and iPT groups. At T4, the median IL-8 concentration continued to remain stable for women delivering term. However, for the sPT group, a reduction in median IL-8 concentration was seen at T4. There was no significant difference between IL-8 concentrations in T versus sPT women at T4 (>0.9999).

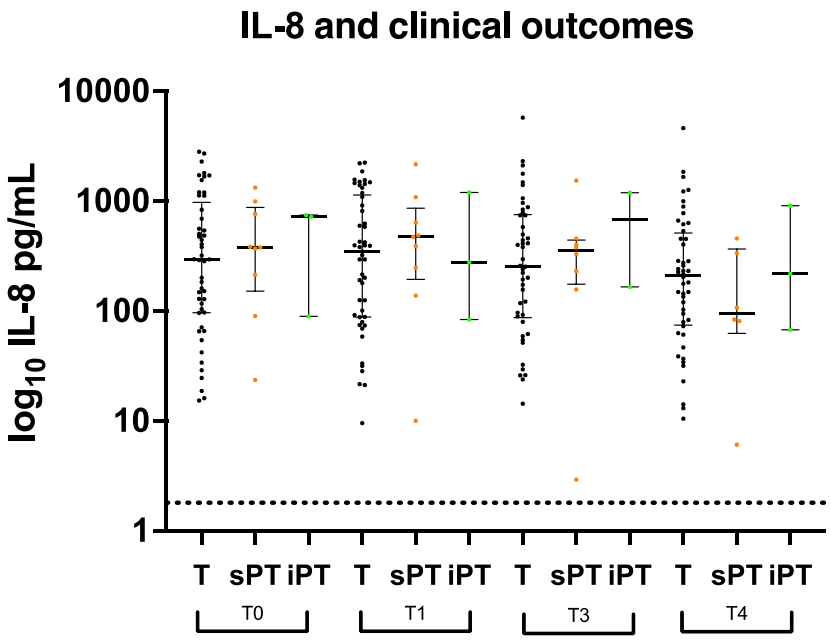


Figure 5.17 – IL-8 concentrations according to clinical outcome. At T0 and T1 results are presented for n=49, n=9, n=3 for T, sPT and iPT groups respectively. At T3 results are reported for n=46, n=8 and n=2 for T, sPT and iPT groups respectively. At T4 results are reported for n=48, n=6 and n=3 for T, sPT and iPT groups respectively. Median T0 IL-8 concentrations were 295.1, 377.8 and 719.1 pg/mL for T, sPT and iPT groups respectively. Median T1 IL-8 concentrations were 347.9, 473.8 and 277.2 pg/mL respectively for T, sPT and iPT groups. Median T3 IL-8 concentrations were 255.5, 253.4 and 677.7 pg/mL for T, sPT and iPT groups respectively. Median T4 IL-8 concentrations were 209.6, 95.6, 218.2 pg/mL for T, sPT and iPT groups respectively. IL-8 concentrations did not differ significantly in T and sPT groups at T0 ($p>0.9999$), T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p>0.9999$). Statistics: Kruskal-Wallis test with Dunn's correction. The dotted line represents the sensitivity of detection. Data is presented as median values with IQR. Abbreviations: T=term, sPT = spontaneous preterm, iPT = iatrogenic preterm.

Figure 5.18 shows the IL-6 response at each timepoint according to clinical outcome. At T0 the median IL-6 concentrations in women delivering term, sPT and iPT were 0.85, 13.0 and 3.6 pg/mL respectively. sPT women had higher starting median IL-6 concentrations at T0 than term women (p=0.1763).

At T1 the median IL-6 concentrations in term, sPT and iPT were 1.2, 2.8 and 0.85 pg/mL respectively. The median IL-6 concentrations increased from T0 (0.85 pg/mL) to T1 (1.2 pg/mL) in women delivering term. For sPT women, median IL-6 concentrations reduced from T0 (13.0 pg/mL) to T1 (2.8 pg/mL). At T1 there was no significant difference in IL-6 concentrations between term and sPT groups (p=0.7624).

At T3 the median IL-6 concentrations in term, sPT and iPT groups were 0.85, 1.5 and 4,804 pg/mL respectively. At T3 The median IL-6 concentrations returned to baseline for women who delivered term. The median IL-6 concentrations reduced at T3 for sPT women. At T3 there was no significant difference in IL-6 concentrations between term and sPT groups (>0.9999).

The median IL-6 concentrations at T4 were 0.85, 0.85 and 10.2 pg/mL for term, sPT and iPT groups. At T4, the median IL-6 concentration remained stable for women that delivered term. However, sPT women showed a further reduction in median IL-6 concentration. There was no significant difference between IL-6 concentrations in term and sPT women at T4 (p>0.9999).

Overall, the median IL-6 concentrations in women delivering term remained stable from T0 to T4. In contrast, in sPT women, the IL-6 median concentration reduced from T0 to T4.

Women with iPT are presented separately in Figures 5.16-5.18 to highlight their exclusion from the analysis of term and sPT women. Although cytokine the analysis of concentrations in women with iatrogenic PTB was outside the scope of this study, this group of women displayed high cytokine concentrations. The median IL-1ß concentration was higher in iPT women at all timepoints, when compared to term and sPT women (Figure 5.16). This was also seen with IL-8 at T0, T3 and T4 (Figure 5.17) and IL-6 at T3 and T4 (Figure 5.18).

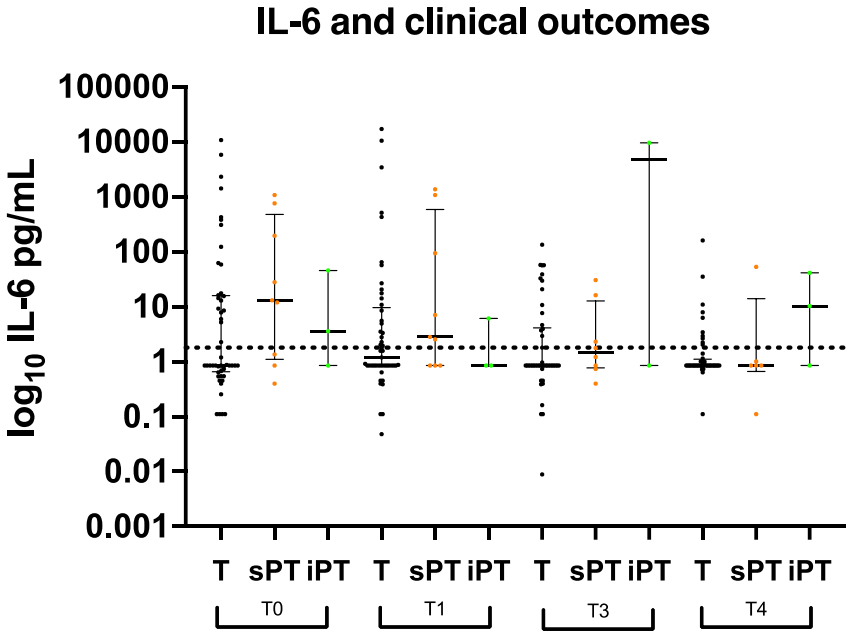


Figure 5.18 – IL-6 concentrations according to clinical outcome. At T0 and T1 results are presented for n=49, n=9, n=3 for T, sPT and iPT groups respectively. At T3 results are reported for n=46, n=8 and n=2 for T, sPT and iPT groups respectively. At T4 results are reported for n=48, n=6 and n=3 for T, sPT and iPT groups respectively. Median T0 IL-6 concentrations were 0.85, 13.0 and 3.6 pg/mL for T, sPT and iPT groups respectively. Median T1 IL-6 concentrations were 1.2, 2.8 and 0.85 pg/mL respectively for T, sPT and iPT groups respectively. Median T3 IL-6 concentrations were 0.85, 1.5 and 4,804 pg/mL for T, sPT and iPT groups respectively. Median T4 IL-6 concentrations were 0.85, 0.85 and 10.2 pg/mL for T, sPT and iPT groups respectively. IL-6 concentrations did not differ significantly in T and sPT groups at T0 (p=0.1763), T1 (p=0.7624), T3 (p>0.9999) or T4 (p>0.9999). Statistics: Kruskal-Wallis test with Dunn's correction. The dotted line represents the sensitivity of detection. Data is presented as median values with IQR. Abbreviations: T=term, sPT = spontaneous preterm, iPT = iatrogenic preterm.

5.3.7 LACTIN-V response according to ethnicity

This study was not powered to detect differences according to ethnicity. Whether ethnicity influenced immune response to LACTIN-V was included for transparency and clarity (Figures 5.19-5.21). At T0 and T1 data is reported for n=37, n=8, n=11 and n=6 for White, Black, Asian and other ethnic groups respectively. At T3 data is reported for n=36, n=7, n=8 and n=5 for White, Black, Asian and other ethnic groups respectively.

At T4 results are reported for n=37, n=5, n=9 and n=6 for White, Black, Asian and other ethnic groups respectively.

IL-1ß concentrations were assessed according to ethnicity (Figure 5.19). At T0 the median IL-1ß concentrations for White, Black, Asian and other women were 226.7, 204.5, 631.3 and 548.1 pg/mL respectively. Asian women had the highest median IL-1ß concentration at baseline.

At T1 the median IL-1ß concentrations for White, Black, Asian and other women were 242.0, 952.6, 247.4 and 142.8 pg/mL respectively. Median IL-1ß concentrations reduced for all women, except for Black women. Instead, the median IL-1ß concentration increased at T1 in Black women.

At T3 the median IL-1ß concentration for White, Black, Asian and other women was 68.5, 180.3, 244.7 and 254.3 pg/mL respectively. At T3, the median IL-1ß concentration reduced for White, Black and Asian women.

At T4 the median IL-1ß concentrations for White, Black, Asian and other women were 38.8, 110.5, 43.3 and 11.1 pg/mL respectively. The median IL-1ß concentrations reduced for women of all ethnicities by T4.

Overall, women from all ethnic groups showed a trend to reduce IL-1ß concentrations from T0-T4. Black women showed an increase in IL-1ß concentration at T1. There was no significant difference between IL-1ß concentrations at T0, T1, T3 or T4 according to ethnicity ($p>0.9999$).

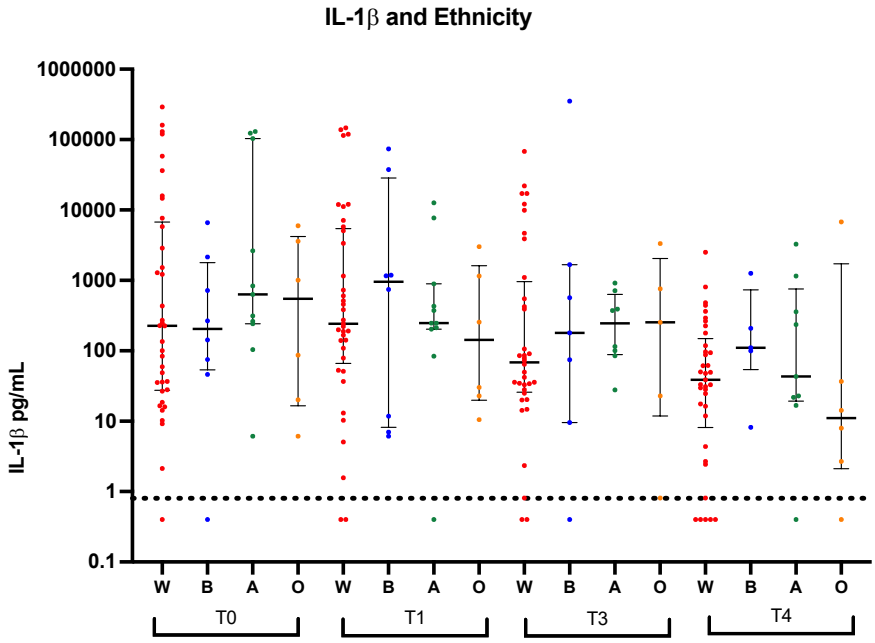


Figure 5.19 – IL-1ß concentrations according to ethnicity. At T0 and T1 data is reported for n=37, n=8, n=11 and n=6 for White, Black, Asian and other groups respectively. At T3 data is reported for n=36, n=7, n=8 and n=5 for White, Black, Asian and other groups respectively. At T4 results are reported for n=37, n=5, n=9 and n=6 for White, Black, Asian and other groups respectively. Median T0 IL-1ß concentrations were 226.7, 204.5, 631.3 and 548.1 pg/mL for White, Black, Asian and other ethnicities. Median T1 IL-1ß concentrations were 242.0, 952.6, 247.4 and 142.8 pg/mL respectively for White, Black, Asian and other ethnicities. Median T3 IL-1ß concentrations were 68.5, 180.3, 244.7 and 254.3 pg/mL for White, Black, Asian and other ethnicities. Median T4 IL-1ß concentrations were 38.8, 110.5, 43.3 and 11.1 pg/mL for White, Black, Asian and other ethnicities. IL-1ß concentrations did not differ significantly according to ethnicity at T0 ($p>0.9999$), T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p>0.9999$). Statistics: Kruskal-Wallis test with Dunn's correction. The dotted line represents the sensitivity of detection. Data is presented as median values with IQR. Abbreviations: W=White, B=Black, A=Asian, O=other.

The IL-8 concentrations at each timepoint were assessed according to ethnicity (Figure 5.20). At T0 the median IL-8 concentrations for White, Black, Asian and other women were 226.7, 204.5, 631.3 and 548.1 pg/mL respectively. Asian women had the highest median IL-8 concentration at baseline, although this was not significantly higher than White ($p>0.9999$) or Black women ($p>0.9999$).

At T1 the median IL-8 concentrations for White, Black, Asian and other women were 295.0, 436.3, 473.8 and 1,040 pg/mL respectively. The median IL-8 concentrations increased in White, Black and other ethnic groups. The median IL-8 reduced at T1 for Asian women. There was no significant difference in IL-8 concentrations between different ethnicities at T1 ($p>0.9999$).

At T3 the median IL-8 concentrations for White, Black, Asian and other women were 215.6, 836.5, 354.8 and 962.7 pg/mL respectively. At T3 the median IL-8 concentration reduced for women of all ethnic groups, except for Black women who showed an increase in the median IL-8. There was no significant difference in IL-8 concentrations between different ethnicities at T3 ($p>0.9999$).

At T4 the median IL-8 concentrations for White, Black, Asian and other women were 204.1, 403.3, 240.5 and 101.5 pg/mL respectively. The median IL-8 concentrations remained stable for White women. For Black, Asian and other ethnic groups, the median IL-8 concentration reduced. At T4 IL-8 concentrations did not differ between ethnic groups ($p>0.9999$).

Overall, Black, Asian and women from other ethnic groups showed a trend for median IL-8 concentrations to reduce by T4. In White women, the median IL-8 concentration at T4 (204.1 pg/mL) was similar to that at T0 (226.7 pg/mL). There was no significant difference between IL-8 concentrations at T0, T1, T3 or T4 according to ethnicity ($p>0.9999$). Interestingly, Asian women displayed higher median IL-8 concentrations at T0 and T1 when compared to Black and White women. In Asian women, IL-8 concentrations had a narrow range suggesting less variability between women of Asian ethnicity.

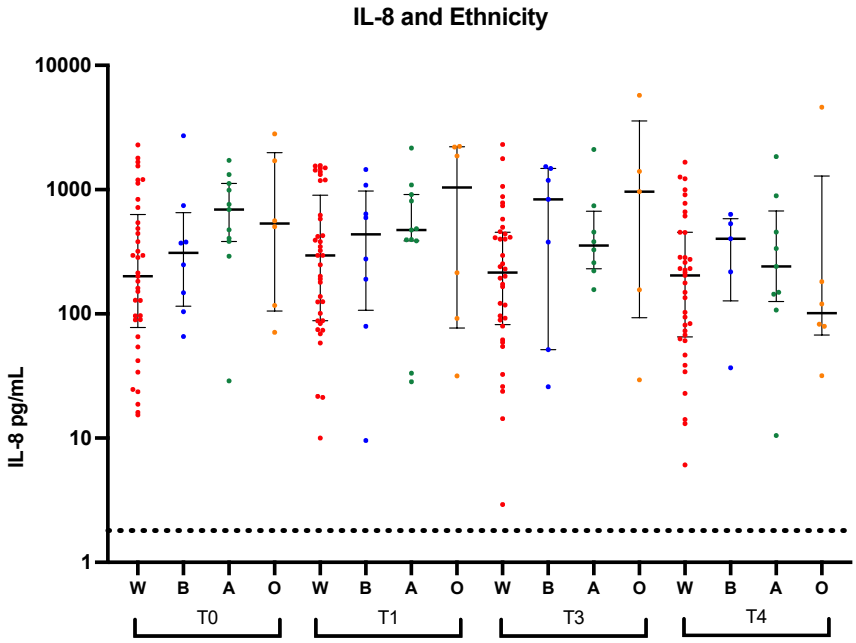


Figure 5.20 – IL-8 concentrations according to ethnicity. At T0 and T1 data is reported for n=37, n=8, n=11 and n=6 for White, Black, Asian and other groups respectively. At T3 data is reported for n=36, n=7, n=8 and n=5 for White, Black, Asian and other groups respectively. At T4 results are reported for n=37, n=5, n=9 and n=6 for White, Black, Asian and other groups respectively. Median T0 IL-8 concentrations were 226.7, 204.5, 631.3 and 548.1 pg/mL for White, Black, Asian and other ethnicities. Median T1 IL-8 concentrations were 295.0, 436.3, 473.8 and 1,040 pg/mL respectively for White, Black, Asian and other ethnicities. Median T3 IL-8 concentrations were 215.6, 836.5, 354.8 and 962.7 pg/mL for White, Black, Asian and other ethnicities. Median T4 IL-8 concentrations were 204.1, 403.3, 240.5 and 101.5 pg/mL for White, Black, Asian and other ethnicities. IL-8 concentrations did not differ significantly according to ethnicity at T0 ($p>0.9999$), T1 ($p>0.9999$), T3 ($p>0.9999$) or T4 ($p>0.9999$). Statistics: Kruskal-Wallis test with Dunn's correction. The dotted line represents the sensitivity of detection. Data is presented as median values with IQR. Abbreviations: W=White, B=Black, A=Asian, O=other.

Figure 5.21 demonstrates the proportions of women with detectable and undetectable IL-6 according to ethnicity at T0-T4. At all timepoint, the detectability of IL-6 did not differ significantly according to ethnicity (p>0.9999). Interestingly, Asian women displayed the highest proportion of IL-6 detectability at T0 (73%). IL-6 detectability showed a trend to reduce from T0-T4 in most ethnic groups. For White women, IL-6 detectability reduced from 41% at T0 to 22% by T4. The IL-6 detectability in Black women was comparable at T0 (38%) to T4 (40%). Interestingly, an increase in IL-6 detectability occurred at T1 (63%) in Black women. Asian women displayed the most marked decline in IL-6 detectability with LACTIN-V use, with IL-6 detectability reducing from 73% at T0 to 22% by T4. Women of other ethnic groups also displayed reduced IL-6 detectability across the study.

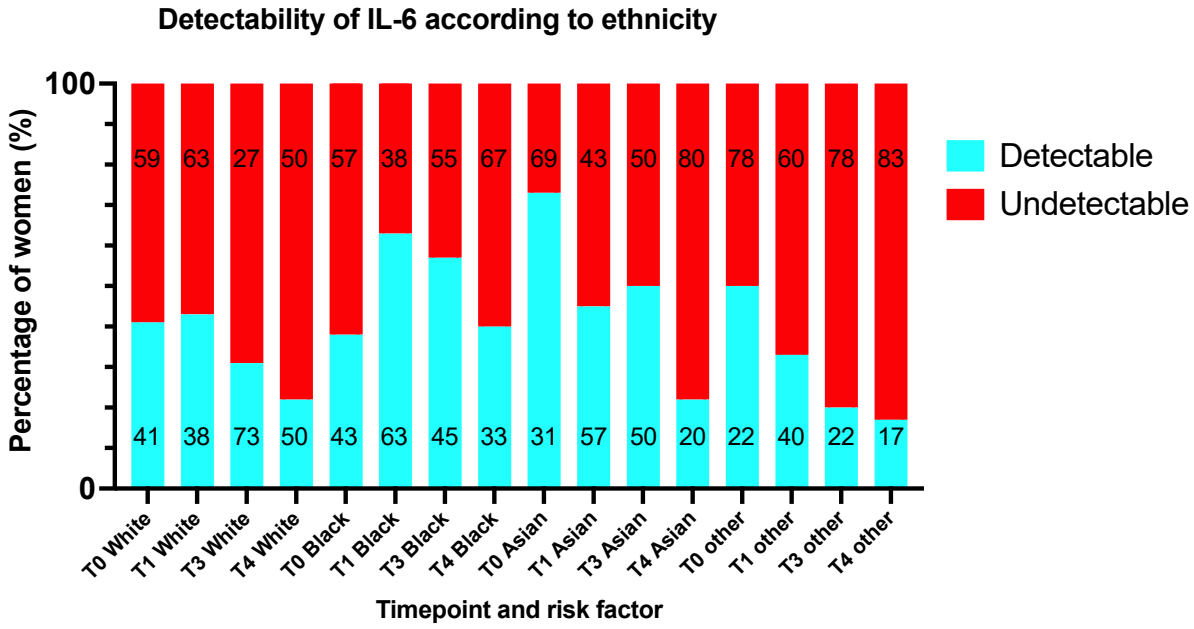


Figure 5.21 – IL-6 detectability according to ethnicity. At T0 and T1 data is reported for n=37, n=8, n=11 and n=6 for White, Black, Asian and other groups respectively. At T3 data is reported for n=36, n=7, n=8 and n=5 for White, Black, Asian and other groups respectively. At T4 results are reported for n=37, n=5, n=9 and n=6 for White, Black, Asian and other groups respectively. At T0 41%, 38%, 73% and 50% of White, Black, Asian and other women had detectable IL-6. At T1 43%, 63%, 45% and 33% of White, Black, Asian and other women had detectable IL-6. At T3 31%, 57%, 50% and 20% of White, Black, Asian and other women had detectable IL-6. At T4 22%, 40%, 22% and 17% of White, Black, Asian and other women had detectable IL-6. IL-6 detectability did not differ significantly according to ethnicity at T0 (p>0.9999), T1 (p>0.9999), T3 (p>0.9999) or T4 (p>0.9999). Statistics: one-way Chi-squared testing.

5.4 Discussion

LACTIN-V reduces pro-inflammatory cytokines

LACTIN-V leads to a reduction in pro-inflammatory cytokine concentrations at the cervicovaginal interface. Although IL-1ß concentrations remained stable between T0 to T1, reducing concentrations were seen from T3. The IL-1ß concentration at T4 was significantly lower than that at T0 (p=0.0002). IL-8 concentrations followed a similar trend, although statistical significance was not reached. Detectability of IL-6 significantly reduced at T3 (p=0.0281) and T4 (p=0.0003). These results are in keeping with the initial hypothesis; that LACTIN-V reduces pro-inflammatory CVF cytokines. Although results for IL-8 did not meet statistical significance, the study of a larger cohort may show a statistically significant reduction.

Matched analysis was performed to allow for comparison of paired cytokine values within individual patients. Analysis of matched data reinforced the association between LACTIN-V and a significant reduction in IL-1ß concentrations at T1 (p=0.0363), T3 (p=0.0254) and T4 (p=0.0002). IL-8 concentrations reduced significantly by T4 (p=0.0191). IL-6 concentrations reduced significantly by T3 (p=0.0139) and T4 (p=0.0055). IL-6 detectability also decreased across the study period.

The reduction in pro-inflammatory cytokines with LACTIN-V can be attributed to the anti-microbial properties of *L. crispatus* CTV-05. LACTIN-V administration and subsequent colonisation with *L. crispatus* CTV-05 contributes to a vaginal milieu that is hostile to pathogens, through lactic acid and H_2O_2 production, restricting pathogen adherence and minimising host response to pathogenic microbes.

The protective role of *L. crispatus* has been reproduced by others. *In-vitro* treatment of human cervical epithelial cells with *L. crispatus* inoculated with *C. trachomatis,* show that *L. crispatus* inhibits *C. trachomatis* adhesion and infectivity (764). In the presence of *L. crispatus*, IL-6, IL-8 and TNF-α production reduced, whilst IL-10 expression increased (764). Animal models also show *Lactobacillus* spp. to play a protective role. Treatment of mice with oral or vaginal lactic acid and H_2O_2 producing bacteria such as *L. johnsonii* reduced expression of pro-inflammatory IL-6, IL-1ß, TNF-α and COX-2

whilst increasing IL-10 expression in vaginal tissue (765). Importantly, authors studied the effects of either orally or vaginally administered *L. johnsonii* and route of administration was not considered in the analysis of results.

To assess the effect of *L. rhamnosus* GR-1, sPTB rates in mice were assessed after intra-uterine injection of lipopolysaccharide with and without previous *L. rhamnosus* GR-1 supernatant treatment (766). Immune assays demonstrated a decreased release of pro-inflammatory cytokines in plasma, at the myometrium and in amniotic fluid in mice treated with *L. rhamnosus* GR-1 supernatant. Moreover, mice that received intra-uterine *L. rhamnosus* GR-1 supernatant showed a significant reduction of PTB by 43%.

In human trials, non-pregnant women with BV were randomised to receive oral or vaginal multi-strain probiotics containing *L. acidophilus*, *L. rhamnosus* GG, *B. bifidum* and *B. longum,* or a placebo intervention (767). Authors reported no difference in CVF IL-1α concentrations between probiotic and placebo groups. Once again, outcomes were analysed together irrespective of whether probiotics were administered orally or vaginally. Separate analysis based on mode of administration may yield different results.

Vitali et al (2012) offered pregnant women oral probiotics containing four strains of *Lactobacillus* (*L. paracasei, L. plantarum, L. acidophilus, L. delbrueckii subsp. bulgaricus*), three strains of *Bifidobacterium* (*B. longum, B. breve, B. infantis*) and one strain of *Streptococcus thermophilus* (768). Women were recruited between 33^{+0} and 37^{+0} weeks' gestation. Authors demonstrated a significant increase in CVF anti-inflammatory IL-4 and IL-10 and decreased eotaxin in probiotic users (n=15), when compared to a non-randomised placebo control group (n=12) (768). However, authors also showed no significant alterations in the vaginal microbial composition. Authors concluded that dietary probiotics exhibit local anti-inflammatory effects. Arguably, in the absence of any alteration on the vaginal microbiome, this association requires further assessment in a larger sample size with a placebo-randomised intervention. Vitali et al (2012) did not comment on demographics, such as ethnicity, which could influence CVF cytokine measurements. The potential for probiotics to prevent sPTB

was highlighted, however, assessment of this requires intervention prior to 33^{+0} weeks'.

One study assessed 23 vaginal *Lactobacillus* isolates from South African women. Following culture of isolates with human ectocervical epithelial cells, cytokine immune assays were performed, showing that most isolates including *L. crispatus*, induced pro-inflammatory cytokine release. However, when co-cultured in the presence of *G. vaginalis*, most of the studied isolates including *L. crispatus*, supressed pro-inflammatory cytokine release (769).

Similarly to the results presented within this chapter, a trial randomised women with BV to receive vaginal tablets containing *L. brevis* CD2, *L. salivarius subsp. salicinius* and *L. plantarum* or a vaginal pH tablet and demonstrated a significant reduction in IL-1β and IL-6 CVF cytokines in those receiving probiotics (770).

Most clinical trials involving LACTIN-V focus on its safety, acceptability and colonisation dynamics. Therefore, little is known regarding the immunomodulatory effect of LACTIN-V. Armstrong et al (2022) explored the local immune response to LACTIN-V in non-pregnancy. Women with BV were randomised to receive LACTIN-V (once daily for five days, followed by twice weekly for ten weeks) or a placebo intervention, after five days of vaginal metronidazole. Sampling of women 13 weeks after LACTIN-V use demonstrated a significant reduction in CVF IL-1α concentrations. IL-8 and IL-6 were higher in placebo groups when compared to LACTIN-V groups, although statistical significance was not reached. Although Armstrong et al (2022) chose to study IL-1α, both IL-1α and IL-1ß exert similar biological functions (771). Results presented in this chapter are in keeping with results from Armstrong et al (2022).

IL-1ß is the most responsive cytokine to LACTIN-V

Interestingly, results from this chapter identify IL-1ß to be most affected by LACTIN-V. IL-1ß concentrations reduced sooner than IL-8 and IL-6. In analysis of matched data, IL-1ß was the only cytokine to reduce as early as T1.

In the presence of infection, IL-1ß is the initial cytokine to be released, followed by IL-6 and IL-8 (461). Results from this chapter suggest that IL-1ß is a 'first responder' to LACTIN-V, whilst reductions in IL-6 and IL-8 take longer to develop. Performing cytokine assays at the complete timepoint sample set (T0-T6), would permit closer assessment of the cytokine response to LACTIN-V.

IL-1ß concentrations also showed the greatest reduction with LACTIN-V, when compared to IL-8 and IL-6. Butler, Silvestroni and Stapleton (2016) co-cultured *L. crispatus* CTV-05 with vaginal epithelial cells that were either infected or non-infected with *E. coli,* a common urogenital pathogen (772). Firstly, authors showed that *L. crispatus* CTV-05 reduced the adherence of *E. coli* to vaginal epithelial cells. Secondly, a reduction in pro-inflammatory vaginal epithelial cell function was shown, as demonstrated by a decrease in IL-1ß production. Changes in IL-6 and IL-10 concentrations were not seen. This *in-vitro* assessment allowed for the assessment of a specific and single cell type, vaginal epithelial cells. However, the study of immune responses within the *in-vitro* environment is limited by removal of other cell types and influencers of immune pathways, and therefore does not fully reflect *in-vivo* immune responses. Unlike Butler, Silvestroni and Stapleton (2016), results from this chapter do demonstrate a reduction in IL-8 concentrations and IL-6 detectability with *L. crispatus* CTV-05. A decreased IL-1ß production with LACTIN-V use, is in keeping with results from Butler, Silvestroni and Stapleton (2016), whose work suggest that the immune modulation seen with *L. crispatus* CTV-05 occurs because of its effect on vaginal epithelial cells. *In-vivo* work by Armstrong et al (2022) showed LACTIN-V to enhance epithelial barrier integrity, due to the reduction in CVF E-cadherin, a biomarker of epithelial disruption. Furthermore, *in-vitro* treatment of ectocervical and endocervical epithelial cells with *L. crispatus*, *L. iners* and *G. vaginalis* bacteria-free supernatants, show reduced epithelial cell pro-inflammatory cytokine release in the presence of *L. crispatus*, when compared to *L. iners* and *G. vaginalis* (757). *In-vitro* work with ectocervical, endocervical and vaginal cell lines have also shown that the introduction of *L. crispatus* reduces pro-inflammatory cytokine release, with the converse seen with *G. vaginalis* (773).

The IL-1ß response is more profound than IL-6 and IL-8, due the impact of *L. crispatus* CTV-05 on cervicovaginal epithelial cells, the predominant source of IL-1ß (772).

Although epithelial cells contribute to IL-8 production, neutrophils are the predominant cell type to produce IL-8 (774). This explains why some reduction in IL-8 is seen in the FLIP-1 cohort. A limited influence of LACTIN-V on neutrophil function, may explain why IL-8 concentrations reduce to a lesser effect. To investigate whether LACTIN-V influences neutrophil function, future work should investigate whether LACTIN-V alters MMP concentrations. Neutrophils recruited to the cervix release MMP-8 and MMP-9, enzymes that contribute to cervical dilatation (775). A reduction in MMP-8 and MMP-9 with LACTIN-V this would suggest an impact on neutrophil cell function. Armstrong et al (2022) report a trend for lower MMP-9 concentrations in women with BV using LACTIN-V when compared to placebo, although statistical significance was not reached. Further work in pregnancy is required to determine neutrophil response to LACTIN-V.

Baseline pro-inflammatory cytokines are elevated in women with CST III and IV

At enrolment, the most common CST was CST I (44%). The remaining women were classified as CST III (24%), CST IV (16%), CST II (10%) and CST V (6%). These observed CST distributions are comparable to that seen in pregnant women of similar demographics (654).

Higher pro-inflammatory cytokines were seen at T0 in women starting with a high-risk microbial community. The IL-1ß concentrations were significantly higher (p=0.0075) for women with a CST III/IV at T0, when compared to those with CST I/II/V. The median IL-8 concentration at T0 was also higher in women starting with CST III/IV (p=0.1016), as was IL-6 detectability (p=0.0019) when compared to those with CST I/II/V. At subsequent time points, LACTIN-V use diminished the difference in cytokine concentrations between those starting with high and low-risk microbial communities.

The elevated T0 cytokines seen in women starting with a high-risk microbial community, is in-keeping with previously published work. Kindinger et al (2016) were the first to report that vaginal dysbiosis in pregnancy associated with higher levels of pro-inflammatory cytokines (IL-1ß, GM-CSF and TNF-α) (269). In contrast, *L. crispatus* abundance associates with lower CVF eotaxin, IL-1β, IL-6 and MIP-1β

concentrations (370). Studies associating elevated CVF pro-inflammatory cytokines with a sub-optimal vaginal microbial profile, also associate with sPTB (498, 776) .

Through production of anti-microbial compounds and maintaining an acidic environment, *Lactobacillus* spp. dominance is the hallmark of vaginal health. Evaluation of human CVF samples shows that diminished lactobacilli presence corresponds with increased IL-1β and IL-8 concentrations (461). Reduced lactobacilli decreases lactate levels, increasing the vaginal pH and consequently increasing susceptibility to invading microorganisms (461). Pathogen presence stimulates IL-1β release, which in turn stimulates macrophage production of IL-8. *L. crispatus* confers protective properties through its ability to produce lactic acid and H_2O_2 (546, 773). H_2O_2 kills pathogenic bacteria by damaging bacterial DNA through oxidative stress (777). However, not all *Lactobacillus* species act in the same way. *L. iners* provides limited protection against reproductive tract pathology. Production of D-lactic acid reduces the permeability of the cervicovaginal mucus and provides protection from pathogens (778). As *L. iners* is not a producer of D-lactic acid it therefore offers limited protection (778). Clinical studies have shown that *L. iners* associates with preterm delivery (352, 353). Results from this chapter therefore identify women starting with CST III or IV, as a sub-group who may benefit from LACTIN-V, in view of their higher baseline pro-inflammatory cytokines.

Starting CST influences cytokine response to LACTIN-V

Women with a baseline high-risk vaginal microbial composition (CST III/IV) show a significant IL-1ß reduction. This is coupled with a transition to a *L. crispatus* dominant state. In comparison, women with a baseline low-risk vaginal microbial composition (CST I/II/V) display similar median IL-1ß concentrations at T0 and T4, suggesting that in this group of women, LACTIN-V has a limited influence on the cervicovaginal immune milieu. In women with pre-existing *L. crispatus* dominance, endogenous *L. crispatus* may be superior to exogenous *L. crispatus.* Endogenous *L. crispatus* may have advantageous survival properties including release of anti-microbial substances, prohibiting *L. crispatus* CTV-05 adherence and growth. Endogenous *L. crispatus* may also out-compete exogenous *L. crispatus* for energy sources.

The response in IL-8 concentrations appears consistent between women with baseline low and high-risk vaginal microbial compositions. Of note, women with a baseline of CST IV show a large reduction in median IL-8 concentrations at T3, that is not seen with other baseline CSTs. Statistical analysis is limited by a small sample size, especially as only eight women had CST IV observed at baseline. Future studies with larger sample sizes should explore this trend further.

The IL-8 trend in women with a baseline CST III/IV was similar to the IL-8 trend in women with a baseline CST of I/II/V. As most women beginning with CST III/IV transitioned to a *L. crispatus* dominant state with LACTIN-V use, it can be concluded that in women with a high-risk microbial community at T0, LACTIN-V dampens down the pro-inflammatory response to a state similar to women with endogenously dominant *L. crispatus* vaginal communities.

Analysis according to starting CST shows a trend for IL-6 concentrations to decrease throughout the study in all starting CST groups. As the starting median IL-6 concentration was highest in those starting with CST III/IV, the IL-6 reduction was more profound in these women and occurred with a concomitant switch to CST I. The detectability of IL-6 reduced significantly in women starting with CST III. Women with a baseline of CST I/II/V also displayed a significant reduction in IL-6 detectability.

Results from this chapter firstly show that women with a baseline of CST III/IV, would benefit from LACTIN-V intervention due to the higher starting pro-inflammatory levels seen. Secondly it is shown that this group of women respond the most to LACTIN-V. Work from the MacIntyre/Takats group have utilised rapid metabolic profiling of CVF to describe metabolome signatures that associate with different CSTs and can consequently identify women at high-risk of sPTB. Clinical integration of this technique could rapidly identify women who would most benefit from LACTIN-V intervention, in under three minutes (736).

A baseline CST III influenced IL-6 detectability more than a baseline CST IV. At baseline, only 15 and ten women had CST III and CST IV observed respectively, restricting statistical analysis. However, for women with CST III at baseline, IL-6 detectability significantly reduced by T4 ($p=0.0175$), whereas IL-6 detectability did not

show a substantial reduction in women starting with CST IV. An over-representation of *L. iners* (CST III) is associated with shortening of the cervix and elevated CVF IL-6 (498). Results from this chapter, although require reproducibility in larger sample sizes, offer a therapeutic intervention to reduce IL-6. In women with *L. iners* dominance at 14^{+0} LACTIN-V successfully reduces IL-6 detectability from 60% to 21% from T0 to T4 (p=0.0175).

Intra-amniotic IL-6 concentrations act as independent predictors of sPTB and neonatal morbidity such as cerebral palsy (779, 780). CVF cytokines measurements have been explored as biomarkers of sPTB. IL-6 has been shown to be as effective as fFN as a marker for sPTB (743). Bedside IL-6 testing involving 100µL of vaginal fluid pipetted into a lateral flow immunoassay has been trialled and shown to guide the management of patients with threatened preterm labour (781). Bedside IL-6 testing has also shown capability to detect chorioamnionitis (782). Based on work published by Chan et al (2022), demonstrating elevated IL-6 levels in women with *L. crispatus* depletion and its association with cervical shortening and sPTB, implementation of bedside IL-6 testing could identify women early in pregnancy, that would benefit from LACTIN-V intervention (498).

A transient increase in pro-inflammatory cytokines is seen in women with baseline *L. crispatus* dominance following LACTIN-V use

In women with CST I at baseline, a transient increase in IL-1ß and IL-8 concentrations and an increase in IL-6 detectability is seen on completion of the loading dose. An initial immune response may occur upon introduction of exogenous *L. crispatus* CTV-05.

Vaginal *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were trialled for UTI prevention in post-menopausal women. An increase in the relative *Lactobacilli* abundance was seen with a simultaneous increase in IL-5, IL-1ß, IL-6, TNF-α and GM-CSF (761). Although the studied population included post-menopausal women, a group of women with limited comparability against pregnant women, in view of the altered endocrine status and microbial dynamics that occur during menopause, it is interesting that other

authors report an increase in pro-inflammatory cytokines with vaginally administered exogenous *Lactobacillus* spp.

Immune response to LACTIN-V is limited in non-colonisers

Colonisers displayed a reduction in IL-1ß and IL-8 concentrations across the study. Similarly, IL-6 detectability reduced for colonisers. These results are in keeping with the hypothesis that *L. crispatus* CTV-05 reduces pro-inflammatory cytokines.

In non-colonisers, the median IL-1ß and IL-8 concentrations at T0 and T4 were comparable, and a cytokine reduction was not seen. Similarly, IL-6 detectability in non-colonisers was comparable at T0 (30%) and T4 (25%). Without *L. crispatus* CTV-05 colonisation, women would not be subject to the anti-inflammatory effects of LACTIN-V. These results highlight the importance of strain selection when considering probiotic agents. The first step in achieving benefit lies in the ability of the selected strain to adhere and colonise. Reasons why some women do not achieve *L. crispatus* CTV-05 colonisation are discussed in Chapter 4.

Although it was hypothesised that non-colonisers would display higher cytokine concentrations when compared to colonisers, this was not the case for IL-1ß (T0, T3, T4) or IL-8 (T0, T1). IL-6 detectability was lower in non-colonisers when compared to colonisers (T0-T4). As shown in Chapter 4, non-colonisers were statistically more likely to have *L. crispatus* dominance at the study start. If non-colonisers begin with a favourable vaginal microbial community which subsequently out-competes exogenous *L. crispatus* CTV-05, a cervicovaginal milieu characterised by low pro-inflammatory cytokines is not surprising.

Women delivering preterm show a reduction in pro-inflammatory cytokines

Cytokine concentrations were compared between 49 women delivering term and nine women delivering preterm.

At T0, women delivering sPT showed higher IL-1ß, IL-8 and IL-6 concentrations, when compared to women delivering term. Although statistical significance was not reached

in the study of a small cohort size, these results are in keeping with the well-established association between elevated pro-inflammatory cytokines seen in women who eventually deliver sPT (494, 584, 585). First trimester CVF cytokines have been identified as strong predictors of sPTB (783). In Chapter 4, analysis of the nine women that delivered sPT showed that the majority (7/9, 78%) displayed a *L. crispatus* dominant state with LACTIN-V use. Results from Chapter 3 show two key findings. Firstly, the pro-inflammatory profile at T0 differs in those that deliver term versus sPT. Secondly, a reduction in median IL-1ß, IL-8 and IL-6 concentrations occurs from T0 to T4 in women that deliver sPT. In a sub-group of women compliant to LACTIN-V sPTB may still occur despite colonisation, optimisation of the vaginal community and a reduction in inflammation. In these women, sPTB may occur due to early pregnancy elevation of pro-inflammatory cytokines. Commencement of LACTIN-V prior to 14^{+0} weeks of pregnancy may permit earlier reduction in inflammation with potential to reduce sPTB risk. Importantly, whether these women would have delivered even earlier without LACTIN-V remains unknown. Additionally, the contribution of non-microbial causes of sPTB requires consideration. Among the nine women with sPTB, three participants had concomitant gestational diabetes or pre-eclampsia. Such comorbidities associate with an increased risk of sPTB (784, 785). Furthermore, one woman had DCDA twins which is also known to increase sPTB risk (99).

Ethnicity

A reduction in IL-1ß concentrations from T0-T4 was seen in all ethnic groups. Although this study was not powered to assess host-microbial response according to ethnicity, Asian women displayed higher median IL-8 concentrations at T0 and T1 when compared to Black and White women. Throughout the timepoints, less variability between Asian women was seen. Ethnic differences in host-microbial responses exist and further work is required to determine whether ethnicity impacts response to LACTIN-V (654).

Ethnicity is an important determinant of the cervicovaginal immune milieu. The greatest difference in cervicovaginal immunity seen between White and non-White pregnant women is seen in the second trimester (786). Women of African descent are more likely to have increased vaginal microbial diversity when compared to White

women (654, 787). However, many Black women lacking *Lactobacillus* spp. deliver term. Irrespective of ethnicity, *Lactobacillus* spp. abundance does not guarantee term delivery. Host immune factors, such as local ß-defensin-2 concentrations, can influence the risk of sPTB that is associated with *Lactobacillus* spp. depletion in an ethnicity-dependent manner (501). Ethnic variations in the cervicovaginal immune milieu emphasise the importance of conducting larger clinical trials with representation from a multi-ethnic cohort, to determine LACTIN-V response among different ethnicities.

Strengths

The strengths of this study include the use of Luminex Multiplex Immunoassays, a robust technique to allow comprehensive assessment of CVF cytokines. The Luminex Multiplex Immunoassay is advantageous in that the assessment of multiple analytes can be performed using small volumes of analytes. Results showed low intra and inter-plate variability, contributing to accurate results. Cytokine concentrations were reported as pg/mL rather than pg/swab to reduce swab-to-swab variation.

The extensive sample collection, including analysis of the vaginal microbiome and cytokine concentrations at corresponding timepoints allowed for an integrated and in-depth analysis of the microbial-host immune response to LACTIN-V.

Another strength included the choice to study CVF rather than peripheral cytokines. Significantly higher levels of CVF cytokines including IL-6, IL-8 and TNF-α are seen in women delivering preterm as opposed to term, and these trends are not consistent across sampling of different biologic pools such as serum samples (462, 788).

Limitations

The main limitation includes the small sample size, challenging statistical analysis. This was particularly the case for sub-analyses of women, including those delivering preterm and response according to ethnicity. Samples from T0, T1, T3 and T4 were selected for study as represented key timepoints during the LACTIN-V dosing regimen and clinically relevant points. However, conducting immune assays across all

timepoints would provide a more detailed understanding of the immune response to LACTIN-V.

The median IL-1ß and IL-8 concentrations were highest in women experiencing iatrogenic PTB at all timepoints, except for IL-8 at T1, when compared to women delivering term or sPT. Although iatrogenic PTB occurred in a small number (n=3), whether other obstetric pathologies influence CVF cytokine concentrations requires consideration. For example, a local pro-inflammatory state is also associated with pre-eclampsia (789).

Participants were not screened for STIs at the study start. Urogenital infections can cause elevated cervicovaginal cytokines (790). For example, *Chlamydia trachomatis* leads to higher IFN-γ detection in cervical washes when compared to women without *C. trachomatis* infection (791). The influence of concomitant yeasts, BV and UTIs on the cervicovaginal inflammatory profile requires consideration, as well as antibiotic therapy. Other confounders that require consideration include smoking, sexual intercourse and semen exposure (792, 793). Research has also shown that weight gain during pregnancy can alter CVF IL-1α and IL-6 concentrations (794).

During the study, 14 women (23%) required a cervical cerclage. Although most were monofilament cerclages (11, 79%), three women (21%) received a braided cervical cerclage. The impact of suture material on the cervicovaginal milieu requires consideration. Braided sutures increase bacterial diversity, leading to elevated CVF IL-1β, IL-8, IL-6, TNF-α and MMP-1 (269). Consequently, this altered immune profile contributes to cervical remodelling, leading to an increased risk of sPTB and non-viable pregnancy (269). Future studies should consistently offer monofilament cerclages, to exclude an inflammatory effect caused by the placement of a braided cerclage.

Unfortunately, the COVID-19 pandemic challenged sample collection and some samples were missing from the total cohort due to halting of clinical trial activity. This limited analysis of matched data. Interestingly, the impact of COVID-19 infection on CVF cytokines has not been studied. Analysis of cervicovaginal samples from 16 post-menopausal women admitted to intensive care with COVID-19 showed an

upregulation in IL-1α (795). Although post-menopausal women display a different microbial and immune profile when compared to pregnant women, this study highlights that severe COVID-19 infection may influence the cervicovaginal immune milieu.

Notably, the cytokine concentrations for women varied substantially. The great variability seen between participants requires thought. Others also report variations in CVF cytokine measurements during pregnancy (783). Variability may be reflective of the aforementioned factors that can influence CVF cytokine concentrations. Environmental pollutants such as carbon monoxide and particulate matter, can also alter CVF TNF-α, IL-6, MIP-1β and eotaxin concentrations (796). Unfortunately, in this study IL-10 was undetectable in most samples. Other authors similarly report IL-10 to be undetectable in CVF of pregnant women, with IL-10 more likely to be undetectable in White women (786). Although it is difficult to elucidate the cause of such variation between women, further understanding into this is required prior to the clinical implementation of LACTIN-V. Rather than offering LACTIN-V to all women at high-risk of sPTB, with further work, identification of a sub-group of women for targeted LACTIN-V therapy may be possible.

Future work

Future study design should incorporate randomisation with a placebo-control group to provide an in-depth understanding of LACTIN-V impact on the host immune response in pregnant women at high-risk of sPTB. Assessment of cytokine concentrations at all timepoints will provide a closer understanding into the LACTIN-V response across the study period.

Assessment of additional immune factors in response to LACTIN-V should be conducted. Elevated MMP-9 and complement proteins (C3a and C4a) in amniotic fluid are associated with intra-amniotic infection and sPTB (490). Higher levels of complement proteins (C3a, C4a and C5a) within the CVF are seen in sPTB, suggesting that activation of the complement system relates to sPTB (490). Expanding the panel of assessed immune factors will also determine which cell types are affected by LACTIN-V; measurement of CVF MMP-8 and MMP-9 will elucidate whether neutrophil function is altered. High diversity and depletion of *Lactobacillus* spp. leads

to elevated IgM and IgG levels and increased sPTB risk (498). Understanding the impact of LACTIN-V on local immunoglobulin levels, will deepen the understanding of LACTIN-V impact on host immune response.

5.5 Conclusion

In conclusion LACTIN-V associates with a reduction in pro-inflammatory cytokines. Starting CST influences response to LACTIN-V, with a more pronounced anti-inflammatory response seen in women with a high-risk microbial community at baseline. Further work is required to establish the impact of LACTIN-V on a wider panel of immune mediators and in a larger and randomised cohort of women at risk of sPTB.

6 Discussion

6.1 Introduction

PTB is a global health concern, affecting women and children worldwide. Despite advances in antenatal and neonatal care, the PTB incidence remains unchanged (8). PTB is associated with considerable neonatal mortality and morbidity. Lifelong consequences include cerebral palsy, speech and language difficulties and poor growth (18). Beyond the medical sequelae, the impact of PTB extends to women and their families, affecting psychosocial wellbeing (26).

PTB prevention is a global public health priority. The implementation of PTB prevention clinics as part of the NHS Long Term Plan has enabled prediction and prevention approaches to be streamlined by specialist teams (797). Nevertheless, current preventative strategies are limited and a need to explore new preventative strategies exists.

The development of culture-independent techniques has led to analysis of the vaginal microbiota in its relation to sPTB. High diversity and *L. iners* dominance predispose to sPTB, whilst *L. crispatus* dominance is protective (352, 353). Analyses of published datasets have verified this association, and the vaginal microbial profile can accurately predict sPTB (375, 376).

Modulation of the vaginal microbiome with live biotherapeutics has been explored as a means to prevent sPTB (503). Orally administered live biotherapeutics are limited in that studied strains such as *L. rhamnosus* are uncommon colonisers of the vagina (707-709). Targeting low-risk women further limits existing clinical trials (535). Most clinical trials exploring oral live biotherapeutics report on clinical outcomes, circumventing assessment of the vaginal microbiota (524, 619). Trials that do assess the vaginal microbiota are restricted by culture-based methodology or determination of overall *lactobacilli* abundance, overlooking whether vaginal colonisation with the ingested strain or alterations in microbial communities occur (527, 529). Where next generation sequencing has been utilised, oral probiotic use in pregnancy has not shown vaginal colonisation or changes to the vaginal microbial composition (530).
L. crispatus CTV-05 (LACTIN-V) is a live vaginal biotherapeutic that has been studied in its ability to modulate the vaginal microbiome and prevent pathology. *L. crispatus*

CTV-05 is unique in its ability to adhere to epithelial cells, promote vaginal acidity through lactic acid production and displace pathogens (548). Clinical trials involving non-pregnant women demonstrate LACTIN-V to be safe and acceptable with evidence of vaginal colonisation and reduced BV and UTI recurrence (551, 554).

In the FLIP-1 trial presented in this thesis, pregnant women at high-risk of sPTB were given LACTIN-V. Firstly I sought to determine its safety and acceptability in pregnancy. Further, vaginal colonisation, modulation of microbial communities and impact on local inflammation were studied.

6.2 Summary of findings

In Chapter three, LACTIN-V safety, acceptability and tolerability were explored. Solicited adverse events were reported for 13 women (19%) and mostly included altered vaginal discharge affecting seven women (10%). Clinical trials assessing LACTIN-V in non-pregnancy similarly report vaginal discharge as the most common adverse event (509, 554). Importantly, all adverse events were mild and self-resolved. LACTIN-V was tolerable; only one woman withdrew due to a mild adverse event. Fetal adverse events were also explored as a marker of safety. There were no IUDs or NNDs among women receiving LACTIN-V.

The impressive study uptake highlighted LACTIN-V acceptability among pregnant women. Notably, 97% of surveyed women would use LACTIN-V in future pregnancies if benefits and safety were confirmed. Although vaginal interventions can be regarded as more invasive and less convenient than oral preparations, results in Chapter 3 indicate vaginal administration to be unproblematic.

The prevalence of sPTB in women using LACTIN-V was compared to women at equally high-risk of sPTB. Although not powered to assess clinical outcomes, the incidence of PTB $<34^{+0}$ weeks in women using LACTIN-V was 3.3%, more than half that observed in comparison cohorts.

In Chapter 4, persistent *L. crispatus* CTV-05 colonisation was reported in 65% and 58% of women at T4 and T5 respectively. HBLC, defined as *L. crispatus* CTV-05 load $>10^6$/swab, was seen in 52% and 44% of women at T4 and T5 respectively. Overall, impressive rates of colonisation were achieved, higher than reported in non-pregnant women. *L. crispatus* CTV-05 optimises the vaginal microbial community by displacing BV-related pathogens and increasing *L. crispatus* dominance. At T0 44% of women had CST I observed, increasing to 86% and 88% at T4 and T5 respectively. Colonisers were more likely to have a baseline of CST III/IV, whilst non-colonisers were more likely to have baseline CST I observed, highlighting that women in need of microbial modulation are likely to achieve colonisation. Antibiotic use interfered with *L. crispatus* CTV-05 load, although further work is required to determine *L. crispatus* CTV-05 sensitivity to different antibiotic classes. Moreover, the absence of *L. crispatus* CTV-05 in placenta and fetal membranes obtained at ELCS support the safety profile of LACTIN-V.

In Chapter 5, a reduction in CVF pro-inflammatory cytokines was demonstrated with LACTIN-V use. By T4, a significant reduction in IL-1ß concentration was seen. A reduction in IL-8 was observed, although significance was not met. The small sample size limited statistical analysis and assessment of a larger sample size would strengthen analysis. IL-6 detectability significantly reduced by T4, with concentrations reducing as early as T3. Baseline CST influenced immune response to LACTIN-V; women with CST III/IV observed at baseline showed a more profound immune response when compared to women starting with CST I/II/V.

6.3 Strengths

This is the first in-pregnancy clinical trial to investigate vaginally administered LACTIN-V in women at high-risk of sPTB. The strain *L. crispatus* CTV-05 was selected for its advantageous properties including adherence to vaginal epithelial cells, colonisation *in-vivo*, safety profile, anti-microbial properties and unique DNA sequence facilitating its study. Regular vaginal sampling at seven timepoints across pregnancy, allowed close assessment of response. In comparison to trials assessing oral probiotics in pregnancy, this research studied that impact of LACTIN-V on the vaginal microbiome

and local inflammation. Despite the challenges posed by COVID-19, a near-complete sample set was obtained.

6.4 Limitations

The main limitation of the FLIP-1 study was its small sample size, limiting statistical analysis of assessed outcomes. FLIP-1 was not powered to assess clinical outcomes and a larger sample size is required to elucidate whether LACTIN-V reduces sPTB. A small sample size also limited the exploration of ethnicity in response to LACTIN-V, as well as sub-group analyses, including women with sPTB, PPROM or antibiotic use.

Self-reported compliance to LACTIN-V was a limitation and results from chapters four and five require consideration of this. The inclusion of trypan blue staining to verify intra-vaginal use would benefit future work (686). Confounding factors, including ethnicity, sexual intercourse, douching, use of lubricants and smoking were not accounted for. As assessment of sPTB was not a primary aim, two twin pregnancies were included. Future work assessing clinical outcomes should be restricted to singleton pregnancies, in view of the increased risk of sPTB associated with multiple pregnancies (99).

6.5 Future work

In 1993, intravaginal use of *Lactobacillus*-containing yoghurt in pregnant women with BV was shown to restore pH (536). Authors concluded by recommending future exploration of live vaginal biotherapeutics in pregnancy. Thirty years later, advancements have been minimal. Although further work is needed to build on the findings of FLIP-1, the presented results are promising. Based on data presented in this thesis, my hypothesise for future work is that through encouraging *L. crispatus* dominance, displacement of pathogens and reducing local inflammation, LACTIN-V has potential to reduce sPTB in women at high-risk.

To further explore the microbial response to LACTIN-V, whole genome shot gun sequencing, although costly and requiring extensive data analysis, would provide greater insight into *L. crispatus* CTV-05 detection in samples with high accuracy (734). The vaginal microbiota and host interactions are reflected by the metabolic milieu at the cervicovaginal mucosa (736). Future work should assess LACTIN-V impact on the CVF chemical composition and particularly address whether CVF metabolites can predict colonisation.

Assessment of the host immune response to LACTIN-V was limited to CVF IL-1ß, IL-8 and IL-6. Future work should widen the panel of assessed immune proteins to include complement, MMPs and immunoglobulins. *Lactobacillus* spp. deplete environments and high diversity associate with increased CVF MBL, IgM, IgG, C3b and C5 and subsequently an increased risk of sPTB (498). In non-pregnant women, LACTIN-V reduces soluble E-cadherin, a marker of mucosal inflammation and epithelial barrier disruption (762). Expanding the study of immune proteins will elucidate which cell types are influenced by *L. crispatus* CTV-05 colonisation, highlighting inflammatory pathways that are altered by LACTIN-V.

A single strain approach involving *L. crispatus* CTV-05 was selected for this study based on its advantageous properties and availability. The comparison of *L. crispatus* CTV-05 with other strains was outside the scope of this study. Multi-strain approaches have been studied. Advantages of multi-strain approaches include synergistic effects, enhanced adhesion and reduced antagonism from endogenous microbiota (798). However, multi-strain approaches can also create antagonistic effects between combined strains. Further work should explore whether a multi-strain live biotherapeutic involving *L. crispatus* CTV-05 would enhance efficacy.

The effect of LACTIN-V in terms of its safety, impact on microbial composition and host immune response requires verification in larger, randomised placebo-control trials, powered to assess clinical outcomes including MTL, sPTB and PPROM. Whether LACTIN-V use can prolong gestation in cases of PPROM, without compromising neonatal morbidity should also be investigated.

6.6 Clinical implementation

The clinical implementation of LACTIN-V is promising if future randomised placebo-control trials reproduce the microbial, immune and clinical benefits of LACTIN-V that have been shown in FLIP-1.

Results from Chapter 4 suggest that the dosing regimen implemented is sufficient for *L. crispatus* CTV-05 colonisation. However, antibiotics may influence colonisation and *L. crispatus* CTV-05 load. As antibiotics are the most frequently prescribed drug during pregnancy this requires careful consideration (704). Additional LACTIN-V dosing following antibiotic use may be required. Importantly, extra doses of LACTIN-V may increase adverse events.

During FLIP-1, participants were asked to abstain from sexual intercourse. As this may not be practical in the implementation of LACTIN-V, work exploring the impact of sexual intercourse on LACTIN-V response is required. Additional LACTIN-V dosing may be also required if sexual intercourse detriments colonisation.

Investigating the microbial and immune response in women delivering preterm despite LACTIN-V use will enhance efficient implementation of LACTIN-V. In Chapter 4, reasons for sPTB with LACTIN-V use are discussed and include, non-colonisation, late commencement of LACTIN-V, non-compliance and non-microbial causes of sPTB.

Based on results presented in this thesis, targeted use of LACTIN-V involving the identification of women that would most benefit from LACTIN-V, should be incorporated into clinical application. Direct on-swab desorption electrospray ionization mass spectrometry (DESI-MS) metabolic profiling of CVF, can predict sPTB through characterisation of the vaginal microbiota composition and host inflammatory profile (736). Results from Chapter 4 demonstrate that baseline CST relates to colonisation. Utilisation of DESI-MS in the form of a bedside point-of-care test may therefore facilitate targeted LACTIN-V use based on baseline metabolome signatures.

In Chapter 5, women delivering spontaneously preterm are shown to have higher IL-1ß, IL-8 and IL-6 concentrations observed at T0, when compared to women that deliver term. In a sub-group of women, sPTB occurs despite colonisation, optimisation of the vaginal microbial community and reduction in inflammation. This may be attributed to the timing of LACTIN-V intervention, which in the FLIP-1 trial, commenced at 14^{+0} weeks of pregnancy. Pro-inflammatory cytokines associated with sPTB are elevated as early as the first trimester (783). In these women, earlier intervention may be required to tackle immune pathways predisposing women to sPTB.

Bedside IL-6 testing has been developed and recent research has shown its potential to guide the management of PPROM and threatened preterm labour (781, 799). First trimester CVF IL-6 concentrations associate with sPTB risk (783). Identifying and targeting women with elevated first trimester IL-6 using bedside tests may guide LACTIN-V intervention.

In Chapter 4, a group of non-colonisers were identified. Although non-colonisers were more likely to have pre-existing *L. crispatus* at the study start, this was not the case for all women. Some women with baseline CST III or IV, did not engraft *L. crispatus* CTV-05 and a group of women resistant to colonisation may exist. Colonisation resistance may be attributed to biofilms. Biofilms are ineffectively eliminated by the immune system and incompletely destroyed by antibiotics, explaining the high recurrence of BV. Colonisation efficacy in clinical practice may be enhanced with the use of adjuvant biofilm disruptors. For example, amphoteric tensides are compounds that disrupt biofilms *in-vitro* and enhance metronidazole efficacy against BV (800, 801). Other biofilm disruptors such as the bactericidal antiseptic dequalinium chloride, may also enhance *L. crispatus* CTV-05 colonisation (802). Further work is required to assess biofilm disruptors in non-colonisers.

An alternative concept that has been recently investigated includes vaginal microbiota transplantation (VMT). Vaginal dysbiosis has been implicated in women experiencing infertility and chromosomally normal miscarriage (803, 804). In 2019, a case series describes the VMT treatment of five women with recurrent and intractable BV (805). Results were encouraging, with four women displaying long-term remission, improved symptoms, Amsel criteria and replenishment of *Lactobacillus*. More recently, a 30

year-old woman experiencing recurrent pregnancy loss with a vaginal microbiome composition dominated by *Gardnerella* spp. underwent VMT (806). A shift to *L. crispatus* dominance was seen, accompanied by an improvement in vaginal symptoms and a successful pregnancy ensued. Future RCTs are required to build on results from these case reports. VMT have not been explored in pregnant women at risk of PTB but should be considered.

Whilst exploration of VMT in pregnancy would be of great interest, the potential for LACTIN-V to modulate the vaginal microbiome is at a more advanced stage of development. Facilities are already in place for its large-scale production, as evidenced by the provision of LACTIN-V by Osel.

Although large RCTs are required to determine LACTIN-V impact on PTB, the main barriers in its clinical implementation include budget, regulatory hurdles, logistics of importing the product, cold chain maintenance, safety regulations and quality control (807). 90% of PTBs occur in low and middle-income countries where these challenges are more pronounced (2). Refrigeration of LACTIN-V during its transportation and storage is essential for maintaining *L. crispatus* CTV-05 viability. Improper storage can increase the cost burden on developing countries (808). If such challenges can be overcome, LACTIN-V presents a cost-effective intervention that is easily self-administered by women, without requiring regular monitoring from healthcare professionals. Importantly, results from the FLIP-1 study reflect host-microbe responses to LACTIN-V in pregnant women of the developed world. Further work is required to explore ethnic and socio-demographic variations that may influence LACTIN-V response in developing countries.

Live biotherapeutics require regulation. Contaminants have been identified in commercially available probiotics, including *Enterococcus faecium*, *Clostridium sporogenes* and *Streptococcus mitts* (809). Furthermore, some products do not contain the specified organisms. Screening of commercially available probiotics has shown antibiotic resistance, including resistance to vancomycin highlighting the need for regulated production to minimise the risk of transferrable resistance (700, 810). Clinical implementation of LACTIN-V will require stringent quality control and product regulation. Key considerations include product purity, viability and quantity.

Importantly, the risk of genetic drift, defined as a divergence of DNA strain caused by DNA mutation during the production process also requires consideration (811).

6.7 Conclusion

In summary, LACTIN-V use in pregnancy is safe and tolerable. Compliance and willingness for future use suggest high patient acceptance. LACTIN-V causes persistent *L. crispatus* CTV-05 colonisation, displaces less favourable bacteria and optimises the vaginal microbial community in pregnancy. Additionally, the immune milieu is improved to a more protective state. Future work should involve randomised placebo-control trials with a larger sample size with multi-ethnic representation, to confirm microbial and immune benefits. Ability to reduce sPTB in high-risk women should be determined. If benefits are demonstrated, clinical implementation with the appropriate regulations in place offer new hope for PTB prevention.

7 References

1. Jamie Perin AM, Diana Yeung, Francisco Villavicencio, Gerard Lopez, Kathleen L Strong, David Prieto-Merino, Simon Cousens, Robert E Black, Li Liu. Global, regional, and national progress towards Sustainable Development Goal 3.2 for neonatal and child health: all-cause and cause-specific mortality findings from the Global Burden of Disease Study 2019. Lancet. 2021;398(10303):870-905.
2. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller A-B, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. The lancet. 2012;379(9832):2162-72.
3. Ohuma EO, Moller A-B, Bradley E, Chakwera S, Hussain-Alkhateeb L, Lewin A, et al. National, regional, and global estimates of preterm birth in 2020, with trends from 2010: a systematic analysis. The Lancet. 2023;402(10409):1261-71.
4. Field D, Draper ES, Fenton A, Papiernik E, Zeitlin J, Blondel B, et al. Rates of very preterm birth in Europe and neonatal mortality rates. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2009;94(4):F253-F6.
5. Delnord M, Hindori-Mohangoo AD, Smith LK, Szamotulska K, Richards JL, Deb-Rinker P, et al. Variations in very preterm birth rates in 30 high-income countries: are valid international comparisons possible using routine data? BJOG: An International Journal of Obstetrics & Gynaecology. 2017;124(5):785-94.
6. Caul S. Pregnancy and ethnic factors influencing births and infant mortality: 2013. 2015.
7. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). Bmj. 2012;345.
8. Boel L, Banerjee S, Clark M, Greenwood A, Sharma A, Goel N, et al. Temporal trends of care practices, morbidity, and mortality of extremely preterm infants over 10-years in South Wales, UK. Scientific Reports. 2020;10(1):18738.
9. WHO. Preterm Birth 2023 https://www.who.int/news-room/fact-sheets/detail/preterm-birth, accessed June 2023.
10. Rees S, Inder T. Fetal and neonatal origins of altered brain development. Early human development. 2005;81(9):753-61.
11. Santhakumaran S, Statnikov Y, Gray D, Battersby C, Ashby D, Modi N. Survival of very preterm infants admitted to neonatal care in England 2008–2014: time trends and regional variation. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2018;103(3):F208-F15.
12. Ancel P-Y, Goffinet F, Kuhn P, Langer B, Matis J, Hernandorena X, et al. Survival and morbidity of preterm children born at 22 through 34 weeks' gestation in France in 2011: results of the EPIPAGE-2 cohort study. JAMA pediatrics. 2015;169(3):230-8.
13. Gkiougki E, Chatziioannidis I, Pouliakis A, Iacovidou N. Periviable birth: A review of ethical considerations. Hippokratia. 2021;25(1):1.

14. Younge N, Goldstein RF, Bann CM, Hintz SR, Patel RM, Smith PB, et al. Survival and neurodevelopmental outcomes among periviable infants. New England Journal of Medicine. 2017;376(7):617-28.
15. Inder TE, de Vries LS, Ferriero DM, Grant PE, Ment LR, Miller SP, et al. Neuroimaging of the preterm brain: review and recommendations. The Journal of Pediatrics. 2021;237:276-87. e4.
16. Greisen G. Autoregulation of cerebral blood flow in newborn babies. Early human development. 2005;81(5):423-8.
17. Stavsky M, Mor O, Mastrolia SA, Greenbaum S, Than NG, Erez O. Cerebral palsy—trends in epidemiology and recent development in prenatal mechanisms of disease, treatment, and prevention. Frontiers in pediatrics. 2017;5:21.
18. Ream MA, Lehwald L. Neurologic consequences of preterm birth. Current neurology and neuroscience reports. 2018;18:1-10.
19. de Kieviet JF, Piek JP, Aarnoudse-Moens CS, Oosterlaan J. Motor development in very preterm and very low-birth-weight children from birth to adolescence: a meta-analysis. Jama. 2009;302(20):2235-42.
20. Stoll BJ, Hansen NI, Bell EF, Walsh MC, Carlo WA, Shankaran S, et al. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993-2012. Jama. 2015;314(10):1039-51.
21. Bazacliu C, Neu J. Necrotizing enterocolitis: long term complications. Current Pediatric Reviews. 2019;15(2):115-24.
22. Camacho-Gonzalez A, Spearman PW, Stoll BJ. Neonatal infectious diseases: evaluation of neonatal sepsis. Pediatric Clinics. 2013;60(2):367-89.
23. Vergnano S, Menson E, Kennea N, Embleton N, Russell AB, Watts T, et al. Neonatal infections in England: the NeonIN surveillance network. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2011;96(1):F9-F14.
24. Medina IMF, Granero-Molina J, Fernández-Sola C, Hernández-Padilla JM, Ávila MC, Rodríguez MdML. Bonding in neonatal intensive care units: Experiences of extremely preterm infants' mothers. Women and Birth. 2018;31(4):325-30.
25. Korja R, Latva R, Lehtonen L. The effects of preterm birth on mother–infant interaction and attachment during the infant's first two years. Acta obstetricia et gynecologica Scandinavica. 2012;91(2):164-73.
26. Bener A. Psychological distress among postpartum mothers of preterm infants and associated factors: a neglected public health problem. Brazilian Journal of Psychiatry. 2013;35:231-6.
27. Meijssen D, Wolf MJ, Koldewijn K, van Baar A, Kok J. Maternal psychological distress in the first two years after very preterm birth and early intervention. Early Child Development and Care. 2011;181(1):1-11.
28. Pierrehumbert B, Nicole A, Muller-Nix C, Forcada-Guex M, Ansermet F. Parental post-traumatic reactions after premature birth: implications for sleeping and eating problems in the infant. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2003;88(5):F400-F4.
29. Caporali C, Pisoni C, Gasparini L, Ballante E, Zecca M, Orcesi S, et al. A global perspective on parental stress in the neonatal intensive care unit: a meta-analytic study. Journal of Perinatology. 2020;40(12):1739-52.
30. Crump C, Sundquist J, Sundquist K. Preterm delivery and long term mortality in women: national cohort and co-sibling study. bmj. 2020;370.

31. NICE. Preterm Labour and Birth 2022 https://www.nice.org.uk/guidance/ng25, accessed June 2023.
32. Mangham LJ, Petrou S, Doyle LW, Draper ES, Marlow N. The cost of preterm birth throughout childhood in England and Wales. Pediatrics. 2009;123(2):e312-27.
33. Álvarez-Fuente M, Arruza L, Muro M, Zozaya C, Avila A, López-Ortego P, et al. The economic impact of prematurity and bronchopulmonary dysplasia. European journal of pediatrics. 2017;176:1587-93.
34. Cavallo MC, Gugiatti A, Fattore G, Gerzeli S, Barbieri D, Zanini R. Cost of care and social consequences of very low birth weight infants without premature-related morbidities in Italy. Italian journal of pediatrics. 2015;41(1):1-12.
35. Fox H, Callander E. Cost of preterm birth to Australian mothers: Assessing the financial impact of a birth outcome with an increasing prevalence. Journal of Paediatrics and Child Health. 2021;57(5):618-25.
36. Thornton JM, Browne B, Ramphul M. Mechanisms and management of normal labour. Obstetrics, Gynaecology & Reproductive Medicine. 2020;30(3):84-90.
37. Word RA, Li X-H, Hnat M, Carrick K, editors. Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. Seminars in reproductive medicine; 2007: Copyright© 2007 by Thieme Publishers, Inc., 333 Seventh Avenue, New York, NY ….
38. Nott JP, Bonney EA, Pickering JD, Simpson NA. The structure and function of the cervix during pregnancy. Translational Research in Anatomy. 2016;2:1-7.
39. Carvalho M, Bittar R, Brizot M, Maganha P, Borges da Fonseca E, Zugaib M. Cervical length at 11–14 weeks' and 22–24 weeks' gestation evaluated by transvaginal sonography, and gestational age at delivery. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2003;21(2):135-9.
40. Read CP, Word RA, Ruscheinsky MA, Timmons BC, Mahendroo MS. Cervical remodeling during pregnancy and parturition: molecular characterization of the softening phase in mice. Reproduction. 2007;134(2):327-40.
41. Yoshimatsu K, Sekiya T, Ishihara K, Fukami T, Otabe T, Araki T. Detection of the cervical gland area in threatened preterm labor using transvaginal sonography in the assessment of cervical maturation and the outcome of pregnancy. Gynecologic and obstetric investigation. 2002;53(3):149-56.
42. Havelock JC, Keller P, Muleba N, Mayhew BA, Casey BM, Rainey WE, et al. Human myometrial gene expression before and during parturition. Biology of reproduction. 2005;72(3):707-19.
43. Moriyama A, Shimoya K, Ogata I, Kimura T, Nakamura T, Wada H, et al. Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle. Molecular human reproduction. 1999;5(7):656-61.
44. Nold C, Anton L, Brown A, Elovitz M. Inflammation promotes a cytokine response and disrupts the cervical epithelial barrier: a possible mechanism of premature cervical remodeling and preterm birth. American journal of obstetrics and gynecology. 2012;206(3):208. e1-. e7.
45. Bokström H, Brännström M, Alexandersson M, Norström A. Leukocyte subpopulations in the human uterine cervical stroma at early and term pregnancy. Human reproduction (Oxford, England). 1997;12(3):586-90.

46. Barclay CG, Brennand JE, Kelly RW, Galder AA. Interleukin-8 production by the human cervix. American journal of obstetrics and gynecology. 1993;169(3):625-32.
47. Leppert PC. Anatomy and physiology of cervical ripening. Clinical obstetrics and gynecology. 1995;38(2):267-79.
48. Uchiyama T, Matsumoto T, Suzuki Y, Ishida M, Obara T, Kanayama T. Endogenous hyaluronan: a cytokine-like factor present in rabbit uterine cervix during pregnancy. Biological and Pharmaceutical Bulletin. 2004;27(12):1907-12.
49. Obara M, Hirano H, Ogawa M, Tsubaki H, Hosoya N, Yoshida Y, et al. Changes in molecular weight of hyaluronan and hyaluronidase activity in uterine cervical mucus in cervical ripening. Acta Obstetricia et Gynecologica Scandinavica. 2001;80(6):492-6.
50. Mowa C, Jesmin S, Sakuma I, Usip S, Togashi H, Yoshioka M, et al. Characterization of vascular endothelial growth factor (VEGF) in the uterine cervix over pregnancy: effects of denervation and implications for cervical ripening. Journal of Histochemistry & Cytochemistry. 2004;52(12):1665-74.
51. Osmers R, Rath W, Adelmann-Grill B, Fittkow C, Kuloczik M, Szeverenyi M, et al. Origin of cervical collagenase during parturition. American Journal of Obstetrics and Gynecology. 1992;166(5):1455-60.
52. Lindstrom TM, Bennett PR. The role of nuclear factor kappa B in human labour. Reproduction. 2005;130(5):569-81.
53. Bourne GL. The microscopic anatomy of the human amnion and chorion. American journal of obstetrics and gynecology. 1960;79(6):1070-3.
54. Manabe Y, Himeno N, Fukumoto M. Tensile strength and collagen content of amniotic membrane do not change after the second trimester or during delivery. Obstetrics & Gynecology. 1991;78(1):24-7.
55. Oyen ML, Calvin SE, Landers DV. Premature rupture of the fetal membranes: is the amnion the major determinant? American journal of obstetrics and gynecology. 2006;195(2):510-5.
56. Arikat S, Novince RW, Mercer BM, Kumar D, Fox JM, Mansour JM, et al. 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. 2006;194(1):211-7.
57. Strauss III JF. Extracellular matrix dynamics and fetal membrane rupture. Reproductive Sciences. 2013;20(2):140-53.
58. Gomez-Lopez N, Laresgoiti-Servitje E, Olson DM, Estrada-Gutiérrez G, Vadillo-Ortega F. The role of chemokines in term and premature rupture of the fetal membranes: a review. Biology of reproduction. 2010;82(5):809-14.
59. Reti NG, Lappas M, Riley C, Wlodek ME, Permezel M, Walker S, et al. Why do membranes rupture at term? Evidence of increased cellular apoptosis in the supracervical fetal membranes. American journal of obstetrics and gynecology. 2007;196(5):484. e1-. e10.
60. Woessner Jr JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. The FASEB Journal. 1991;5(8):2145-54.
61. Malak T, Bell S. Structural characteristics of term human fetal membranes: a novel zone of extreme morphological alteration within the rupture site. BJOG: An International Journal of Obstetrics & Gynaecology. 1994;101(5):375-86.
62. McLaren J, Taylor D, Bell S. Prostaglandin E2-dependent production of latent matrix metalloproteinase-9 in cultures of human fetal membranes. Molecular human reproduction. 2000;6(11):1033-40.

63. Johnson JW, Daikoku NH, Niebyl JR, Johnson Jr TR, Khouzami VA, Witter FR. Premature rupture of the membranes and prolonged latency. Obstetrics & Gynecology. 1981;57(5):547-56.
64. Aguilar HN, Mitchell B. Physiological pathways and molecular mechanisms regulating uterine contractility. Human reproduction update. 2010;16(6):725-44.
65. Young RC. Myocytes, myometrium, and uterine contractions. Annals of the new york academy of sciences. 2007;1101(1):72-84.
66. Sanborn BM. Hormones and calcium: mechanisms controlling uterine smooth muscle contractile activity. Experimental physiology. 2001;86(2):223-37.
67. Wray S, Jones K, Kupittayanant S, Li Y, Matthew A, Monir-Bishty E, et al. Calcium signaling and uterine contractility. The Journal of the Society for Gynecologic Investigation: JSGI. 2003;10(5):252-64.
68. Mitchell B, Schmid B. Oxytocin and its receptor in the process of parturition. Journal of the Society for Gynecologic Investigation. 2001;8(3):122-33.
69. Kim SH, Bennett PR, Terzidou V. Advances in the role of oxytocin receptors in human parturition. Molecular and cellular endocrinology. 2017;449:56-63.
70. Sanborn BM, Ku C-Y, Shlykov S, Babich L. Molecular signaling through G-protein-coupled receptors and the control of intracellular calcium in myometrium. The Journal of the Society for Gynecologic Investigation: JSGI. 2005;12:479-87.
71. Kim SH, Bennett PR, Terzidou V. Diverse roles of oxytocin. Inflammation and Cell Signaling. 2015;2.
72. Fuchs A-R, Fuchs F, Husslein P, Soloff MS, Fernström MJ. Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor. Science. 1982;215(4538):1396-8.
73. Petraglia F, Sutton S, Vale W. Neurotransmitters and peptides modulate the release of immunoreactive corticotropin-releasing factor from cultured human placental cells. American Journal of Obstetrics and Gynecology. 1989;160(1):247-51.
74. Sfakianaki AK, Norwitz ER. Mechanisms of progesterone action in inhibiting prematurity. The Journal of Maternal-Fetal & Neonatal Medicine. 2006;19(12):763-72.
75. Challis J, Lye S. Characteristics of parturition. Maternal-fetal medicine: principles and practice. 1999:79-87.
76. Tan H, Yi L, Rote NS, Hurd WW, Mesiano S. Progesterone receptor-A and-B have opposite effects on proinflammatory gene expression in human myometrial cells: implications for progesterone actions in human pregnancy and parturition. The Journal of Clinical Endocrinology. 2012;97(5):E719-E30.
77. Grammatopoulos DK, Hillhouse EW. Role of corticotropin-releasing hormone in onset of labour. The Lancet. 1999;354(9189):1546-9.
78. Li W, Challis JR. Corticotropin-releasing hormone and urocortin induce secretion of matrix metalloproteinase-9 (MMP-9) without change in tissue inhibitors of MMP-1 by cultured cells from human placenta and fetal membranes. The Journal of Clinical Endocrinology & Metabolism. 2005;90(12):6569-74.
79. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A placental clock controlling the length of human pregnancy. Nature medicine. 1995;1(5):460-3.
80. Smith R, Mesiano S, McGrath S. Hormone trajectories leading to human birth. Regulatory peptides. 2002;108(2-3):159-64.

81. Herrera CL, Bowman ME, McIntire DD, Nelson DB, Smith R. Revisiting the placental clock: Early corticotrophin-releasing hormone rise in recurrent preterm birth. PloS one. 2021;16(9):e0257422.
82. Papatheodorou DC, Karagiannidis LK, Paltoglou G, Margeli A, Kaparos G, Valsamakis G, et al. Pulsatile interleukin-6 leads CRH secretion and is associated with myometrial contractility during the active phase of term human labor. The Journal of Clinical Endocrinology & Metabolism. 2013;98(10):4105-12.
83. Bolt R, Van Weissenbruch M, Lafeber H, Delemarre-Van de Waal H. Development of the hypothalamic-pituitary-adrenal axis in the fetus and preterm infant. Journal of Pediatric Endocrinology and Metabolism. 2002;15(6):759-70.
84. Wijaya JC, Khanabdali R, Georgiou HM, Kalionis B. Ageing in human parturition: impetus of the gestation clock in the decidua. Biology of Reproduction. 2020;103(4):695-710.
85. Condon JC, Jeyasuria P, Faust JM, Mendelson CR. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. Proceedings of the National Academy of Sciences. 2004;101(14):4978-83.
86. Menon R, Bonney EA, Condon J, Mesiano S, Taylor RN. Novel concepts on pregnancy clocks and alarms: redundancy and synergy in human parturition. Human reproduction update. 2016;22(5):535-60.
87. VIRGO BB, BELLWARD GD. Serum progesterone levels in the pregnant and postpartum laboratory mouse. Endocrinology. 1974;95(5):1486-90.
88. Feinshtein V, Ben-Zvi Z, Sheiner E, Amash A, Sheizaf B, Holcberg G. Progesterone levels in cesarean and normal delivered term placentas. Archives of gynecology and obstetrics. 2010;281:387-92.
89. Astle S, Slater DM, Thornton S. The involvement of progesterone in the onset of human labour. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2003;108(2):177-81.
90. Karalis K, Goodwin G, Majzoub JA. Cortisol blockade of progesterone: a possible molecular mechanism involved in the initiation of human labor. Nature medicine. 1996;2(5):556-60.
91. Aghaeepour N, Ganio EA, Mcilwain D, Tsai AS, Tingle M, Van Gassen S, et al. An immune clock of human pregnancy. Science immunology. 2017;2(15):eaan2946.
92. Romero R, Erez O, Maymon E, Chaemsaithong P, Xu Z, Pacora P, et al. The maternal plasma proteome changes as a function of gestational age in normal pregnancy: a longitudinal study. American journal of obstetrics and gynecology. 2017;217(1):67. e1-. e21.
93. Mitsuya K, Singh N, Sooranna SR, Johnson MR, Myatt L. Epigenetics of human myometrium: DNA methylation of genes encoding contraction-associated proteins in term and preterm labor. Biology of reproduction. 2014;90(5):98, 1-8.
94. Wang X-M, Tian F-Y, Fan L-J, Xie C-B, Niu Z-Z, Chen W-Q. Comparison of DNA methylation profiles associated with spontaneous preterm birth in placenta and cord blood. BMC Medical Genomics. 2019;12:1-14.
95. Leguizamón G, Smith J, Younis H, Nelson DM, Sadovsky Y. Enhancement of amniotic cyclooxygenase type 2 activity in women with preterm delivery associated with twinsor polyhydramnios. American journal of obstetrics and gynecology. 2001;184(2):117-22.
96. Nemeth E, Tashima LS, Yu Z, Bryant-Greenwood GD. Fetal membrane distention: I. Differentially expressed genes regulated by acute distention in amniotic epithelial (WISH) cells. American journal of obstetrics and gynecology. 2000;182(1):50-9.

97. Kirkinen P, Jouppila P, editors. Polyhydramnion. A clinical study. Annales Chirurgiae et Gynaecologiae; 1978.
98. Girault A, Le Ray C, Chapron C, Goffinet F, Marcellin L. Leiomyomatous uterus and preterm birth: an exposed/unexposed monocentric cohort study. American Journal of Obstetrics and Gynecology. 2018;219(4):410. e1-. e7.
99. Stock S, Norman J, editors. Preterm and term labour in multiple pregnancies. Seminars in Fetal and Neonatal Medicine; 2010: Elsevier.
100. Kim M-A, Kim HS, Kim Y-H. Reproductive, obstetric and neonatal outcomes in women with congenital uterine anomalies: a systematic review and meta-analysis. Journal of clinical medicine. 2021;10(21):4797.
101. Berghella V, Saccone G. Fetal fibronectin testing for reducing the risk of preterm birth. Cochrane Database of Systematic Reviews. 2019(7).
102. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. The lancet. 2008;371(9606):75-84.
103. Peaceman AM, Lai Y, Rouse DJ, Spong CY, Mercer BM, Varner MW, et al. Length of latency with preterm premature rupture of membranes before 32 weeks' gestation. American journal of perinatology. 2015;32(01):057-62.
104. Romero R, Quintero R, Oyarzun E, Wu YK, Sabo V, Mazor M, et al. Intraamniotic infection and the onset of labor in preterm premature rupture of the membranes. American journal of obstetrics and gynecology. 1988;159(3):661-6.
105. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. BJOG: An International Journal of Obstetrics & Gynaecology. 2006;113:17-42.
106. Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. BJOG: An International Journal of Obstetrics & Gynaecology. 2003;110:71-5.
107. Lockwood CJ. Predicting premature delivery—no easy task. Mass Medical Soc; 2002. p. 282-4.
108. Hirsch E, Saotome I, Hirsch D. A model of intrauterine infection and preterm delivery in mice. American journal of obstetrics and gynecology. 1995;172(5):1598-603.
109. Gibbs RS, McDuffie Jr RS, Kunze M, Barr JM, Wolf DM, Sze C-I, et al. Experimental intrauterine infection with Prevotella bivia in New Zealand White rabbits. American journal of obstetrics and gynecology. 2004;190(4):1082-6.
110. McDuffie Jr R, Sherman MP, Gibbs RS. Amniotic fluid tumor necrosis factor-α and interleukin-1 in a rabbit model of bacterially induced preterm pregnancy loss. American journal of obstetrics and gynecology. 1992;167(6):1583-8.
111. Elphinstone RE, Weckman AM, McDonald CR, Tran V, Zhong K, Madanitsa M, et al. Early malaria infection, dysregulation of angiogenesis, metabolism and inflammation across pregnancy, and risk of preterm birth in Malawi: a cohort study. PLoS medicine. 2019;16(10):e1002914.
112. Chen Y-H, Keller J, Wang I-T, Lin C-C, Lin H-C. Pneumonia and pregnancy outcomes: a nationwide population-based study. American journal of obstetrics and gynecology. 2012;207(4):288. e1-. e7.
113. Farkash E, Weintraub AY, Sergienko R, Wiznitzer A, Zlotnik A, Sheiner E. Acute antepartum pyelonephritis in pregnancy: a critical analysis of risk factors and outcomes. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2012;162(1):24-7.

114. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. Jama. 1986;256(14):1899-903.
115. Cotch MF, Pastorek JG, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. Trichomonas vaginalis associated with low birth weight and preterm delivery. Sexually transmitted diseases. 1997:353-60.
116. Thomsen A, Mørup L, Hansen KB. Antibiotic elimination of group-B streptococci in urine in prevention of preterm labour. The Lancet. 1987;329(8533):591-3.
117. Goldenberg RL, Andrews WW, Hauth JC. Choriodecidual infection and preterm birth. Nutrition reviews. 2002;60(suppl_5):S19-S25.
118. CASSELL GH, DAVIS RO, WAITES KB, BROWN MB, MARRIOTT PA, STAGNO S, et al. Isolation of Mycoplasma hominis and Ureaplasma urealyticum from amniotic fluid at 16-20 weeks of gestation: potential effect on outcome of pregnancy. Sexually transmitted diseases. 1983:294-302.
119. Horowitz S, Mazor M, Romero R, Horowitz J, Glezerman M. Infection of the amniotic cavity with Ureaplasma urealyticum in the midtrimester of pregnancy. The Journal of reproductive medicine. 1995;40(5):375-9.
120. Andrews WW, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. American journal of obstetrics and gynecology. 2000;183(3):662-8.
121. Vallely LM, Egli-Gany D, Wand H, Pomat WS, Homer CS, Guy R, et al. Adverse pregnancy and neonatal outcomes associated with Neisseria gonorrhoeae: systematic review and meta-analysis. Sexually transmitted infections. 2021;97(2):104-11.
122. Goldenberg RL, Andrews WW, Goepfert AR, Faye-Petersen O, Cliver SP, Carlo WA, et al. The Alabama Preterm Birth Study: umbilical cord blood Ureaplasma urealyticum and Mycoplasma hominis cultures in very preterm newborn infants. American journal of obstetrics and gynecology. 2008;198(1):43. e1-. e5.
123. Noda-Nicolau NM, Tantengco OAG, Polettini J, Silva MC, Bento GF, Cursino GC, et al. Genital mycoplasmas and biomarkers of inflammation and their Association with spontaneous Preterm Birth and Preterm Prelabor rupture of membranes: A systematic review and Meta-analysis. Frontiers in Microbiology. 2022;13:859732.
124. Miyoshi Y, Suga S, Sugimi S, Kurata N, Yamashita H, Yasuhi I. Vaginal Ureaplasma urealyticum or Mycoplasma hominis and preterm delivery in women with threatened preterm labor. The Journal of Maternal-Fetal & Neonatal Medicine. 2022;35(5):878-83.
125. Meijer WJ, van Noortwijk AG, Bruinse HW, Wensing AM. Influenza virus infection in pregnancy: a review. Acta obstetricia et gynecologica Scandinavica. 2015;94(8):797-819.
126. Gibson CS, Goldwater P, MacLennan A, Haan E, Priest K, Dekker G, et al. Fetal exposure to herpesviruses may be associated with pregnancy-induced hypertensive disorders and preterm birth in a Caucasian population. BJOG: An International Journal of Obstetrics & Gynaecology. 2008;115(4):492-500.
127. KOI H, ZHANG J, PARRY S. The mechanisms of placental viral infection. Annals of the New York Academy of Sciences. 2001;943(1):148-56.
128. Conner SN, Frey HA, Cahill AG, Macones GA, Colditz GA, Tuuli MG. Loop electrosurgical excision procedure and risk of preterm birth: a systematic review and meta-analysis. Obstetrics and gynecology. 2014;123(4):752.

129. Bearfield C, Davenport ES, Sivapathasundaram V, Allaker RP. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. BJOG: an international journal of obstetrics and gynaecology. 2002;109(5):527-33.
130. Khader YS, Ta'ani Q. Periodontal diseases and the risk of preterm birth and low birth weight: A meta-analysis. Journal of periodontology. 2005;76(2):161-5.
131. Romero R, Espinoza J, Chaiworapongsa T, Kalache K, editors. Infection and prematurity and the role of preventive strategies. Seminars in Neonatology; 2002: Elsevier.
132. Romero R, Gonzalez R, Sepulveda W, Brandt F, Ramirez M, Sorokin Y, et al. Infection and labor: VIII. Microbial invasion of the amniotic cavity in patients with suspected cervical incompetence: prevalence and clinical significance. American journal of obstetrics and gynecology. 1992;167(4):1086-91.
133. Hassan S, Romero R, Hendler I, Gomez R, Khalek N, Espinoza J, et al. A sonographic short cervix as the only clinical manifestation of intra-amniotic infection. Journal of perinatal medicine. 2006;34(1):13-9.
134. Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, et al. 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. 1989;161(3):817-24.
135. Romero R, Mazor M, Morrotti R, Avila C, Oyarzun E, Insunza A, et al. Infection and labor: VII. Microbial invasion of the amniotic cavity in spontaneous rupture of membranes at term. American journal of obstetrics and gynecology. 1992;166(1):129-33.
136. Yoon BH, Romero R, Lim J-H, Shim S-S, Hong J-S, Shim J-Y, et al. The clinical significance of detecting Ureaplasma urealyticum by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. American journal of obstetrics and gynecology. 2003;189(4):919-24.
137. Kase NG, Glass RH. Clinical gynecologic endocrinology and infertility: Williams & Wilkins; 1994.
138. Sideris IG, Nicolaides KH. Amniotic fluid pressure during pregnancy. Fetal diagnosis and therapy. 1990;5(2):104-8.
139. Laudański T, Rocki W. The effects on stretching and prostaglandin F2alpha on the contractile and bioelectric activity of the uterus in rat. Acta Physiologica Polonica. 1975;26(4):385-93.
140. Kloeck F, Jung H. In vitro release of prostaglandins from the human myometrium under the influence of stretching. American journal of obstetrics and gynecology. 1973;115(8):1066-9.
141. Ou C-W, Chen Z-Q, Qi S, Lye SJ. Increased expression of the rat myometrial oxytocin receptor messenger ribonucleic acid during labor requires both mechanical and hormonal signals. Biology of reproduction. 1998;59(5):1055-61.
142. Maehara K, Kanayama N, Maradny EE, Uezato T, Fujita M, Terao T. Mechanical stretching induces interleukin-8 gene expression in fetal membranes: a possible role for the initiation of human parturition. European Journal of Obstetrics & Gynecology and Reproductive Biology. 1996;70(2):191-6.
143. El Maradny E, Kanayama N, Halim A, Maehara K, Terao T. Stretching of fetal membranes increases the concentration of interleukin-8 and collagenase activity. American journal of obstetrics and gynecology. 1996;174(3):843-9.
144. Kanayama N, Fukamizu H. Mechanical stretching increases prostaglandin E2 in cultured human amnion cells. Gynecologic and obstetric investigation. 1989;28(3):123-6.

145. Obstetricians ACo, Gynecologists. Cervical insufficiency. ACOG practice bulletin. 2003(48):716-24.
146. Roman A, Suhag A, Berghella V. Overview of cervical insufficiency: diagnosis, etiologies, and risk factors. Clinical obstetrics and gynecology. 2016;59(2):237-40.
147. Brown R, Gagnon R, Delisle M-F. No. 373-cervical insufficiency and cervical cerclage. Journal of Obstetrics and Gynaecology Canada. 2019;41(2):233-47.
148. Romero R, Espinoza J, Erez O, Hassan S. The role of cervical cerclage in obstetric practice: can the patient who could benefit from this procedure be identified? American journal of obstetrics and gynecology. 2006;194(1):1-9.
149. Kristensen J, Langhoff-Roos J, Wittrup M, Bock J. Cervical conization and preterm delivery/low birth weight: a systematic review of the literature. Acta obstetricia et gynecologica scandinavica. 1993;72(8):640-4.
150. Watson HA, Carter J, David AL, Seed PT, Shennan AH. Full dilation cesarean section: a risk factor for recurrent second-trimester loss and preterm birth. Acta Obstetricia et Gynecologica Scandinavica. 2017;96(9):1100-5.
151. Zhou H, Lai K-F, Xiang Q, Zhang L-L, Xu Y, Cheng C, et al. Second-stage cesarean delivery and preterm birth in subsequent pregnancy: A large multi-institutional cohort study. Journal of Gynecology Obstetrics and Human Reproduction. 2022;51(8):102447.
152. Schulz K, Grimes D, Cates Jr W. Measures to prevent cervical injury during suction curettage abortion. The lancet. 1983;321(8335):1182-5.
153. Cong A, de Vries B, Ludlow J. Does previous caesarean section at full dilatation increase the likelihood of subsequent spontaneous preterm birth? Australian and New Zealand Journal of Obstetrics and Gynaecology. 2018;58(3):267-73.
154. Wang M, Kirby A, Gibbs E, Gidaszewski B, Khajehei M, Chua SC. Risk of preterm birth in the subsequent pregnancy following caesarean section at full cervical dilatation compared with mid-cavity instrumental delivery. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2020;60(3):382-8.
155. Levine LD, Sammel MD, Hirshberg A, Elovitz MA, Srinivas SK. Does stage of labor at time of cesarean delivery affect risk of subsequent preterm birth? American journal of obstetrics and gynecology. 2015;212(3):360. e1-. e7.
156. Vahanian SA, Vintzileos AM. The role of second-stage cesarean delivery in contributing to preterm delivery. American Journal of Obstetrics & Gynecology. 2020;222(6):636-7.
157. Bitensky S, Pariente G, Rotem R, Sergienko R, Weintraub AY. Risk for complications in the subsequent pregnancy following first versus second-stage cesarean delivery: 25 years follow-up in a large cohort. The Journal of Maternal-Fetal & Neonatal Medicine. 2022;35(23):4485-90.
158. Han Y, Li M, Ma H, Yang H. Cervical insufficiency: a noteworthy disease with controversies. Journal of Perinatal Medicine. 2020;48(7):648-55.
159. Lind J, Wallenburg HC. Pregnancy and the Ehlers–Danlos syndrome: a retrospective study in a Dutch population. Acta Obstetricia et Gynecologica Scandinavica. 2002;81(4):293-300.
160. Anum EA, Hill LD, Pandya A, Strauss III JF. Connective tissue and related disorders and preterm birth: clues to genes contributing to prematurity. Placenta. 2009;30(3):207-15.
161. GOLDSTEIN DP. Incompetent cervix in offspring exposed to diethylstilbestrol in utero. Obstetrics & Gynecology. 1978;52(1):73s-5s.

162. Arias F, Rodriquez L, Rayne SC, Kraus FT. Maternal placental vasculopathy and infection: two distinct subgroups among patients with preterm labor and preterm ruptured membranes. American journal of obstetrics and gynecology. 1993;168(2):585-91.
163. Giorgione V, Quintero Mendez O, Pinas A, Ansley W, Thilaganathan B. Routine first-trimester pre-eclampsia screening and risk of preterm birth. Ultrasound in Obstetrics & Gynecology. 2022;60(2):185-91.
164. Campos G, Guerra F, Israel E. Angiotensin II induced release of prostaglandins from rat uterus. Archivos de Biologia y Medicina Experimentales. 1983;16(1):43-9.
165. Woods LL, Brooks VL. Role of the renin-angiotensin system in hypertension during reduced uteroplacental perfusion pressure. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1989;257(1):R204-R9.
166. Yart L, Roset Bahmanyar E, Cohen M, Martinez de Tejada B. Role of the uteroplacental renin–angiotensin system in placental development and function, and its implication in the preeclampsia pathogenesis. Biomedicines. 2021;9(10):1332.
167. Elovitz MA, Ascher-Landsberg J, Saunders T, Phillippe M. The mechanisms underlying the stimulatory effects of thrombin on myometrial smooth muscle. American journal of obstetrics and gynecology. 2000;183(3):674-81.
168. Elovitz MA, Saunders T, Ascher-Landsberg J, Phillippe M. Effects of thrombin on myometrial contractions in vitro and in vivo. American journal of obstetrics and gynecology. 2000;183(4):799-804.
169. Duhamel-Clérin E, Orvain C, Lanza Fo, Cazenave J-P, Klein-Soyer C. Thrombin receptor-mediated increase of two matrix metalloproteinases, MMP-1 and MMP-3, in human endothelial cells. Arteriosclerosis, thrombosis, and vascular biology. 1997;17(10):1931-8.
170. Hashem A, Sarsam SD. The impact of incidental ultrasound finding of subchorionic and retroplacental hematoma in early pregnancy. The Journal of Obstetrics and Gynecology of India. 2019;69:43-9.
171. Al-Memar M, Vaulet T, Fourie H, Bobdiwala S, Farren J, Saso S, et al. First-trimester intrauterine hematoma and pregnancy complications. Ultrasound in Obstetrics & Gynecology. 2020;55(4):536-45.
172. Porter TF, Fraser AM, Hunter CY, Ward RH, Varner MW. The risk of preterm birth across generations. Obstetrics & Gynecology. 1997;90(1):63-7.
173. Winkvist A, Mogren I, Högberg U. Familial patterns in birth characteristics: impact on individual and population risks. International journal of epidemiology. 1998;27(2):248-54.
174. Schaaf JM, Liem SM, Mol BWJ, Abu-Hanna A, Ravelli AC. Ethnic and racial disparities in the risk of preterm birth: a systematic review and meta-analysis. American journal of perinatology. 2012:433-50.
175. Frey HA, Stout MJ, Pearson LN, Tuuli MG, Cahill AG, Strauss III JF, et al. Genetic variation associated with preterm birth in African-American women. American journal of obstetrics and gynecology. 2016;215(2):235. e1-. e8.
176. Manuck TA, editor Racial and ethnic differences in preterm birth: a complex, multifactorial problem. Seminars in perinatology; 2017: Elsevier.
177. Zhang G, Feenstra B, Bacelis J, Liu X, Muglia LM, Juodakis J, et al. Genetic associations with gestational duration and spontaneous preterm birth. New England Journal of Medicine. 2017;377(12):1156-67.
178. Mozurkewich EL, Luke B, Avni M, Wolf FM. Working conditions and adverse pregnancy outcome: a meta-analysis. Obstetrics & Gynecology. 2000;95(4):623-35.

179. Van Melick M, Van Beukering M, Mol BW, Frings-Dresen M, Hulshof C. Shift work, long working hours and preterm birth: a systematic review and meta-analysis. International archives of occupational and environmental health. 2014;87:835-49.
180. Kader M, Bigert C, Andersson T, Selander J, Bodin T, Skröder H, et al. Shift and night work during pregnancy and preterm birth—a cohort study of Swedish health care employees. International journal of epidemiology. 2021;50(6):1864-74.
181. Hoirisch-Clapauch S, Brenner B, Nardi AE. Adverse obstetric and neonatal outcomes in women with mental disorders. Thrombosis Research. 2015;135:S60-S3.
182. Staneva A, Bogossian F, Pritchard M, Wittkowski A. The effects of maternal depression, anxiety, and perceived stress during pregnancy on preterm birth: A systematic review. Women and birth. 2015;28(3):179-93.
183. Wadhwa PD, Culhane JF, Rauh V, Barve SS. Stress and preterm birth: neuroendocrine, immune/inflammatory, and vascular mechanisms. Maternal and child health journal. 2001;5:119-25.
184. Amabebe E, Anumba DO. Psychosocial stress, cortisol levels, and maintenance of vaginal health. Frontiers in endocrinology. 2018;9:403253.
185. Jones GL, Mitchell CA, Hirst JE, Anumba DO, Obstetricians RCo, Gynaecologists. Understanding the relationship between social determinants of health and maternal mortality: scientific impact paper no. 67. BJOG: An International Journal of Obstetrics & Gynaecology. 2022;129(7):1211-28.
186. Mercer BM, Goldenberg RL, Moawad AH, Meis PJ, Iams JD, Das AF, et al. The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. American journal of obstetrics and gynecology. 1999;181(5):1216-21.
187. Yang J, Baer RJ, Berghella V, Chambers C, Chung P, Coker T, et al. Recurrence of preterm birth and early term birth. Obstetrics and gynecology. 2016;128(2):364.
188. Smith GC, Pell JP, Dobbie R. Interpregnancy interval and risk of preterm birth and neonatal death: retrospective cohort study. Bmj. 2003;327(7410):313.
189. Kasuga Y, Miyakoshi K, Nishio H, Akiba Y, Otani T, Fukutake M, et al. Mid-trimester residual cervical length and the risk of preterm birth in pregnancies after abdominal radical trachelectomy: a retrospective analysis. BJOG: An International Journal of Obstetrics & Gynaecology. 2017;124(11):1729-35.
190. Vats H, Saxena R, Sachdeva MP, Walia GK, Gupta V. Impact of maternal pre-pregnancy body mass index on maternal, fetal and neonatal adverse outcomes in the worldwide populations: A systematic review and meta-analysis. Obesity Research & Clinical Practice. 2021;15(6):536-45.
191. Salihu H, Mbah A, Alio A, Clayton H, Lynch O. Low pre-pregnancy body mass index and risk of medically indicated versus spontaneous preterm singleton birth. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2009;144(2):119-23.
192. Allen LH. Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth. The Journal of nutrition. 2001;131(2):581S-9S.
193. Liu B, Xu G, Sun Y, Qiu X, Ryckman KK, Yu Y, et al. Maternal cigarette smoking before and during pregnancy and the risk of preterm birth: A dose–response analysis of 25 million mother–infant pairs. PLoS medicine. 2020;17(8):e1003158.
194. Regan AK, Bombard JM, O'Hegarty MM, Smith RA, Tong VT. Adverse birth outcomes associated with prepregnancy and prenatal electronic cigarette use. Obstetrics & Gynecology. 2021;138(1):85-94.

195. Siega-Riz AM, Keim-Malpass J, Lyons GR, Alhusen J. The association between legalization of recreational marijuana use and birth outcomes in Colorado and Washington state. Birth defects research. 2020;112(9):660-9.
196. Laughon SK, Albert PS, Leishear K, Mendola P. The NICHD Consecutive Pregnancies Study: recurrent preterm delivery by subtype. American journal of obstetrics and gynecology. 2014;210(2):131. e1-. e8.
197. Adams MM, Elam-Evans LD, Wilson HG, Gilbertz DA. Rates of and factors associated with recurrence of preterm delivery. Jama. 2000;283(12):1591-6.
198. Ananth CV, Getahun D, Peltier MR, Salihu HM, Vintzileos AM. Recurrence of spontaneous versus medically indicated preterm birth. American journal of obstetrics and gynecology. 2006;195(3):643-50.
199. To M, Skentou C, Royston P, Yu C, Nicolaides K. Prediction of patient-specific risk of early preterm delivery using maternal history and sonographic measurement of cervical length: a population-based prospective study. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2006;27(4):362-7.
200. Owen J, Yost N, Berghella V, Thom E, Swain M, Dildy III GA, et al. Mid-trimester endovaginal sonography in women at high risk for spontaneous preterm birth. Jama. 2001;286(11):1340-8.
201. Heath V, Southall T, Souka A, Elisseou A, Nicolaides K. Cervical length at 23 weeks of gestation: prediction of spontaneous preterm delivery. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 1998;12(5):312-7.
202. Berghella V, Talucci M, Desai A. Does transvaginal sonographic measurement of cervical length before 14 weeks predict preterm delivery in high-risk pregnancies? Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2003;21(2):140-4.
203. Antsaklis P, Daskalakis G, Pilalis A, Papantoniou N, Mesogitis S, Antsaklis A. The role of cervical length measurement at 11–14 weeks for the prediction of preterm delivery. The Journal of Maternal-Fetal & Neonatal Medicine. 2011;24(3):465-70.
204. McIntosh J, Feltovich H, Berghella V, Manuck T, Medicine SfM-F. The role of routine cervical length screening in selected high-and low-risk women for preterm birth prevention. American journal of obstetrics and gynecology. 2016;215(3):B2-B7.
205. Gomez R, Galasso M, Romero R, Mazor M, Sorokin Y, Gonçalves L, et al. Ultrasonographic examination of the uterine cervix is better than cervical digital examination as a predictor of the likelihood of premature delivery in patients with preterm labor and intact membranes. American journal of obstetrics and gynecology. 1994;171(4):956-64.
206. Clement S, Candy B, Heath V, To M, Nicolaides K. Transvaginal ultrasound in pregnancy: its acceptability to women and maternal psychological morbidity. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2003;22(5):508-14.
207. Whitworth M, Bricker L, Mullan C. Ultrasound for fetal assessment in early pregnancy. Cochrane database of systematic reviews. 2015(7).
208. Berghella V, Bega G, Tolosa JE, Berghella M. Ultrasound assessment of the cervix. Clinical obstetrics and gynecology. 2003;46(4):947-62.

209. Hassan SS, Romero R, Berry SM, Dang K, Blackwell SC, Treadwell MC, et al. Patients with an ultrasonographic cervical length≤ 15 mm have nearly a 50% risk of early spontaneous preterm delivery. American journal of obstetrics and gynecology. 2000;182(6):1458-67.
210. Iams JD, Grobman WA, Lozitska A, Spong CY, Saade G, Mercer BM, et al. Adherence to criteria for transvaginal ultrasound imaging and measurement of cervical length. American journal of obstetrics and gynecology. 2013;209(4):365. e1-. e5.
211. Gascón A, Goya M, Mendoza M, Gracia-Perez-Bonfils A, Higueras T, Calero I, et al. Intraobserver and interobserver variability in first-trimester transvaginal ultrasound cervical length. The Journal of Maternal-Fetal & Neonatal Medicine. 2020;33(1):136-41.
212. Kuusela P, Wennerholm UB, Fadl H, Wesström J, Lindgren P, Hagberg H, et al. Second trimester cervical length measurements with transvaginal ultrasound: A prospective observational agreement and reliability study. Acta obstetricia et gynecologica Scandinavica. 2020;99(11):1476-85.
213. Sonek JD, Iams J, Blumenfeld M, Johnson F, Landon M, Gabbe S. Measurement of cervical length in pregnancy: comparison between vaginal ultrasonography and digital examination. Obstetrics and gynecology. 1990;76(2):172-5.
214. Owen J, Iams JD, Health NIoC, Network HDM-FMU, editors. What we have learned about cervical ultrasound. Seminars in perinatology; 2003: Elsevier.
215. Pagani G, Stagnati V, Fichera A, Prefumo F. Cervical length at mid gestation for the screening of pre-term birth in twin pregnancies. Ultrasound Obstet Gynecol. 2015.
216. Gyamfi C, Lerner V, Holzman I, Stone JL. Routine cervical length in twins and perinatal outcomes. American Journal of Perinatology. 2006:065-9.
217. NICE. Biomarker tests to help diagnose preterm labour with intact membranes 2018 https://www.nice.org.uk/guidance/dg33, accessed June 2023.
218. Matsuura H, Hakomori S-I. The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. Proceedings of the National Academy of Sciences. 1985;82(19):6517-21.
219. Goldenberg RL, Iams JD, Mercer BM, Meis PJ, Moawad AH, Copper R, et al. The preterm prediction study: the value of new vs standard risk factors in predicting early and all spontaneous preterm births. NICHD MFMU Network. American journal of public health. 1998;88(2):233-8.
220. Genc MR, Ford CE. The clinical use of inflammatory markers during pregnancy. Current opinion in obstetrics and gynecology. 2010;22(2):116-21.
221. Dutta D, Norman J. The efficacy of fetal fibronectin testing in minimising hospital admissions, length of hospital stay and cost savings in women presenting with symptoms of pre-term labour. Journal of obstetrics and gynaecology. 2010;30(8):768-73.
222. Foster C, Shennan AH. Fetal fibronectin as a biomarker of preterm labor: a review of the literature and advances in its clinical use. Biomarkers in medicine. 2014;8(4):471-84.
223. Lockwood CJ, Senyei AE, Dische MR, Casal D, Shah KD, Thung SN, et al. Fetal fibronectin in cervical and vaginal secretions as a predictor of preterm delivery. New England Journal of Medicine. 1991;325(10):669-74.
224. Iams JD, Casal D, McGregor JA, Goodwin TM, Kreaden US, Lowensohn R, et al. Fetal fibronectin improves the accuracy of diagnosis of preterm labor. American journal of obstetrics and gynecology. 1995;173(1):141-5.

225. Yoneda S, Sakai M, Sasaki Y, Shiozaki A, Hidaka T, Saito S. Interleukin-8 and glucose in amniotic fluid, fetal fibronectin in vaginal secretions and preterm labor index based on clinical variables are optimal predictive markers for preterm delivery in patients with intact membranes. Journal of Obstetrics and Gynaecology Research. 2007;33(1):38-44.
226. Morrison JC, Allbert JR, McLaughlin BN, Whitworth NS, Roberts WE, Martin RW. Oncofetal fibronectin in patients with false labor as a predictor of preterm delivery. American journal of obstetrics and gynecology. 1993;168(2):538-42.
227. Inglis SR, Jeremias J, Kuno K, Lescale K, Peeper Q, Chervenak FA, et al. Detection of tumor necrosis factor-α, interleukin-6, and fetal fibronectin in the lower genital tract during pregnancy: Relation to outcome. American journal of obstetrics and gynecology. 1994;171(1):5-10.
228. Peaceman AM, Andrews WW, Thorp JM, Cliver SP, Lukes A, Iams JD, et al. Fetal fibronectin as a predictor of preterm birth in patients with symptoms: a multicenter trial. American journal of obstetrics and gynecology. 1997;177(1):13-8.
229. Sunagawa S, Takagi K, Ono K, Miyachi K, Kikuchi A. Comparison of biochemical markers and cervical length for predicting preterm delivery. Journal of Obstetrics and Gynaecology Research. 2008;34(5):812-9.
230. Bartnicki J, Casal D, Kreaden US, Saling E, Vetter K. Fetal fibronectin in vaginal specimens predicts preterm delivery and very-low-birth-weight infants. American journal of obstetrics and gynecology. 1996;174(3):971-4.
231. Lowe MP, Zimmerman B, Hansen W. Prospective randomized controlled trial of fetal fibronectin on preterm labor management in a tertiary care center. American journal of obstetrics and gynecology. 2004;190(2):358-62.
232. Giles W, Bisits A, Knox M, Madsen G, Smith R. The effect of fetal fibronectin testing on admissions to a tertiary maternal-fetal medicine unit and cost savings. American journal of obstetrics and gynecology. 2000;182(2):439-42.
233. Goldenberg RL, Mercer BM, Meis PJ, Copper RL, Das A, McNellis D. The preterm prediction study: fetal fibronectin testing and spontaneous preterm birth. Obstetrics & Gynecology. 1996;87(5):643-8.
234. Bolt LA, Chandiramani M, De Greeff A, Seed P, Shennan AH. Does fetal fibronectin testing change patient management in women at risk of preterm labour? European Journal of Obstetrics & Gynecology and Reproductive Biology. 2009;146(2):180-3.
235. Goepfert AR, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The preterm prediction study: quantitative fetal fibronectin values and the prediction of spontaneous preterm birth. American journal of obstetrics and gynecology. 2000;183(6):1480-3.
236. Abbott DS, Radford SK, Seed PT, Tribe RM, Shennan AH. Evaluation of a quantitative fetal fibronectin test for spontaneous preterm birth in symptomatic women. American journal of obstetrics and gynecology. 2013;208(2):122. e1-. e6.
237. Lu GC, Goldenberg RL, Cliver SP, Kreaden US, Andrews WW. Vaginal fetal fibronectin levels and spontaneous preterm birth in symptomatic women. Obstetrics & Gynecology. 2001;97(2):225-8.
238. Kurtzman J, Chandiramani M, Briley A, Poston L, Das A, Shennan A. Quantitative fetal fibronectin screening in asymptomatic high-risk patients and the spectrum of risk for recurrent preterm delivery. American journal of obstetrics and gynecology. 2009;200(3):263. e1-. e6.
239. Gomez R, Romero R, Medina L, Nien JK, Chaiworapongsa T, Carstens M, et al. Cervicovaginal fibronectin improves the prediction of preterm delivery based on

sonographic cervical length in patients with preterm uterine contractions and intact membranes. American journal of obstetrics and gynecology. 2005;192(2):350-9.
240. Kuhrt K, Smout E, Hezelgrave N, Seed P, Carter J, Shennan A. Development and validation of a tool incorporating cervical length and quantitative fetal fibronectin to predict spontaneous preterm birth in asymptomatic high-risk women. Ultrasound in Obstetrics & Gynecology. 2016;47(1):104-9.
241. Watson H, Carter J, Seed P, Tribe R, Shennan A. The QUiPP App: a safe alternative to a treat-all strategy for threatened preterm labor. Ultrasound in Obstetrics & Gynecology. 2017;50(3):342-6.
242. Watson H, Seed P, Carter J, Hezelgrave N, Kuhrt K, Tribe R. Development and validation of the predictive models for the QUiPP App vol 2: a tool for predicting preterm birth in high-risk asymptomatic women. Ultrasou Obstet Gynecol. 2019.
243. Carter J, Seed P, Watson H, David A, Sandall J, Shennan A, et al. Development and validation of predictive models for QUiPP App v. 2: tool for predicting preterm birth in women with symptoms of threatened preterm labor. Ultrasound in obstetrics & gynecology. 2020;55(3):357-67.
244. Goodfellow L, Care A, Sharp A, Ivandic J, Poljak B, Roberts D, et al. Effect of QUiPP prediction algorithm on treatment decisions in women with a previous preterm birth: a prospective cohort study. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(13):1569-75.
245. Medicine BAoP. Antenatal Optimisation for Preterm Infants less than 34 weeks A Quality Improvement Toolkit 2020 http://hubble-live-assets.s3.amazonaws.com/bapm/redactor2_assets/files/843/AO_Toolkit_FULLTOOLKIT_11-2-21.docx.pdf, accessed November 2022.
246. England N. Saving babies' lives: version 3 2023 https://www.england.nhs.uk/long-read/saving-babies-lives-version-3/, accessed Dec 2023.
247. Rutanen E-M. Insulin-like growth factors in obstetrics. Current Opinion in Obstetrics and Gynecology. 2000;12(3):163-8.
248. Erdemoglu E, Mungan T. Significance of detecting insulin-like growth factor binding protein-1 in cervicovaginal secretions: comparison with nitrazine test and amniotic fluid volume assessment. Acta obstetricia et gynecologica Scandinavica. 2004;83(7):622-6.
249. Tripathi R, Tyagi S, Mala YM, Singh N, Pandey NB, Yadav P. Comparison of rapid bedside tests for phosphorylated insulin-like growth factor-binding protein 1 and fetal fibronectin to predict preterm birth. International Journal of Gynecology & Obstetrics. 2016;135(1):47-50.
250. Hillman-Cooper C, Ghag K, Dempsey A, Denbow M, Bernal AL. PPO. 26 Actim partus–the first year at St. Michael's Hospital, Bristol. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2014;99(Suppl 1):A158-A9.
251. Honest H, Forbes C, Durée K, Norman G, Duffy S, Tsourapas A, et al. Screening to prevent spontaneous preterm birth: systematic reviews of accuracy and effectiveness literature with economic modelling. Health Technol Assess. 2009;13(43):1-627.
252. Conde-Agudelo A, Romero R. Cervical phosphorylated insulin-like growth factor binding protein-1 test for the prediction of preterm birth: a systematic review and metaanalysis. American journal of obstetrics and gynecology. 2016;214(1):57-73.
253. Bolotskikh V, Borisova V. Combined value of placental alpha microglobulin-1 detection and cervical length via transvaginal ultrasound in the diagnosis of preterm labor in symptomatic patients. Journal of Obstetrics and Gynaecology Research. 2017;43(8):1263-9.

254. Cousins LM, Smok DP, Lovett SM, Poeltler DM. AmniSure placental alpha microglobulin-1 rapid immunoassay versus standard diagnostic methods for detection of rupture of membranes. American journal of perinatology. 2005:317-20.
255. Dochez V, Ducarme G, Gueudry P, Joueidi Y, Boivin M, Boussamet L, et al. Methods of detection and prevention of preterm labour and the PAMG-1 detection test: a review. Journal of Perinatal Medicine. 2021;49(2):119-26.
256. Cnota W, Jagielska A, Janowska E, Banas E, Kierach R, Nycz-Reska M, et al. Prediction of preterm birth using PAMG-1 test: a single centre experience—preliminary report. Ginekologia Polska. 2022;93(7):574-7.
257. Nikolova T, Bayev O, Nikolova N, Di Renzo GC. Evaluation of a novel placental alpha microglobulin-1 (PAMG-1) test to predict spontaneous preterm delivery. Journal of perinatal medicine. 2014;42(4):473-7.
258. Soneji S, Beltrán-Sánchez H. Association of maternal cigarette smoking and smoking cessation with preterm birth. JAMA network open. 2019;2(4):e192514-e.
259. Stock SJ, Bauld L. Maternal smoking and preterm birth: An unresolved health challenge. PLoS Medicine. 2020;17(9):e1003386.
260. Alfirevic Z, Stampalija T, Medley N. Cervical stitch (cerclage) for preventing preterm birth in singleton pregnancy. Cochrane database of systematic reviews. 2017(6).
261. Iams JD, Goldenberg RL, Mercer BM, Moawad A, Thom E, Meis PJ, et al. The Preterm Prediction Study: recurrence risk of spontaneous preterm birth. American journal of obstetrics and gynecology. 1998;178(5):1035-40.
262. Mohan AR, Sooranna SR, Lindstrom TM, Johnson MR, Bennett PR. The effect of mechanical stretch on cyclooxygenase type 2 expression and activator protein-1 and nuclear factor-κB activity in human amnion cells. Endocrinology. 2007;148(4):1850-7.
263. Althuisius SM, Dekker GA, Hummel P, Bekedam DJ, van Geijn HP. Final results of the Cervical Incompetence Prevention Randomized Cerclage Trial (CIPRACT): therapeutic cerclage with bed rest versus bed rest alone. American journal of obstetrics and gynecology. 2001;185(5):1106-12.
264. Rust OA, Atlas RO, Jones KJ, Benham BN, Balducci J. A randomized trial of cerclage versus no cerclage among patients with ultrasonographically detected second-trimester preterm dilatation of the internal os. American journal of obstetrics and gynecology. 2000;183(4):830-5.
265. To MS, Alfirevic Z, Heath VC, Cicero S, Cacho AM, Williamson PR, et al. Cervical cerclage for prevention of preterm delivery in woman with short cervix: randomised controlled trial. The Lancet. 2004;363(9424):1849-53.
266. Owen J, Hankins G, Iams JD, Berghella V, Sheffield JS, Perez-Delboy A, et al. Multicenter randomized trial of cerclage for preterm birth prevention in high-risk women with shortened midtrimester cervical length. American journal of obstetrics and gynecology. 2009;201(4):375. e1-. e8.
267. Simcox R, Bennett P, Shennan A. Cervical cerclage for prevention of preterm delivery in women with short cervix. The Lancet. 2004;364(9449):1934-5.
268. Israfil-Bayli F, Toozs-Hobson P, Lees C, Slack M, Daniels J, Vince A, et al. Cervical cerclage and type of suture material: a survey of UK consultants' practice. The Journal of Maternal-Fetal & Neonatal Medicine. 2014;27(15):1584-8.
269. Kindinger LM, MacIntyre DA, Lee YS, Marchesi JR, Smith A, McDonald JA, et al. Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage. Science translational medicine. 2016;8(350):350ra102-350ra102.

270. Morton VH, Toozs-Hobson P, Moakes CA, Middleton L, Daniels J, Simpson NA, et al. Monofilament suture versus braided suture thread to improve pregnancy outcomes after vaginal cervical cerclage (C-STICH): a pragmatic randomised, controlled, phase 3, superiority trial. The Lancet. 2022;400(10361):1426-36.
271. McDONALD IA. Suture of the cervix for inevitable miscarriage. Obstetrical & Gynecological Survey. 1957;12(5):673-4.
272. Shirodkar V. A new method of operative treatment for habitual abortions in the second trimester of pregnancy. Antiseptic. 1955;52:299-300.
273. Rozenberg P, Senat M, Gillet A, Ville Y. Comparison of two methods of cervical cerclage by ultrasound cervical measurement. Journal of Maternal-Fetal & Neonatal Medicine. 2003;13(5):314.
274. McAuliffe L, Issah A, Diacci R, Williams KP, Aubin AM, Phung J, et al. McDonald versus Shirodkar cerclage technique in the prevention of preterm birth: A systematic review and meta-analysis. BJOG: An International Journal of Obstetrics & Gynaecology. 2023.
275. Kindinger LM, David AL, editors. The role of the obstetrician in the prevention of retinopathy of prematurity. Seminars in Perinatology; 2019: Elsevier.
276. Gundabattula SR, Marakani LR, Dasari S, Surampudi K, Pochiraju M, Nirmalan PK. Outcomes of pregnancy in women who had rescue cerclage for cervical insufficiency: a single-center retrospective study. Journal of Obstetrics and Gynaecology Research. 2013;39(8):1293-300.
277. Namouz S, Porat S, Okun N, Windrim R, Farine D. Emergency cerclage: literature review. Obstetrical & gynecological survey. 2013;68(5):379-88.
278. Althuisius SM, Dekker GA, Hummel P, van Geijn HP. Cervical incompetence prevention randomized cerclage trial: emergency cerclage with bed rest versus bed rest alone. American journal of obstetrics and gynecology. 2003;189(4):907-10.
279. Hodgetts-Morton V, Hewitt CA, Jones L, Leighton L, Pilarski N, Molloy E, et al. C-STICH2: emergency cervical cerclage to prevent miscarriage and preterm birth—study protocol for a randomised controlled trial. Trials. 2021;22(1):1-11.
280. Carter J, Chandiramani M, Seed P, Shennan A. MAVRIC: multicentre abdominal vs vaginal randomised investigation of cerclage. BJOG. 2015;122:1-7.
281. Umstad MP, Quinn MA, Ades A. Transabdominal cervical cerclage. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2010;50(5):460-4.
282. Debbs RH, Vega GAD, Pearson S, Sehdev H, Marchiano D, Ludmir J. Transabdominal cerclage after comprehensive evaluation of women with previous unsuccessful transvaginal cerclage. American journal of obstetrics and gynecology. 2007;197(3):317. e1-. e4.
283. Shennan A, Chandiramani M, Bennett P, David AL, Girling J, Ridout A, et al. MAVRIC: a multicenter randomized controlled trial of transabdominal vs transvaginal cervical cerclage. American Journal of Obstetrics and Gynecology. 2020;222(3):261. e1-. e9.
284. Johnson JW, Austin KL, Jones GS, Davis GH, King TM. Efficacy of 17α-hydroxyprogesterone caproate in the prevention of premature labor. New England Journal of Medicine. 1975;293(14):675-80.
285. Gynecologists TACoOa. Updated Clinical Guidance for the Use of Progesterone Supplementation for the Prevention of Recurrent Preterm Birth, https://www.acog.org/clinical/clinical-guidance/practice-advisory/articles/2023/04/updated-guidance-use-of-progesterone-supplementation-for-prevention-of-recurrent-preterm-birth, accessed March 2024. 2023.

286. Anderson L, Martin W, Higgins C, Nelson SM, Norman JE. The effect of progesterone on myometrial contractility, potassium channels, and tocolytic efficacy. Reproductive sciences. 2009;16(11):1052-61.
287. Soloff MS, Jeng Y-J, Izban MG, Sinha M, Luxon BA, Stamnes SJ, et al. Effects of progesterone treatment on expression of genes involved in uterine quiescence. Reproductive Sciences. 2011;18(8):781-97.
288. Macintyre DA, Chan E-C, Smith R. Myometrial activation–coordination, connectivity and contractility. Fetal and Maternal Medicine Review. 2007;18(4):333-56.
289. Conde-Agudelo A, Romero R, Nicolaides K, Chaiworapongsa T, O'Brien JM, Cetingoz E, et al. Vaginal progesterone vs cervical cerclage for the prevention of preterm birth in women with a sonographic short cervix, previous preterm birth, and singleton gestation: a systematic review and indirect comparison metaanalysis. American journal of obstetrics and gynecology. 2013;208(1):42. e1-. e18.
290. Committee SfM-FMP. The choice of progestogen for the prevention of preterm birth in women with singleton pregnancy and prior preterm birth. Am J Obstet Gynecol. 2017;216(3):B11-B3.
291. Practice FWGOB, Medicine IM-F, of Gynecology IF. Best practice in maternal-fetal medicine. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2015;128(1):80-2.
292. Norman JE, Marlow N, Messow C-M, Shennan A, Bennett PR, Thornton S, et al. Vaginal progesterone prophylaxis for preterm birth (the OPPTIMUM study): a multicentre, randomised, double-blind trial. The lancet. 2016;387(10033):2106-16.
293. Gyamfi-Bannerman C, Biggio Jr J, Chauhan S, Hughes B, Louis J, Manuck T, et al. 17-OHPC to prevent recurrent preterm birth in singleton gestations (PROLONG study): a multicenter, international, randomized double-blind trial. American journal of perinatology. 2020;37(02):127-36.
294. Combs CA, Garite TJ, Maurel K, Abril D, Das A, Clewell W, et al. 17-hydroxyprogesterone caproate for preterm rupture of the membranes: a multicenter, randomized, double-blind, placebo-controlled trial. American journal of obstetrics and gynecology. 2015;213(3):364. e1-. e12.
295. Romero R, Nicolaides K, Conde-Agudelo A, O'Brien J, Cetingoz E, Da Fonseca E, et al. Vaginal progesterone decreases preterm birth≤ 34 weeks of gestation in women with a singleton pregnancy and a short cervix: an updated meta-analysis including data from the OPPTIMUM study. Ultrasound in Obstetrics & Gynecology. 2016;48(3):308-17.
296. Hassan S, Romero R, Vidyadhari D, Fusey S, Baxter J, Khandelwal M, et al. Vaginal progesterone reduces the rate of preterm birth in women with a sonographic short cervix: a multicenter, randomized, double-blind, placebo-controlled trial. Ultrasound in Obstetrics & Gynecology. 2011;38(1):18-31.
297. Sykes L, Bennett PR. Efficacy of progesterone for prevention of preterm birth. Best Practice & Research Clinical Obstetrics & Gynaecology. 2018;52:126-36.
298. Arabin B, Halbesma JR, Vork F, Hübener M, Eyck Jv. Is treatment with vaginal pessaries an option in patients with a sonographically detected short cervix? 2003.
299. Saccone G, Ciardulli A, Xodo S, Dugoff L, Ludmir J, Pagani G, et al. Cervical pessary for preventing preterm birth in singleton pregnancies with short cervical length: a systematic review and meta-analysis. Journal of Ultrasound in Medicine. 2017;36(8):1535-43.
300. Hibbard L, Thrupp L, Summeril S, Smale M, Adams R. Treatment of pyelonephritis in pregnancy. American Journal of Obstetrics and Gynecology. 1967;98(5):609-15.

301. Romero R, Oyarzun E, Mazor M, Sirtori M, Hobbins JC, Bracken M. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. Obstetrics & Gynecology. 1989;73(4):576-82.
302. Benedetti TJ, Valle R, Ledger WJ. Antepartum pneumonia in pregnancy. American journal of obstetrics and gynecology. 1982;144(4):413-7.
303. Care A, Ingleby L, Alfirevic Z, Sharp A. The influence of the introduction of national guidelines on preterm birth prevention practice: UK experience. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(6):763-9.
304. Carlisle N, Care A, Anumba DO, Dalkin S, Sandall J, Shennan AH. How are hospitals in England caring for women at risk of preterm birth in 2021? The influence of national guidance on preterm birth care in England: a national questionnaire. BMC Pregnancy and Childbirth. 2023;23(1):47.
305. Celik E, To M, Gajewska K, Smith G, Nicolaides K. Cervical length and obstetric history predict spontaneous preterm birth: development and validation of a model to provide individualized risk assessment. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2008;31(5):549-54.
306. O'Brien ET, Quenby S, Lavender T. Women's views of high risk pregnancy under threat of preterm birth. Sexual & Reproductive Healthcare. 2010;1(3):79-84.
307. Newnham JP, White SW, Meharry S, Lee H-S, Pedretti MK, Arrese CA, et al. Reducing preterm birth by a statewide multifaceted program: an implementation study. American journal of obstetrics and gynecology. 2017;216(5):434-42.
308. Hendler I, Goldenberg RL, Mercer BM, Iams JD, Meis PJ, Moawad AH, et al. The Preterm Prediction Study: association between maternal body mass index and spontaneous and indicated preterm birth. American journal of obstetrics and gynecology. 2005;192(3):882-6.
309. Baer RJ, Yang J, Berghella V, Chambers CD, Coker TR, Kuppermann M, et al. Risk of preterm birth by maternal age at first and second pregnancy and race/ethnicity. Journal of perinatal medicine. 2018;46(5):539-46.
310. Haram K, Mortensen JHS, Morrison JC. Tocolysis for acute preterm labor: does anything work. The Journal of Maternal-Fetal & Neonatal Medicine. 2015;28(4):371-8.
311. Vogel JP, Oladapo OT, Manu A, Gülmezoglu AM, Bahl R. New WHO recommendations to improve the outcomes of preterm birth. The Lancet Global Health. 2015;3(10):e589-e90.
312. McGoldrick E, Stewart F, Parker R, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database of Systematic Reviews. 2020(12).
313. Melamed N, Shah J, Soraisham A, Yoon EW, Lee SK, Shah PS, et al. Association between antenatal corticosteroid administration-to-birth interval and outcomes of preterm neonates. Obstetrics & Gynecology. 2015;125(6):1377-84.
314. Norberg H, Kowalski J, Maršál K, Norman M. Timing of antenatal corticosteroid administration and survival in extremely preterm infants: a national population-based cohort study. BJOG: An International Journal of Obstetrics & Gynaecology. 2017;124(10):1567-74.
315. Razaz N, Skoll A, Fahey J, Allen VM, Joseph K. Trends in optimal, suboptimal, and questionably appropriate receipt of antenatal corticosteroid prophylaxis. Obstetrics & Gynecology. 2015;125(2):288-96.

316. Shields A, Thomson M, Winter V, Coalson J, Rees S. Repeated courses of antenatal corticosteroids have adverse effects on aspects of brain development in naturally delivered baboon infants. Pediatric research. 2012;71(6):661-7.
317. French NP, Hagan R, Evans SF, Mullan A, Newnham JP. Repeated antenatal corticosteroids: effects on cerebral palsy and childhood behavior. American journal of obstetrics and gynecology. 2004;190(3):588-95.
318. Spinillo A, Viazzo F, Colleoni R, Chiara A, Cerbo RM, Fazzi E. Two-year infant neurodevelopmental outcome after single or multiple antenatal courses of corticosteroids to prevent complications of prematurity. American journal of obstetrics and gynecology. 2004;191(1):217-24.
319. Aviram A, Murphy K, McDonald S, Asztalos E, Zaltz A, Redelmeier D, et al. Antenatal corticosteroids and neurodevelopmental outcomes in late preterm births. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2022;107(3):250-5.
320. Wolf HT, Huusom LD, Henriksen TB, Hegaard HK, Brok J, Pinborg A. Magnesium sulphate for fetal neuroprotection at imminent risk for preterm delivery: a systematic review with meta-analysis and trial sequential analysis. BJOG: An International Journal of Obstetrics & Gynaecology. 2020;127(10):1180-8.
321. Werter DE, Dehaene I, Gurney L, Buján MV, Kazemier BM. Differences in clinical practice regarding screening and treatment of infections associated with spontaneous preterm birth: An international survey. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2021;266:83-8.
322. Kenyon S, Taylor D, Tarnow-Mordi W. Broad-spectrum antibiotics for preterm, prelabour rupture of fetal membranes: the ORACLE I randomised trial. The Lancet. 2001;357(9261):979-88.
323. Kenyon S, Taylor D, Tarnow-Mordi Wf. Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. The Lancet. 2001;357(9261):989-94.
324. Kenyon S, Pike K, Jones D, Brocklehurst P, Marlow N, Salt A, et al. Childhood outcomes after prescription of antibiotics to pregnant women with spontaneous preterm labour: 7-year follow-up of the ORACLE II trial. The Lancet. 2008;372(9646):1319-27.
325. Flenady V, Hawley G, Stock OM, Kenyon S, Badawi N. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. Cochrane Database of Systematic Reviews. 2013(12).
326. Kenyon S, Boulvain M, Neilson JP, Pregnancy C, Group C. Antibiotics for preterm rupture of membranes. Cochrane database of systematic reviews. 1996;2013(12).
327. Kenyon S, Pike K, Jones D, Brocklehurst P, Marlow N, Salt A, et al. Childhood outcomes after prescription of antibiotics to pregnant women with preterm rupture of the membranes: 7-year follow-up of the ORACLE I trial. The Lancet. 2008;372(9646):1310-8.
328. Money D, Allen VM, Yudin MH, Bouchard C, Boucher M, Caddy S, et al. The prevention of early-onset neonatal group B streptococcal disease. Journal of Obstetrics and Gynaecology Canada. 2013;35(10):939-48.
329. Shitara Y, Kakiuchi S, Mukai T, Kashima K, Kato M, Takahashi N. Case Report: Treatment of Extremely Preterm Infants With Birthweight Below 300 g: Case Series. Frontiers in Pediatrics. 2021;9:758683.
330. Arimitsu T, Wakabayashi D, Tamaoka S, Takahashi M, Hida M, Takahashi T. Case report: intact survival of a marginally viable male infant born weighing 268 grams at 24 weeks gestation. Frontiers in Pediatrics. 2021;8:628362.

331. Medicine BAoP. Perinatal Management of Extreme Preterm Birth before 27 weeks of gestation 2019 https://hubble-live-assets.s3.eu-west-1.amazonaws.com/bapm/file_asset/file/30/Extreme_Preterm_28-11-19_FINAL.pdf, accessed Nov 2022.
332. Hall M, Valencia CM, Soma-Pillay P, Luyt K, Jacobsson B, Shennan A, et al. Effective and simple interventions to improve outcomes for preterm infants worldwide: The FIGO PremPrep-5 initiative. International Journal of Gynecology & Obstetrics. 2024.
333. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007;449(7164):804-10.
334. Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal. Microbiome. 2015;3:31.
335. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of microbiological methods. 2007;69(2):330-9.
336. MacIntyre DA, Sykes L, Bennett PR. The human female urogenital microbiome: complexity in normality. Emerging Topics in Life Sciences. 2017;1(4):363-72.
337. Hill G, Claire KS, Gutman L. Anaerobes predominate among the vaginal microflora of prepubertal girls. Clinical infectious diseases. 1995;20(Supplement_2):S269-S70.
338. Linhares IM, Summers PR, Larsen B, Giraldo PC, Witkin SS. Contemporary perspectives on vaginal pH and lactobacilli. American journal of obstetrics and gynecology. 2011;204(2):120. e1-. e5.
339. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences. 2011;108(supplement_1):4680-7.
340. Hickey RJ, Zhou X, Pierson JD, Ravel J, Forney LJ. Understanding vaginal microbiome complexity from an ecological perspective. Translational Research. 2012;160(4):267-82.
341. Antonio MA, Rabe LK, Hillier SL. Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis. The Journal of infectious diseases. 2005;192(3):394-8.
342. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, et al. Prevalence of hydrogen peroxide-producing Lactobacillus species in normal women and women with bacterial vaginosis. Journal of clinical microbiology. 1989;27(2):251-6.
343. Ness RB, Hillier SL, Kip KE, Soper DE, Stamm CA, McGregor JA, et al. Bacterial vaginosis and risk of pelvic inflammatory disease. Obstetrics & Gynecology. 2004;104(4):761-9.
344. Wang S, Wang Q, Yang E, Yan L, Li T, Zhuang H. Antimicrobial compounds produced by vaginal Lactobacillus crispatus are able to strongly inhibit Candida albicans growth, hyphal formation and regulate virulence-related gene expressions. Frontiers in microbiology. 2017;8:564.
345. France MT, Ma B, Gajer P, Brown S, Humphrys MS, Holm JB, et al. VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. Microbiome. 2020;8:1-15.
346. Chaban B, Links MG, Jayaprakash TP, Wagner EC, Bourque DK, Lohn Z, et al. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. Microbiome. 2014;2(1):1-12.

347. Eschenbach DA, Thwin SS, Patton DL, Hooton TM, Stapleton AE, Agnew K, et al. Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. Clinical Infectious Diseases. 2000;30(6):901-7.
348. Roy EJ, Mackay R. The concentration of oestrogens in blood during pregnancy. BJOG: An International Journal of Obstetrics & Gynaecology. 1962;69(1):13-7.
349. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proceedings of the National Academy of Sciences. 2015;112(35):11060-5.
350. BENIRSCHKE K. Routes and types of infection in the fetus and the newborn. AMA Journal of Diseases of Children. 1960;99(6):714-21.
351. Romero R, Shamma F, Avila C, Jimenez C, Callahan R, Nores J, et al. Infection and labor: VI. Prevalence, microbiology, and clinical significance of intraamniotic infection in twin gestations with preterm labor. American journal of obstetrics and gynecology. 1990;163(3):757-61.
352. Petricevic L, Domig KJ, Nierscher FJ, Sandhofer MJ, Fidesser M, Krondorfer I, et al. Characterisation of the vaginal Lactobacillus microbiota associated with preterm delivery. Scientific reports. 2014;4(1):5136.
353. Kindinger LM, Bennett PR, Lee YS, Marchesi JR, Smith A, Cacciatore S, et al. The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. Microbiome. 2017;5:1-14.
354. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. Journal of clinical microbiology. 1991;29(2):297-301.
355. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. The American journal of medicine. 1983;74(1):14-22.
356. Ison C, Hay P. Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. Sexually transmitted infections. 2002;78(6):413.
357. Patel S, Daniels D. UK National Guideline for the management of Bacterial Vaginosis 2012.
358. Sobel R, Sobel JD. Metronidazole for the treatment of vaginal infections. Expert Opinion on Pharmacotherapy. 2015;16(7):1109-15.
359. Brocklehurst P, Gordon A, Heatley E, Milan SJ. Antibiotics for treating bacterial vaginosis in pregnancy. Cochrane Database of Systematic Reviews. 2013(1).
360. Lamont RF, Nhan-Chang C-L, Sobel JD, Workowski K, Conde-Agudelo A, Romero R. Treatment of abnormal vaginal flora in early pregnancy with clindamycin for the prevention of spontaneous preterm birth: a systematic review and metaanalysis. American journal of obstetrics and gynecology. 2011;205(3):177-90.
361. Lamont RF. Spontaneous preterm labour that leads to preterm birth: An update and personal reflection. Placenta. 2019;79:21-9.
362. Lamont RF, Keelan JA, Larsson PG, Jørgensen JS. The treatment of bacterial vaginosis in pregnancy with clindamycin to reduce the risk of infection-related preterm birth: a response to the Danish Society of Obstetrics and Gynecology guideline group's clinical recommendations. Acta Obstetricia et Gynecologica Scandinavica. 2017;96(2):139-43.
363. Subtil D, Brabant G, Tilloy E, Devos P, Canis F, Fruchart A, et al. Early clindamycin for bacterial vaginosis in pregnancy (PREMEVA): a multicentre, double-blind, randomised controlled trial. The Lancet. 2018;392(10160):2171-9.

364. Klebanoff MA, Brotman RM. Treatment of bacterial vaginosis to prevent preterm birth. The Lancet. 2018;392(10160):2141-2.
365. Amabebe E, Anumba DO. The vaginal microenvironment: the physiologic role of lactobacilli. Frontiers in medicine. 2018;5:181.
366. Hyman RW, Fukushima M, Jiang H, Fung E, Rand L, Johnson B, et al. Diversity of the vaginal microbiome correlates with preterm birth. Reproductive sciences. 2014;21(1):32-40.
367. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Bieda J, et al. The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. Microbiome. 2014;2:1-15.
368. Tabatabaei N, Eren A, Barreiro L, Yotova V, Dumaine A, Allard C, et al. Vaginal microbiome in early pregnancy and subsequent risk of spontaneous preterm birth: a case–control study. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(3):349-58.
369. Callahan BJ, DiGiulio DB, Goltsman DSA, Sun CL, Costello EK, Jeganathan P, et al. 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. 2017;114(37):9966-71.
370. Fettweis JM, Serrano MG, Brooks JP, Edwards DJ, Girerd PH, Parikh HI, et al. The vaginal microbiome and preterm birth. Nature medicine. 2019;25(6):1012-21.
371. Brown RG, Marchesi JR, Lee YS, Smith A, Lehne B, Kindinger LM, et al. Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. BMC medicine. 2018;16(1):1-15.
372. Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D, et al. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Translational Research. 2019;207:30-43.
373. Pereira L, Cotter A, Gómez R, Berghella V, Prasertcharoensuk W, Rasanen J, et al. Expectant management compared with physical examination–indicated cerclage (EM-PEC) in selected women with a dilated cervix at 140/7-256/7 weeks: results from the EM-PEC international cohort study. American journal of obstetrics and gynecology. 2007;197(5):483. e1-. e8.
374. Brown R, Chan D, Terzidou V, Lee Y, Smith A, Marchesi J, et al. Prospective observational study of vaginal microbiota pre-and post-rescue cervical cerclage. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(7):916-25.
375. Huang C, Gin C, Fettweis J, Foxman B, Gelaye B, MacIntyre DA, et al. Meta-analysis reveals the vaginal microbiome is a better predictor of earlier than later preterm birth. BMC biology. 2023;21(1):199.
376. Golob JL, Oskotsky TT, Tang AS, Roldan A, Chung V, Ha CW, et al. Microbiome preterm birth DREAM challenge: Crowdsourcing machine learning approaches to advance preterm birth research. Cell Reports Medicine. 2024;5(1).
377. Mendz GL, Kaakoush NO, Quinlivan JA. Bacterial aetiological agents of intra-amniotic infections and preterm birth in pregnant women. Frontiers in cellular and infection microbiology. 2013;3:58.
378. Fardini Y, Chung P, Dumm R, Joshi N, Han YW. Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection. Infection and immunity. 2010;78(4):1789-96.
379. Macones GA, Parry S, Nelson DB, Strauss JF, Ludmir J, Cohen AW, et al. Treatment of localized periodontal disease in pregnancy does not reduce the occurrence of preterm birth:

results from the Periodontal Infections and Prematurity Study (PIPS). American journal of obstetrics and gynecology. 2010;202(2):147. e1-. e8.
380. Chaim W, Mazor M. Intraamniotic infection with fusobacteria. Archives of gynecology and obstetrics. 1992;251:1-7.
381. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Science translational medicine. 2014;6(237):237ra65-ra65.
382. Lauder AP, Roche AM, Sherrill-Mix S, Bailey A, Laughlin AL, Bittinger K, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome. 2016;4:1-11.
383. Theis KR, Romero R, Winters AD, Greenberg JM, Gomez-Lopez N, Alhousseini A, et al. Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. American journal of obstetrics and gynecology. 2019;220(3):267. e1-. e39.
384. Dzidic M, Boix-Amorós A, Selma-Royo M, Mira A, Collado MC. Gut microbiota and mucosal immunity in the neonate. Medical Sciences. 2018;6(3):56.
385. Rautava S, Luoto R, Salminen S, Isolauri E. Microbial contact during pregnancy, intestinal colonization and human disease. Nature reviews Gastroenterology & hepatology. 2012;9(10):565-76.
386. Yassour M, Jason E, Hogstrom LJ, Arthur TD, Tripathi S, Siljander H, et al. Strain-level analysis of mother-to-child bacterial transmission during the first few months of life. Cell host & microbe. 2018;24(1):146-54. e4.
387. DiGiulio DB, editor Diversity of microbes in amniotic fluid. Seminars in fetal and neonatal medicine; 2012: Elsevier.
388. Mohd Zaki A, Hadingham A, Flaviani F, Haque Y, Mi JD, Finucane D, et al. Neutrophils dominate the cervical immune cell population in pregnancy and their transcriptome correlates with the microbial vaginal environment. Frontiers in microbiology. 2022;13:904451.
389. Iwasaki A. Antiviral immune responses in the genital tract: clues for vaccines. Nature Reviews Immunology. 2010;10(10):699-711.
390. Valore EV, Park CH, Igreti SL, Ganz T. Antimicrobial components of vaginal fluid. American journal of obstetrics and gynecology. 2002;187(3):561-8.
391. Negishi Y, Takahashi H, Kuwabara Y, Takeshita T. Innate immune cells in reproduction. Journal of Obstetrics and Gynaecology Research. 2018;44(11):2025-36.
392. Hedges SR, Agace WW, Svanborg C. Epithelial cytokine responses and mucosal cytokine networks. Trends in Microbiology. 1995;3(7):266-70.
393. Koga K, Mor G. Toll-like receptors at the maternal–fetal interface in normal pregnancy and pregnancy disorders. American journal of reproductive immunology. 2010;63(6):587-600.
394. Nathan C, Sporn M. Cytokines in context. The Journal of cell biology. 1991;113(5):981-6.
395. Wesolowski SR, Kasmi KCE, Jonscher KR, Friedman JE. Developmental origins of NAFLD: a womb with a clue. Nature reviews Gastroenterology & hepatology. 2017;14(2):81-96.
396. Ashkar AA, Di Santo JP, Croy BA. Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. The Journal of experimental medicine. 2000;192(2):259-70.

397. Azizieh FY, Raghupathy RG. Tumor necrosis factor-α and pregnancy complications: a prospective study. Medical principles and practice. 2015;24(2):165-70.
398. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Cytokine & growth factor reviews. 2011;22(4):189-95.
399. Manhanzva MT, Abrahams AG, Gamieldien H, Froissart R, Jaspan H, Jaumdally SZ, et al. Inflammatory and antimicrobial properties differ between vaginal Lactobacillus isolates from South African women with non-optimal versus optimal microbiota. Scientific Reports. 2020;10(1):6196.
400. Yockey LJ, Iwasaki A. Interferons and proinflammatory cytokines in pregnancy and fetal development. Immunity. 2018;49(3):397-412.
401. Cunningham MW, Amaral LM, Campbell NE, Cornelius DC, Ibrahim T, Vaka VR, et al. Investigation of interleukin-2-mediated changes in blood pressure, fetal growth restriction, and innate immune activation in normal pregnant rats and in a preclinical rat model of preeclampsia. Biology of sex Differences. 2021;12:1-10.
402. Meyyazhagan A, Kuchi Bhotla H, Pappuswamy M, Tsibizova V, Al Qasem M, Di Renzo GC. Cytokine see-saw across pregnancy, its related complexities and consequences. International Journal of Gynecology & Obstetrics. 2023;160(2):516-25.
403. Gimeno-Molina B, Muller I, Kropf P, Sykes L. The role of neutrophils in pregnancy, term and preterm labour. Life. 2022;12(10):1512.
404. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 6: a016295. Molecular Therapy: Nucleic Acids. 2014.
405. Prins JR, Gomez-Lopez N, Robertson SA. Interleukin-6 in pregnancy and gestational disorders. Journal of reproductive immunology. 2012;95(1-2):1-14.
406. Ehsani V, Mortazavi M, Ghorban K, Dadmanesh M, Bahramabadi R, Rezayati M-T, et al. Role of maternal interleukin-8 (IL-8) in normal-term birth in the human. Reproduction, Fertility and Development. 2019;31(6):1049-56.
407. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. The Journal of Immunology. 2003;170(6):3369-76.
408. Sakai M, Tsuda H, Tanebe K, Sasaki Y, Saito S. Interleukin-12 secretion by peripheral blood mononuclear cells is decreased in normal pregnant subjects and increased in preeclamptic patients. American journal of reproductive immunology. 2002;47(2):91-7.
409. El Shahaway AA, Abd Elhady RR, Abdelrhman AA, Yahia S. Role of maternal serum interleukin 17 in preeclampsia: diagnosis and prognosis. Journal of inflammation research. 2019:175-80.
410. Chatterjee P, Chiasson VL, Mitchell BM. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. Frontiers in immunology. 2014;5:93233.
411. Roberts CT, White C, Wiemer N, Ramsay A, Robertson S. Altered placental development in interleukin-10 null mutant mice. Placenta. 2003;24:S94-S9.
412. Reis ES, Mastellos DC, Hajishengallis G, Lambris JD. New insights into the immune functions of complement. Nature Reviews Immunology. 2019;19(8):503-16.
413. Warwick CA, Keyes AL, Woodruff TM, Usachev YM. The complement cascade in the regulation of neuroinflammation, nociceptive sensitization, and pain. Journal of Biological Chemistry. 2021;297(3).
414. Aagaard-Tillery KM, Silver R, Dalton J, editors. Immunology of normal pregnancy. Seminars in Fetal and Neonatal Medicine; 2006: Elsevier.

415. Borish LC, Steinke JW. 2. Cytokines and chemokines. Journal of Allergy and Clinical Immunology. 2003;111(2):S460-S75.
416. Greig PC, Murtha AP, Jimmerson CJ, Herbert WN, Roitman-Johnson B, Allen J. Maternal serum interleukin-6 during pregnancy and during term and preterm labor. Obstetrics & Gynecology. 1997;90(3):465-9.
417. Marie C, Pitton C, Fitting C, Cavaillon J. Regulation by anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGFβ) of interleukin-8 production by LPS-and/or TNFα-activated human polymorphonuclear cells. Mediators of inflammation. 1996;5:334-40.
418. Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine & growth factor reviews. 2010;21(5):331-44.
419. Kishimoto T. Interleukin-6: from basic science to medicine—40 years in immunology. Annu Rev Immunol. 2005;23:1-21.
420. Piccinni M-P, Giudizi M-G, Biagiotti R, Beloni L, Giannarini L, Sampognaro S, et al. Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production and membrane CD30 expression in established Th1 cell clones. Journal of immunology (Baltimore, Md: 1950). 1995;155(1):128-33.
421. Huber S, Kupperman J, Newell M. Estradiol prevents and testosterone promotes Fas-dependent apoptosis in CD4+ Th2 cells by altering Bcl 2 expression. Lupus. 1999;8(5):384-7.
422. Xue L, Gyles SL, Wettey FR, Gazi L, Townsend E, Hunter MG, et al. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. The Journal of Immunology. 2005;175(10):6531-6.
423. Kimura A, Naka T, Kishimoto T. IL-6-dependent and-independent pathways in the development of interleukin 17-producing T helper cells. Proceedings of the National Academy of Sciences. 2007;104(29):12099-104.
424. PB M, editor Some immunological and endocrinological problems raised by the evolution of viviparity in vertebarates. Symp Soc Exp Biol; 1953.
425. Yoshinaga K, editor Review of factors essential for blastocyst implantation for their modulating effects on the maternal immune system. Seminars in cell & developmental biology; 2008: Elsevier.
426. Norwitz ER, Bonney EA, Snegovskikh VV, Williams MA, Phillippe M, Park JS, et al. Molecular regulation of parturition: the role of the decidual clock. Cold Spring Harbor perspectives in medicine. 2015:a023143.
427. Dekel N, Gnainsky Y, Granot I, Mor G. Inflammation and implantation. American journal of reproductive immunology. 2010;63(1):17-21.
428. Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. Nature medicine. 2006;12(9):1065-74.
429. Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, et al. Uterine DCs are crucial for decidua formation during embryo implantation in mice. The Journal of clinical investigation. 2008;118(12):3954-65.
430. Bainbridge DR, Ellis SA, Sargent IL. Little evidence of HLA-G mRNA polymorphism in Caucasian or Afro-Caribbean populations. The Journal of Immunology. 1999;163(4):2023-7.
431. Faas MM, De Vos P. Uterine NK cells and macrophages in pregnancy. Placenta. 2017;56:44-52.
432. Vacca P, Cantoni C, Vitale M, Prato C, Canegallo F, Fenoglio D, et al. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and

immunosuppression. Proceedings of the National Academy of Sciences. 2010;107(26):11918-23.
433. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. The Journal of immunology. 2000;164(12):6166-73.
434. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. American journal of reproductive immunology. 2004;51(4):275-82.
435. Erlebacher A. Mechanisms of T cell tolerance towards the allogeneic fetus. Nature Reviews Immunology. 2013;13(1):23-33.
436. Chaouat G. Regulation of T-Cell Activities at the Feto-Placental Interface—by Placenta? American Journal of Reproductive Immunology. 1999;42(4):199-204.
437. Piccinni M-P. T cells in normal pregnancy and recurrent pregnancy loss. Reproductive biomedicine online. 2006;13(6):840-4.
438. Miyaura H, Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. The Journal of Immunology. 2002;168(3):1087-94.
439. Norman JE, Yuan M, Anderson L, Howie F, Harold G, Young A, et al. Effect of prolonged in vivo administration of progesterone in pregnancy on myometrial gene expression, peripheral blood leukocyte activation, and circulating steroid hormone levels. Reproductive sciences. 2011;18:435-46.
440. Akram KM, Frost LI, Anumba DO. Impaired autophagy with augmented apoptosis in a Th1/Th2-imbalanced placental micromilieu is associated with spontaneous preterm birth. Frontiers in Molecular Biosciences. 2022;9:897228.
441. Sykes L, MacIntyre DA, Yap XJ, Teoh TG, Bennett PR. The Th1: th2 dichotomy of pregnancy and preterm labour. Mediators of inflammation. 2012;2012.
442. Jenkins C, Roberts J, Wilson R, MacLean MA, Shilito J, Walker JJ. Evidence of a TH 1 type response associated with recurrent miscarriage. Fertility and sterility. 2000;73(6):1206-8.
443. Chaouat G, Assal Meliani A, Martal J, Raghupathy R, Elliott J, Elliot J, et al. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. Journal of immunology (Baltimore, Md: 1950). 1995;154(9):4261-8.
444. Da Silva J, Spector T. The role of pregnancy in the course and aetiology of rheumatoid arthritis. Clinical rheumatology. 1992;11:189-94.
445. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunology today. 1993;14(7):353-6.
446. Howard FH, Kwan A, Winder N, Mughal A, Collado-Rojas C, Muthana M. Understanding Immune Responses to Viruses—Do Underlying Th1/Th2 Cell Biases Predict Outcome? Viruses. 2022;14(7):1493.
447. Moura E, Mattar R, de Souza E, Torloni MR, Gonçalves-Primo A, Daher S. Inflammatory cytokine gene polymorphisms and spontaneous preterm birth. Journal of reproductive immunology. 2009;80(1-2):115-21.
448. Richani K, Soto E, Romero R, Espinoza J, Chaiworapongsa T, Nien JK, et al. Normal pregnancy is characterized by systemic activation of the complement system. The Journal of Maternal-Fetal & Neonatal Medicine. 2005;17(4):239-45.

449. Le Gars M, Kay AW, Bayless NL, Aziz N, Dekker CL, Swan GE, et al. Increased proinflammatory responses of monocytes and plasmacytoid dendritic cells to influenza A virus infection during pregnancy. The Journal of infectious diseases. 2016;214(11):1666-71.
450. Siegel I, Gleicher N. Peripheral white blood cell alterations in early labor. Diagnostic gynecology and obstetrics. 1981;3(2):123-6.
451. Kühnert M, Strohmeier R, Stegmüller M, Halberstadt E. Changes in lymphocyte subsets during normal pregnancy. European Journal of Obstetrics & Gynecology and Reproductive Biology. 1998;76(2):147-51.
452. Marzi M, Vigano A, Trabattoni D, Villa M, Salvaggio A, Clerici E, et al. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. Clinical & Experimental Immunology. 1996;106(1):127-33.
453. Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV. Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. Biochemical and biophysical research communications. 1998;245(3):933-8.
454. Fu Y, Tang L, Hu M, Xiang Z, Hu Y. Changes of serum interleukin-6 in healthy pregnant women and establishment of relevant reference intervals. Clinica Chimica Acta. 2020;502:116-9.
455. Jarmund AH, Giskeødegård GF, Ryssdal M, Steinkjer B, Stokkeland LMT, Madssen TS, et al. Cytokine patterns in maternal serum from first trimester to term and beyond. Frontiers in immunology. 2021;12:752660.
456. Christian LM, Porter K. Longitudinal changes in serum proinflammatory markers across pregnancy and postpartum: effects of maternal body mass index. Cytokine. 2014;70(2):134-40.
457. Dealtry G, Clark D, Sharkey A, Charnock-Jones D, Smith S. VII international congress of reproductive immunology, New Delhi, 27–30 October 1998: expression and localization of the Th2-type cytokine interleukin-13 and its receptor in the placenta during human pregnancy. American Journal of Reproductive Immunology. 1998;40(4):283-90.
458. Roth I, Corry DB, Locksley RM, Abrams JS, Litton MJ, Fisher SJ. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. The Journal of experimental medicine. 1996;184(2):539-48.
459. Jones CA, Finlay-Jones JJ, Hart PH. Type-1 and type-2 cytokines in human late-gestation decidual tissue. Biology of reproduction. 1997;57(2):303-11.
460. Liu F, Guo J, Tian T, Wang H, Dong F, Huang H, et al. Placental trophoblasts shifted Th1/Th2 balance toward Th2 and inhibited Th17 immunity at fetomaternal interface. Apmis. 2011;119(9):597-604.
461. Donders GG, Bosmans E, Dekeersmaeckerb A, Vereecken A, Van Bulck B, Spitz B. Pathogenesis of abnormal vaginal bacterial flora. American journal of obstetrics and gynecology. 2000;182(4):872-8.
462. Ashford K, Chavan NR, Wiggins AT, Sayre MM, McCubbin A, Critchfield AS, et al. Comparison of serum and cervical cytokine levels throughout pregnancy between preterm and term births. American Journal of Perinatology Reports. 2018;8(02):e113-e20.
463. Heng YJ, Liong S, Permezel M, Rice GE, Quinzio MKD, Georgiou HM. The interplay of the interleukin 1 system in pregnancy and labor. Reproductive Sciences. 2014;21(1):122-30.
464. Ashford KB, Chavan N, Ebersole JL, Wiggins AT, Sharma S, McCubbin MA, et al. Patterns of systemic and cervicovaginal fluid (CVF) inflammatory cytokines throughout pregnancy. American journal of perinatology. 2018;35(5):455.

465. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. MHR: Basic science of reproductive medicine. 2003;9(1):41-5.
466. Omu A, Al-Qattan F, Diejomaoh M, Al-Yatama M. Differential levels of T helper cytokines in preeclampsia, pregnancy, labor and puerperium. Acta obstetricia et gynecologica Scandinavica. 1999;78(8):675-80.
467. Buonocore G, De Filippo M, Gioia D, Picciolini E, Luzzi E, Bocci V, et al. Maternal and neonatal plasma cytokine levels in relation to mode of delivery. Neonatology. 1995;68(2):104-10.
468. Kuwajima T, Suzuki S, Sawa R, Yoneyama Y, Takeshita T, Araki T. Changes in maternal peripheral T helper 1-type and T helper 2-type immunity during labor. The Tohoku Journal of Experimental Medicine. 2001;194(2):137-40.
469. Norman JE, Bollapragada S, Yuan M, Nelson SM. Inflammatory pathways in the mechanism of parturition. BMC pregnancy and childbirth. 2007;7(1):1-4.
470. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, et al. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Human reproduction. 1999;14(1):229-36.
471. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Molecular human reproduction. 2003;9(1):41-5.
472. Maul H, Nagel S, Welsch G, Schäfer A, Winkler M, Rath W. Messenger ribonucleic acid levels of interleukin-1β, interleukin-6 and interleukin-8 in the lower uterine segment increased significantly at final cervical dilatation during term parturition, while those of tumor necrosis factor α remained unchanged. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2002;102(2):143-7.
473. Ledingham M, Thomson A, Jordan F, Young A, Crawford M, Norman J. Cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. Obstetrics & Gynecology. 2001;97(2):235-42.
474. Tribe RM, Moriarty P, Dalrymple A, Hassoni AA, Poston L. Interleukin-1β induces calcium transients and enhances basal and store operated calcium entry in human myometrial smooth muscle. Biology of reproduction. 2003;68(5):1842-9.
475. Oger Sp, Méhats Cl, Dallot E, Ferré Fo, Leroy M-Jp. Interleukin-1β induces phosphodiesterase 4B2 expression in human myometrial cells through a prostaglandin E2-and cyclic adenosine 3', 5'-monophosphate-dependent pathway. The Journal of Clinical Endocrinology & Metabolism. 2002;87(12):5524-31.
476. Gc L. Cervical ripening as an inflammatory reaction. The cervix in pregnancy and labor Clinical and biochemical investigation. 1981:1-9.
477. Ledingham M-A, Thomson AJ, Young A, Macara LM, Greer IA, Norman JE. Changes in the expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition. Molecular human reproduction. 2000;6(11):1041-8.
478. Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Mazor M, et al. Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. American journal of obstetrics and gynecology. 2006;195(2):394-405. e12.

479. Marvin K, Keelan J, Eykholt R, Sato T, Mitchell M. Use of cDNA arrays to generate differential expression profiles for inflammatory genes in human gestational membranes delivered at term and preterm. Molecular human reproduction. 2002;8(4):399-408.
480. Keelan JA, Marvin KW, Sato TA, Coleman M, McCowan LM, Mitchell MD. Cytokine abundance in placental tissues: evidence of inflammatory activation in gestational membranes with term and preterm parturition. American journal of obstetrics and gynecology. 1999;181(6):1530-6.
481. Elliott C, Loudon J, Brown N, Slater D, Bennett P, Sullivan M. IL-1β and IL-8 in human fetal membranes: changes with gestational age, labor, and culture conditions. American Journal of Reproductive Immunology. 2001;46(4):260-7.
482. Opsjøn S-L, Wathen NC, Tingulstad S, Wiedswang G, Sundan A, Waage A, et al. Tumor necrosis factor, interleukin-1, and interleukin-6 in normal human pregnancy. American Journal of Obstetrics and Gynecology. 1993;169(2):397-404.
483. Romero R, Brody DT, Oyarzun E, Mazor M, Wu YK, Hobbins JC, et al. Infection and labor: III. Interleukin-1: A signal for the onset of parturition. American journal of obstetrics and gynecology. 1989;160(5):1117-23.
484. ROMERO R, MAZOR M, BRANDT F, SEPULVEDA W, AVILA C, COTTON DB, et al. Interleukin-1α and Interleukin-1 β in Preterm and Term Human Parturition. American journal of reproductive immunology. 1992;27(3-4):117-23.
485. Fukuda H, Masuzaki H, Ishimaru T. Interleukin-6 and interleukin-1 receptor antagonist in amniotic fluid and cord blood in patients with pre-term, premature rupture of the membranes. International Journal of Gynecology & Obstetrics. 2002;77(2):123-9.
486. Hebisch G, Grauaug AA, Neumaier-Wagner PM, Stallmach T, Huch A, Huch R. The relationship between cervical dilatation, interleukin-6 and interleukin-8 during term labor. Acta Obstetricia et Gynecologica Scandinavica: ORIGINAL ARTICLE. 2001;80(9):840-8.
487. Krediet TG, Wiertsema SP, Vossers MJ, Hoeks SB, Fleer A, Ruven HJ, et al. Toll-like receptor 2 polymorphism is associated with preterm birth. Pediatric research. 2007;62(4):474-6.
488. Hunter PJ, Sheikh S, David AL, Peebles DM, Klein N. Cervical leukocytes and spontaneous preterm birth. Journal of reproductive immunology. 2016;113:42-9.
489. Lynch AM, Gibbs RS, Murphy JR, Giclas PC, Salmon JE, Holers VM. Early elevations of the complement activation fragment C3a and adverse pregnancy outcomes. Obstetrics and gynecology. 2011;117(1):75.
490. Soto E, Romero R, Richani K, Yoon BH, Chaiworapongsa T, Vaisbuch E, et al. Evidence for complement activation in the amniotic fluid of women with spontaneous preterm labor and intra-amniotic infection. The Journal of Maternal-Fetal & Neonatal Medicine. 2009;22(11):983-92.
491. Lappas M, Woodruff TM, Taylor SM, Permezel M. Complement C5A regulates prolabor mediators in human placenta. Biology of reproduction. 2012;86(6):190, 1-9.
492. Makhseed M, Raghupathy R, El-Shazly S, Azizieh F, Al-Harmi J, Al-Azemi M. Pro-inflammatory maternal cytokine profile in preterm delivery. American journal of reproductive immunology. 2003;49(5):308-18.
493. Gargano JW, Holzman C, Senagore P, Thorsen P, Skogstrand K, Hougaard DM, et al. Mid-pregnancy circulating cytokine levels, histologic chorioamnionitis and spontaneous preterm birth. Journal of reproductive immunology. 2008;79(1):100-10.

494. Wei S-Q, Fraser W, Luo Z-C. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. Obstetrics & Gynecology. 2010;116(2 Part 1):393-401.
495. Curry AE, Vogel I, Drews C, Schendel D, Skogstrand K, Flanders WD, et al. Mid-pregnancy maternal plasma levels of interleukin 2, 6, and 12, tumor necrosis factor-alpha, interferon-gamma, and granulocyte-macrophage colony-stimulating factor and spontaneous preterm delivery. Acta obstetricia et gynecologica Scandinavica. 2007;86(9):1103-10.
496. Bahar AM, Ghalib HW, Moosa RA, Zaki ZM, Thomas C, Nabri OA. Maternal serum interleukin-6, interleukin-8, tumor necrosis factor-α and interferon-γ in preterm labor. Acta obstetricia et gynecologica Scandinavica. 2003;82(6):543-9.
497. Curry A, Vogel I, Skogstrand K, Drews C, Schendel D, Flanders W, et al. Maternal plasma cytokines in early-and mid-gestation of normal human pregnancy and their association with maternal factors. Journal of reproductive immunology. 2008;77(2):152-60.
498. Chan D, Bennett PR, Lee YS, Kundu S, Teoh T, Adan M, et al. Microbial-driven preterm labour involves crosstalk between the innate and adaptive immune response. Nature communications. 2022;13(1):975.
499. Kotani H, Matsubara K, Koshizuka T, Nishiyama K, Kaneko H, Tasaka M, et al. Human β-defensin-2 as a biochemical indicator of vaginal environment in pregnant women. Hypertension Research in Pregnancy. 2018;6(2):68-72.
500. Burris HH, Riis VM, Schmidt I, Gerson KD, Brown A, Elovitz MA. Maternal stress, low cervicovaginal β-defensin, and spontaneous preterm birth. American journal of obstetrics & gynecology MFM. 2020;2(2):100092.
501. Elovitz MA, Gajer P, Riis V, Brown AG, Humphrys MS, Holm JB, et al. Cervicovaginal microbiota and local immune response modulate the risk of spontaneous preterm delivery. Nature communications. 2019;10(1):1305.
502. Administration FaD. Early Clinical Trials with Live Biotherapeutic Products: Guidance for Industry 2016 https://www.fda.gov/media/82945/download, accessed July 2023.
503. Charbonneau MR, Isabella VM, Li N, Kurtz CB. Developing a new class of engineered live bacterial therapeutics to treat human diseases. Nature Communications. 2020;11(1):1738.
504. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. PLoS biology. 2016;14(8):e1002533.
505. Czajkowski K, Broś-Konopielko M, Teliga-Czajkowska J. Urinary tract infection in women. Menopause Review/Przegląd Menopauzalny. 2021;20(1):40-7.
506. Epp A, Larochelle A, Lovatsis D, Walter J-E, Easton W, Farrell SA, et al. Recurrent urinary tract infection. Journal of Obstetrics and Gynaecology Canada. 2010;32(11):1082-90.
507. Stamey TA, Sexton CC. The role of vaginal colonization with enterobacteriaceae in recurrent urinary infections. The Journal of urology. 1975;113(2):214-7.
508. Pfau A, Sacks T. The bacterial flora of the vaginal vestibule, urethra and vagina in premenopausal women with recurrent urinary tract infections. The Journal of Urology. 1981;126(5):630-4.
509. Czaja CA, Stapleton AE, Yarova-Yarovaya Y, Stamm WE. Phase I trial of a Lactobacillus crispatus vaginal suppository for prevention of recurrent urinary tract infection in women. Infectious diseases in obstetrics and gynecology. 2007;2007.
510. Baerheim A, Larsen E, Digranes A. Vaginal application of lactobacilli in the prophylaxis of recurrent lower urinary tract infection in women. Scandinavian journal of primary health care. 1994;12(4):239-43.

511. Uehara S, Monden K, Nomoto K, Seno Y, Kariyama R, Kumon H. A pilot study evaluating the safety and effectiveness of Lactobacillus vaginal suppositories in patients with recurrent urinary tract infection. International journal of antimicrobial agents. 2006;28:30-4.
512. Sadahira T, Wada K, Araki M, Mitsuhata R, Yamamoto M, Maruyama Y, et al. Efficacy of Lactobacillus vaginal suppositories for the prevention of recurrent cystitis: A phase II clinical trial. International Journal of Urology. 2021;28(10):1026-31.
513. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, et al. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. The Journal of infectious diseases. 2006;193(11):1478-86.
514. Petricevic L, Witt A. The role of Lactobacillus casei rhamnosus Lcr35 in restoring the normal vaginal flora after antibiotic treatment of bacterial vaginosis. BJOG: An International Journal of Obstetrics & Gynaecology. 2008;115(11):1369-74.
515. Larsson P-G, Stray-Pedersen B, Ryttig KR, Larsen S. Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. BMC women's health. 2008;8:1-8.
516. Bohbot J, Daraï E, Bretelle F, Brami G, Daniel C, Cardot J. Efficacy and safety of vaginally administered lyophilized Lactobacillus crispatus IP 174178 in the prevention of bacterial vaginosis recurrence. Journal of gynecology obstetrics and human reproduction. 2018;47(2):81-6.
517. van de Wijgert JH, Verwijs MC. Lactobacilli-containing vaginal probiotics to cure or prevent bacterial or fungal vaginal dysbiosis: a systematic review and recommendations for future trial designs. BJOG: An International Journal of Obstetrics & Gynaecology. 2020;127(2):287-99.
518. Gopal Rao G, Hiles S, Bassett P, Lamagni T. Differential rates of group B Streptococcus (GBS) colonisation in pregnant women in a racially diverse area of London, UK: a cross-sectional study. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(11):1347-53.
519. Walker O, Kenny CB, Goel N. Neonatal sepsis. Paediatrics and Child Health. 2019;29(6):263-8.
520. No G-tG. Prevention of early onset neonatal group B streptococcal disease. The Public. 2003;2:3.
521. Moghaddam MN. Recto-vaginal colonization of group B Streptococcus in pregnant women referred to a hospital in Iran and its effect on Lactobacillus normal flora. Journal of Biological Sciences. 2010;10(2):166-9.
522. Açikgöz ZC, Gamberzade S, Göçer S, Ceylan P. Inhibitor effect of vaginal lactobacilli on group B streptococci. Mikrobiyoloji Bulteni. 2005;39(1):17-23.
523. Martín V, Cárdenas N, Ocaña S, Marín M, Arroyo R, Beltrán D, et al. Rectal and vaginal eradication of Streptococcus agalactiae (GBS) in pregnant women by using Lactobacillus salivarius CECT 9145, a target-specific probiotic strain. Nutrients. 2019;11(4):810.
524. Myhre R, Brantsæter AL, Myking S, Gjessing HK, Sengpiel V, Meltzer HM, et al. Intake of probiotic food and risk of spontaneous preterm delivery. The American journal of clinical nutrition. 2011;93(1):151-7.

525. Nordqvist M, Jacobsson B, Brantsæter A-L, Myhre R, Nilsson S, Sengpiel V. Timing of probiotic milk consumption during pregnancy and effects on the incidence of preeclampsia and preterm delivery: a prospective observational cohort study in Norway. BMJ open. 2018;8(1).
526. Luoto R, Laitinen K, Nermes M, Isolauri E. Impact of maternal probiotic-supplemented dietary counselling on pregnancy outcome and prenatal and postnatal growth: a double-blind, placebo-controlled study. British journal of nutrition. 2010;103(12):1792-9.
527. Nishijima K, Shukunami K-i, Kotsuji F. Probiotics affects vaginal flora in pregnant women, suggesting the possibility of preventing preterm labor. Journal of clinical gastroenterology. 2005;39(5):447-8.
528. Liu Y, Huang Y, Cai W, Li D, Zheng W, Xiao Y, et al. Effect of oral Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 on vaginal Group B Streptococcus colonization and vaginal microbiome in late pregnancy. Nan Fang yi ke da xue xue bao= Journal of Southern Medical University. 2020;40(12):1753-9.
529. Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, et al. Oral use of Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunology & Medical Microbiology. 2003;35(2):131-4.
530. Husain S, Allotey J, Drymoussi Z, Wilks M, Fernandez-Felix B, Whiley A, et al. Effects of oral probiotic supplements on vaginal microbiota during pregnancy: a randomised, double-blind, placebo-controlled trial with microbiome analysis. BJOG: an International Journal of Obstetrics & Gynaecology. 2020;127(2):275-84.
531. Yang S, Reid G, Challis JR, Gloor GB, Asztalos E, Money D, et al. Effect of oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 on the vaginal microbiota, cytokines and chemokines in pregnant women. Nutrients. 2020;12(2):368.
532. Vásquez A, Ahrné S, Jeppsson B, Molin G. Oral administration of Lactobacillus and Bifidobacterium strains of intestinal and vaginal origin to healthy human females: Re-isolation from faeces and vagina. Microbial Ecology in Health and Disease. 2005;17(1):15-20.
533. Kirihara N, Kamitomo M, Tabira T, Hashimoto T, Taniguchi H, Maeda T. Effect of probiotics on perinatal outcome in patients at high risk of preterm birth. Journal of Obstetrics and Gynaecology Research. 2018;44(2):241-7.
534. Shiozaki A, Yoneda S, Yoneda N, Yonezawa R, Matsubayashi T, Seo G, et al. Intestinal microbiota is different in women with preterm birth: results from terminal restriction fragment length polymorphism analysis. PloS one. 2014;9(11):e111374.
535. Grev J, Berg M, Soll R. Maternal probiotic supplementation for prevention of morbidity and mortality in preterm infants. Cochrane Database of Systematic Reviews. 2018(12).
536. Neri A, Sabah G, Samra Z. Bacterial vaginosis in pregnancy treated with yoghurt. Acta Obstetricia et Gynecologica Scandinavica. 1993;72(1):17-9.
537. Sandeford J, Nippita T, Bhuta T, Patterson J, Morris J, Seeho S. Protocol for probiotic therapy vs placebo for preterm prelabour rupture of membranes to prolong pregnancy duration (Pro-PPROM) trial. Australian and New Zealand Journal of Obstetrics and Gynaecology. 2021;61(2):E12-E7.
538. Yadav J, Das V, Kumar N, Agrawal S, Pandey A, Agrawal A. Vaginal probiotics as an adjunct to antibiotic prophylaxis in the management of preterm premature rupture of the membranes. Journal of Obstetrics and Gynaecology. 2022;42(5):1037-42.

539. Luzi G, Iazzetta E, Cecconi L, Famiani S, Arena S, Carlo G, et al. Lactobacillus rhamnosus BMX 54 vaginal application usefulness as adjuvant therapy for preterm premature rupture of membranes (PPROM) in the second trimester of pregnancy. Int J Pediatr Neonatal Health. 2018;2(1):14-8.
540. Kavak SB, Kavak E, Ilhan R, Atilgan R, Arat O, Deveci U, et al. The efficacy of ampicillin and Lactobacillus casei rhamnosus in the active management of preterm premature rupture of membranes remote from term. Drug design, development and therapy. 2014:1169-73.
541. Kindinger L, MacIntyre D, Lees Y, Marchesi J, Teoh T, Bennett P, editors. The impact of progesterone on the vaginal microbiome in high-risk pregnancy with a short cervix. BJOG-AN INTERNATIONAL JOURNAL OF OBSTETRICS AND GYNAECOLOGY; 2016: WILEY-BLACKWELL 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.
542. Antonio MA, Hillier SL. DNA fingerprinting of Lactobacillus crispatus strain CTV-05 by repetitive element sequence-based PCR analysis in a pilot study of vaginal colonization. Journal of Clinical Microbiology. 2003;41(5):1881-7.
543. Klebanoff SJ, Hillier SL, Eschenbach DA, Waltersdorph AM. Control of the microbial flora of the vagina by H202-generating lactobacilli. Journal of Infectious Diseases. 1991;164(1):94-100.
544. Andreu A, Stapleton AE, Fennell CL, Hillier SL, Stamm WE. Hemagglutination, adherence, and surface properties of vaginal Lactobacillus species. Journal of Infectious Diseases. 1995;171(5):1237-43.
545. Osset J, Bartolomé RM, García E, Andreu A. Assessment of the capacity of Lactobacillus to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. The Journal of infectious diseases. 2001;183(3):485-91.
546. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. 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. 2013;4(4):10.1128/mbio. 00460-13.
547. Edwards VL, Smith SB, McComb EJ, Tamarelle J, Ma B, Humphrys MS, et al. The cervicovaginal microbiota-host interaction modulates Chlamydia trachomatis infection. MBio. 2019;10(4):10.1128/mbio. 01548-19.
548. Lagenaur LA, Hemmerling A, Chiu C, Miller S, Lee PP, Cohen CR, et al. Connecting the dots: Translating the vaginal microbiome into a drug. The Journal of infectious diseases. 2021;223(Supplement_3):S296-S306.
549. Hemmerling A, Harrison W, Schroeder A, Park J, Korn A, Shiboski S, et al. Phase 1 dose-ranging safety trial of Lactobacillus crispatus CTV-05 (LACTIN-V) for the prevention of bacterial vaginosis. Sexually transmitted diseases. 2009;36(9):564.
550. Martinez RM, Hulten KG, Bui U, Clarridge III JE. Molecular analysis and clinical significance of Lactobacillus spp. recovered from clinical specimens presumptively associated with disease. Journal of clinical microbiology. 2014;52(1):30-6.
551. Stapleton AE, Au-Yeung M, Hooton TM, Fredricks DN, Roberts PL, Czaja CA, et al. Randomized, placebo-controlled phase 2 trial of a Lactobacillus crispatus probiotic given intravaginally for prevention of recurrent urinary tract infection. Clinical infectious diseases. 2011;52(10):1212-7.
552. Lupattelli A, Picinardi M, Cantarutti A, Nordeng H. Use and intentional avoidance of prescribed medications in pregnancy: a cross-sectional, web-based study among 926 women in Italy. International journal of environmental research and public health. 2020;17(11):3830.

553. Hemmerling A, Harrison W, Schroeder A, Park J, Korn A, Shiboski S, et al. Phase 2a study assessing colonization efficiency, safety, and acceptability of Lactobacillus crispatus CTV-05 in women with bacterial vaginosis. Sexually transmitted diseases. 2010:745-50.
554. Cohen CR, Wierzbicki MR, French AL, Morris S, Newmann S, Reno H, et al. Randomized trial of lactin-V to prevent recurrence of bacterial vaginosis. New England Journal of Medicine. 2020;382(20):1906-15.
555. Short C-ES, Taylor GP. Antiretroviral therapy and preterm birth in HIV-infected women. Expert review of anti-infective therapy. 2014;12(3):293-306.
556. Stokholm J, Schjørring S, Eskildsen CE, Pedersen L, Bischoff AL, Følsgaard N, et al. Antibiotic use during pregnancy alters the commensal vaginal microbiota. Clinical Microbiology and Infection. 2014;20(7):629-35.
557. Kuperman AA, Koren O. Antibiotic use during pregnancy: how bad is it? BMC medicine. 2016;14:1-7.
558. NICE. Preterm labour and birth. June 2022.
559. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. New England Journal of Medicine. 1996;334(9):567-73.
560. Crane J, Hutchens D. Transvaginal sonographic measurement of cervical length to predict preterm birth in asymptomatic women at increased risk: a systematic review. Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2008;31(5):579-87.
561. FDA U.S. Department of Health and Human Services FaDAGfI. Early clinical trials with live biotherapeutic products: Chemistry, manufacturing, and control information. 2016.
562. Scott S, Clark A, May H, Bhattacharya D. Validation and feasibility of the medication acceptability questionnaire to investigate tablet and liquid alendronic acid with older hospital patients. Pharmacy. 2018;6(3):84.
563. Yaddanapudi S, Yaddanapudi L. How to design a questionnaire. Indian journal of anaesthesia. 2019;63(5):335.
564. Stinson LF, Keelan JA, Payne MS. Comparison of meconium DNA extraction methods for use in microbiome studies. Frontiers in microbiology. 2018;9:270.
565. Coran AG, Caldamone A, Adzick NS, Krummel TM, Laberge J-M, Shamberger R. Pediatric surgery E-book: Elsevier Health Sciences; 2012.
566. Hare JM. Sabouraud agar for fungal growth. Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology. 2013:211-6.
567. Craven RR, Weber CJ, Jennemann RA, Dunne Jr WM. Evaluation of a chromogenic agar for detection of group B streptococcus in pregnant women. Journal of clinical microbiology. 2010;48(9):3370-1.
568. Binghuai L, Yanli S, Shuchen Z, Fengxia Z, Dong L, Yanchao C. Use of MALDI-TOF mass spectrometry for rapid identification of group B Streptococcus on chromID Strepto B agar. International Journal of Infectious Diseases. 2014;27:44-8.
569. Slotved H-C, Facklam RR, Fuursted K. Assessment of a novel bile solubility test and MALDI-TOF for the differentiation of Streptococcus pneumoniae from other mitis group streptococci. Scientific reports. 2017;7(1):7167.
570. England PH. UK Standards for Microbiology Investigations: Identification of Streptococcus species, Enterococcus species and morphologically similar organisms 2021 https://assets.publishing.service.gov.uk/media/614b29fd8fa8f561101f38c5/ID_4i4.pdf, accessed June 2023.

571. Pathology NWLN. HVS/LVS standard operating procedure (2020), unpublished.
572. Liu CM, Aziz M, Kachur S, Hsueh P-R, Huang Y-T, Keim P, et al. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. BMC microbiology. 2012;12(1):1-13.
573. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome research. 1996;6(10):986-94.
574. Stoddard SF, Smith BJ, Hein R, Roller BR, Schmidt TM. rrn DB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. Nucleic acids research. 2015;43(D1):D593-D8.
575. Miles AA, Misra S, Irwin J. The estimation of the bactericidal power of the blood. Epidemiology & Infection. 1938;38(6):732-49.
576. Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. Methods. 2006;38(4):317-23.
577. R&D S. Human Luminex® Discovery Assay.
578. Romero R, Xu Y, Plazyo O, Chaemsaithong P, Chaiworapongsa T, Unkel R, et al. A role for the inflammasome in spontaneous labor at term. American journal of reproductive immunology. 2018;79(6):e12440.
579. Presicce P, Senthamaraikannan P, Alvarez M, Rueda CM, Cappelletti M, Miller LA, et al. Neutrophil recruitment and activation in decidua with intra-amniotic IL-1beta in the preterm rhesus macaque. Biology of reproduction. 2015;92(2):56, 1-13.
580. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1β and tumor necrosis factor-α but not by interleukin-6 or interleukin-8 in a nonhuman primate model. American journal of obstetrics and gynecology. 2006;195(6):1578-89.
581. Genc MR, Witkin SS, Delaney ML, Paraskevas L-R, Tuomala RE, Norwitz ER, et al. A disproportionate increase in IL-1β over IL-1ra in the cervicovaginal secretions of pregnant women with alteredvaginal microflora correlates with preterm birth. American journal of obstetrics and gynecology. 2004;190(5):1191-7.
582. Sennström MB, Ekman G, Westergren-Thorsson G, Malmström A, Byström B, Endresen U, et al. Human cervical ripening, an inflammatory process mediated by cytokines. Molecular human reproduction. 2000;6(4):375-81.
583. Ekman-Ordeberg G, Dubicke A. Preterm cervical ripening in humans. Facts, views & vision in ObGyn. 2012;4(4):245.
584. Sakai M, Ishiyama A, Tabata M, Sasaki Y, Yoneda S, Shiozaki A, et al. Relationship between cervical mucus interleukin-8 concentrations and vaginal bacteria in pregnancy. American Journal of Reproductive Immunology. 2004;52(2):106-12.
585. Rizzo G, Capponi A, Vlachopoulou A, Angelini E, Grassi C, Romanini C. The diagnostic value of interleukin-8 and fetal fibronectin concentrations in cervical secretions in patients with preterm labor and intact membranes. 1997.
586. Taylor BD, Holzman CB, Fichorova RN, Tian Y, Jones NM, Fu W, et al. Inflammation biomarkers in vaginal fluid and preterm delivery. Human reproduction. 2013;28(4):942-52.
587. Moore KW, O'Garra A, Malefyt RW, Vieira P, Mosmann TR. Interleukin-10. Annual review of immunology. 1993;11(1):165-90.
588. Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in

mediating the production of IL-8 triggered by lipopolysaccharide. The Journal of experimental medicine. 1993;178(6):2207-11.
589. Barsig J, Küsters S, Vogt K, Volk HD, Tiegs G, Wendel A. Lipopolysaccharide-induced interleukin-10 in mice: role of endogenous tumor necrosis factor-α. European journal of immunology. 1995;25(10):2888-93.
590. Dubicke A, Fransson E, Centini G, Andersson E, Byström B, Malmström A, et al. Pro-inflammatory and anti-inflammatory cytokines in human preterm and term cervical ripening. Journal of reproductive immunology. 2010;84(2):176-85.
591. Guo X, Li X, Qi T, Pan Z, Zhu X, Wang H, et al. A birth population-based survey of preterm morbidity and mortality by gestational age. BMC pregnancy and childbirth. 2021;21:1-12.
592. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal. 2011;17(1):10-2.
593. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019;37(8):852-7.
594. Callahan B, McMurdie P, Rosen M, Han A, Johnson A, Dada SH. High-resolution sample inference from Illumina amplicon data., 2016, 13. DOI: https://doi org/101038/nmeth.3869:581-3.
595. Fettweis JM, Serrano MG, Sheth NU, Mayer CM, Glascock AL, Brooks JP, et al. Species-level classification of the vaginal microbiome. BMC genomics. 2012;13:1-9.
596. Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nature protocols. 2020;15(3):799-821.
597. Zarasvand S, Bayar E, Adan M, Mountain K, Lewis H, Joash K, et al. Rapid quality improvement in a preterm birth clinic care pathway during the COVID-19 pandemic. BMJ Open Quality. 2020;9(4):e001049.
598. Aagaard K, Riehle K, Ma J, Segata N, Mistretta T-A, Coarfa C, et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PloS one. 2012;7(6):e36466.
599. Goplerud C, Ohm MJ, Galask RP. Aerobic and anaerobic flora of the cervix during pregnancy and the puerperium. American journal of obstetrics and gynecology. 1976;126(7):858-68.
600. Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. Microbiome. 2014;2(1):1-19.
601. Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M. 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. 2009;9(1):1-10.
602. Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ. Microbiota restoration: natural and supplemented recovery of human microbial communities. Nature Reviews Microbiology. 2011;9(1):27-38.
603. Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. The Journal of physiology. 2017;595(2):451-63.
604. Payne MS, Newnham JP, Doherty DA, Furfaro LL, Pendal NL, Loh DE, et al. 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. 2021;224(2):206. e1-. e23.
605. Pybus V, Onderdonk AB. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. Microbes and infection. 1999;1(4):285-92.
606. Trama JP, Pascal KE, Zimmerman J, Self MJ, Mordechai E, Adelson ME. Rapid detection of Atopobium vaginae and association with organisms implicated in bacterial vaginosis. Molecular and cellular probes. 2008;22(2):96-102.
607. Pybus V, Onderdonk AB. A commensal symbiosis between Prevotella bivia and Peptostreptococcus anaerobius involves amino acids: potential significance to the pathogenesis of bacterial vaginosis. FEMS Immunology & Medical Microbiology. 1998;22(4):317-27.
608. Leitich H, Bodner-Adler B, Brunbauer M, Kaider A, Egarter C, Husslein P. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. American journal of obstetrics and gynecology. 2003;189(1):139-47.
609. Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. Best practice & research Clinical obstetrics & gynaecology. 2007;21(3):375-90.
610. Hauth JC, Goldenberg RL, Andrews WW, DuBard MB, Copper RL. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. New England Journal of Medicine. 1995;333(26):1732-6.
611. Lamont RF, Duncan SL, Mandal D, Bassett P. Intravaginal clindamycin to reduce preterm birth in women with abnormal genital tract flora. Obstetrics & Gynecology. 2003;101(3):516-22.
612. McDonald H, O'loughlin J, Vigneswaran R, Jolley P, Harvey J, Bof A, et al. Impact of metronidazole therapy on preterm birth in women with bacterial vaginosis flora (Gardnerella vaginalis): a randomised, placebo controlled trial. BJOG: An International Journal of Obstetrics & Gynaecology. 1997;104(12):1391-7.
613. Sekikubo M, Hedman K, Mirembe F, Brauner A. Antibiotic overconsumption in pregnant women with urinary tract symptoms in Uganda. Clinical Infectious Diseases. 2017;65(4):544-50.
614. Shennan A, Crawshaw S, Briley A, Hawken J, Seed P, Jones G, et al. General obstetrics: A randomised controlled trial of metronidazole for the prevention of preterm birth in women positive for cervicovaginal fetal fibronectin: the PREMET Study. BJOG: An International Journal of Obstetrics & Gynaecology. 2006;113(1):65-74.
615. Health UDo, Services H. Food and Drug Administration Guidance for Industry. Early clinical trials with live biotherapeutic products: Chemistry, manufacturing, and control information. 2016.
616. Goldenberg JZ, Yap C, Lytvyn L, Lo CKF, Beardsley J, Mertz D, et al. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. Cochrane Database of Systematic Reviews. 2017(12).
617. Kajander K, Myllyluoma E, RAJILIĆ-STOJANOVIĆ M, Kyrönpalo S, Rasmussen M, Järvenpää S, et al. Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. Alimentary pharmacology & therapeutics. 2008;27(1):48-57.
618. Wilkins T, Sequoia J. Probiotics for gastrointestinal conditions: a summary of the evidence. American family physician. 2017;96(3):170-8.

619. Nordqvist M, Jacobsson B, Brantsæter A-L, Myhre R, Nilsson S, Sengpiel V. Timing of probiotic milk consumption during pregnancy and effects on the incidence of preeclampsia and preterm delivery: a prospective observational cohort study in Norway. BMJ open. 2018;8(1):e018021.
620. Yefet E, Colodner R, Strauss M, Gam Ze Letova Y, Nachum Z. A randomized controlled open label crossover trial to study vaginal colonization of orally administered Lactobacillus reuteri RC-14 and rhamnosus GR-1 in pregnant women at high risk for preterm labor. Nutrients. 2020;12(4):1141.
621. Deal C. Science and Regulation of Live Microbiome-Based Products Used to Prevent, Treat, or Cure Diseases in Humans. US Food and Drug Administration. 2018.
622. Ross JJ, Boucher PE, Bhattacharyya SP, Kopecko DJ, Sutkowski EM, Rohan PJ, et al. Considerations in the development of live biotherapeutic products for clinical use. Current issues in molecular biology. 2008;10(1-2):13-6.
623. Thomson A, Obstetricians RCo, Gynaecologists. Care of women presenting with suspected preterm prelabour rupture of membranes from 24+ 0 weeks of gestation: green-top guideline no. 73. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(9):e152-e66.
624. Hedermann G, Hedley PL, Bækvad-Hansen M, Hjalgrim H, Rostgaard K, Poorisrisak P, et al. Danish premature birth rates during the COVID-19 lockdown. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2021;106(1):93-5.
625. Philip RK, Purtill H, Reidy E, Daly M, Imcha M, McGrath D, et al. Reduction in preterm births during the COVID-19 lockdown in Ireland: a natural experiment allowing analysis of data from the prior two decades. MedRxiv. 2020:2020.06. 03.20121442.
626. Strömmer S, Lawrence W, Rose T, Vogel C, Watson D, Bottell JN, et al. Improving recruitment to clinical trials during pregnancy: a mixed methods investigation. Social Science & Medicine. 2018;200:73-82.
627. Lindsay KL, Brennan L, McAuliffe FM. Acceptability of and compliance with a probiotic capsule intervention in pregnancy. International Journal of Gynecology and Obstetrics. 2014;3(125):279-80.
628. Townsend R, Chmielewska B, Barratt I, Kalafat E, van der Meulen J, Gurol-Urganci I, et al. Global changes in maternity care provision during the COVID-19 pandemic: a systematic review and meta-analysis. EClinicalMedicine. 2021;37.
629. McMillan A, Rulisa S, Gloor GB, Macklaim JM, Sumarah M, Reid G. Pilot assessment of probiotics for pregnant women in Rwanda. PLoS One. 2018;13(6):e0195081.
630. Shahiwala A. Formulation approaches in enhancement of patient compliance to oral drug therapy. Expert opinion on drug delivery. 2011;8(11):1521-9.
631. Merabet J, Thompson D, Saul Levinson R. Advancing vaginal drug delivery. Expert opinion on drug delivery. 2005;2(4):769-77.
632. O'brien J, Adair C, Lewis D, Hall D, Defranco E, Fusey S, et al. Progesterone vaginal gel for the reduction of recurrent preterm birth: primary results from a randomized, double-blind, placebo-controlled trial. Ultrasound in Obstetrics and Gynecology. 2007;30(5):687-96.
633. Coomarasamy A, Harb HM, Devall AJ, Cheed V, Roberts TE, Goranitis I, et al. Progesterone to prevent miscarriage in women with early pregnancy bleeding: the PRISM RCT. Health Technology Assessment (Winchester, England). 2020;24(33):1.
634. Khaskheli M, Baloch S, Baloch AS, Shah SGS. Vaginal discharge during pregnancy and associated adverse maternal and perinatal outcomes. Pakistan journal of medical sciences. 2021;37(5):1302.

635. Piette PC. The pharmacodynamics and safety of progesterone. Best Practice & Research Clinical Obstetrics & Gynaecology. 2020;69:13-29.
636. Wada K, Uehara S, Ishii A, Sadahira T, Yamamoto M, Mitsuhata R, et al. A Phase II clinical trial evaluating the preventive effectiveness of lactobacillus vaginal suppositories in patients with recurrent cystitis. Acta Medica Okayama. 2016;70(4):299-302.
637. Dugoua J-J, Machado M, Zhu X, Chen X, Koren G, Einarson TR. Probiotic safety in pregnancy: a systematic review and meta-analysis of randomized controlled trials of Lactobacillus, Bifidobacterium, and Saccharomyces spp. Journal of obstetrics and gynaecology Canada. 2009;31(6):542-52.
638. Lee J, Han J, Choi J, Ahn H, Lee S, Kim M, et al. Pregnancy outcome after exposure to the probiotic Lactobacillus in early pregnancy. Journal of Obstetrics and Gynaecology. 2012;32(3):227-9.
639. Sanders ME, Merenstein DJ, Ouwehand AC, Reid G, Salminen S, Cabana MD, et al. Probiotic use in at-risk populations. Journal of the American Pharmacists Association. 2016;56(6):680-6.
640. Villar-García J, Hernández JJ, Güerri-Fernández R, González A, Lerma E, Guelar A, et al. Effect of probiotics (Saccharomyces boulardii) on microbial translocation and inflammation in HIV-treated patients: a double-blind, randomized, placebo-controlled trial. JAIDS Journal of Acquired Immune Deficiency Syndromes. 2015;68(3):256-63.
641. Lee J-Y, Chu S-H, Jeon JY, Lee M-K, Park J-H, Lee D-C, et al. Effects of 12 weeks of probiotic supplementation on quality of life in colorectal cancer survivors: a double-blind, randomized, placebo-controlled trial. Digestive and Liver Disease. 2014;46(12):1126-32.
642. Marrazzo JM, Cook RL, Wiesenfeld HC, Murray PJ, Busse B, Krohn M, et al. Women's satisfaction with an intravaginal Lactobacillus capsule for the treatment of bacterial vaginosis. Journal of Women's Health. 2006;15(9):1053-60.
643. Baldwin EA, Walther-Antonio M, MacLean AM, Gohl DM, Beckman KB, Chen J, et al. Persistent microbial dysbiosis in preterm premature rupture of membranes from onset until delivery. PeerJ. 2015;3:e1398.
644. Veščičík P, Musilová K, Stráník J, Štěpán M, Kacerovský M. Lactobacillus crispatus dominant vaginal microbita in pregnancy. Ceska gynekologie. 2020;85(1):67-70.
645. Ciandrini E, Campana R, Casettari L, Perinelli DR, Fagioli L, Manti A, et al. Characterization of biosurfactants produced by Lactobacillus spp. and their activity against oral streptococci biofilm. Applied microbiology and biotechnology. 2016;100:6767-77.
646. Coman M, Verdenelli M, Cecchini C, Silvi S, Orpianesi C, Caspani M, et al. In vitro evaluation on HeLa cells of protective mechanisms of probiotic lactobacilli against Candida clinical isolates. Journal of applied microbiology. 2015;119(5):1383-90.
647. Kyrgiou M, Valasoulis G, Stasinou S-M, Founta C, Athanasiou A, Bennett P, et al. Proportion of cervical excision for cervical intraepithelial neoplasia as a predictor of pregnancy outcomes. International Journal of Gynecology & Obstetrics. 2015;128(2):141-7.
648. Mitra A, MacIntyre DA, Lee Y, Smith A, Marchesi JR, Lehne B, et al. Cervical intraepithelial neoplasia disease progression is associated with increased vaginal microbiome diversity. Scientific reports. 2015;5(1):16865.
649. Zhang H, Lu J, Lu Y, Cai Q, Liu H, Xu C. Cervical microbiome is altered in cervical intraepithelial neoplasia after loop electrosurgical excision procedure in china. Scientific Reports. 2018;8(1):4923.

650. Khalil A, Von Dadelszen P, Draycott T, Ugwumadu A, O'Brien P, Magee L. Change in the incidence of stillbirth and preterm delivery during the COVID-19 pandemic. Jama. 2020;324(7):705-6.
651. Marques-Fernandez L, Sharma S, Mannu U, Chong HP. Impact of Covid-19 on attendances for a 1st episode of reduced fetal movements: A retrospective observational study. Plos one. 2021;16(6):e0253796.
652. Calvert C, Brockway M, Zoega H, Miller JE, Been JV, Amegah AK, et al. Changes in preterm birth and stillbirth during COVID-19 lockdowns in 26 countries. Nature human behaviour. 2023;7(4):529-44.
653. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UM, Zhong X, et al. Temporal dynamics of the human vaginal microbiota. Science translational medicine. 2012;4(132):132ra52-ra52.
654. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. Scientific reports. 2015;5(1):8988.
655. Ngugi BM, Hemmerling A, Bukusi EA, Kikuvi G, Gikunju J, Shiboski S, et al. Effects of BV-associated bacteria and sexual intercourse on vaginal colonization with the probiotic Lactobacillus crispatus CTV-05. Sexually transmitted diseases. 2011;38(11):1020.
656. Weinstock GM. Genomic approaches to studying the human microbiota. Nature. 2012;489(7415):250-6.
657. Hilton SK, Castro-Nallar E, Pérez-Losada M, Toma I, McCaffrey TA, Johnson WE, et al. Metataxonomic and metagenomic approaches vs. culture-based techniques for clinical pathology. Frontiers in microbiology. 2016;7:184416.
658. Kwok L, Stapleton AE, Stamm WE, Hillier SL, Wobbe CL, Gupta K. Adherence of Lactobacillus crispatus to vaginal epithelial cells from women with or without a history of recurrent urinary tract infection. The Journal of urology. 2006;176(5):2050-4.
659. Onderdonk A, Zamarchi G, Walsh J, Mellor R, Munoz A, Kass E. Methods for quantitative and qualitative evaluation of vaginal microflora during menstruation. Applied and environmental microbiology. 1986;51(2):333-9.
660. Wilks M, Tabaqchali S. Quantitative bacteriology of the vaginal flora during the menstrual cycle. Journal of medical microbiology. 1987;24(3):241-5.
661. Kaur H, Merchant M, Haque MM, Mande SS. Crosstalk between female gonadal hormones and vaginal microbiota across various phases of women's gynecological lifecycle. Frontiers in microbiology. 2020;11:503746.
662. Roberts SA, Brabin L, Diallo S, Gies S, Nelson A, Stewart C, et al. Mucosal lactoferrin response to genital tract infections is associated with iron and nutritional biomarkers in young Burkinabé women. European journal of clinical nutrition. 2019;73(11):1464-72.
663. Akinbiyi A, Watson R, Feyi-Waboso P. Prevalence of Candida albicans and bacterial vaginosis in asymptomatic pregnant women in South Yorkshire, United Kingdom: outcome of a prospective study. Archives of gynecology and obstetrics. 2008;278:463-6.
664. Redondo-Lopez V, Cook RL, Sobel JD. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Reviews of infectious diseases. 1990;12(5):856-72.
665. Reid G, Bruce AW, McGroarty JA, Cheng K, Costerton JW. Is there a role for lactobacilli in prevention of urogenital and intestinal infections? Clinical microbiology reviews. 1990;3(4):335-44.

666. Rybal'chenko O, Bondarenko V, Orlova O, Gusleva O, Larionov I, Fialkina S. Disorganization of biofilms of clinical strains of staphylococci by metabolites of lactobacilli. Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii. 2010(6):66-70.
667. Tuma PL, Hubbard AL. Transcytosis: crossing cellular barriers. Physiological reviews. 2003;83(3):871-932.
668. Atassi F, Brassart D, Grob P, Graf F, Servin AL. Lactobacillus strains isolated from the vaginal microbiota of healthy women inhibit Prevotella bivia and Gardnerella vaginalis in coculture and cell culture. FEMS Immunology & Medical Microbiology. 2006;48(3):424-32.
669. McLEAN NW, Rosenstein IJ. Characterisation and selection of a Lactobacillus species to re-colonise the vagina of women with recurrent bacterial vaginosis. Journal of medical microbiology. 2000;49(6):543-52.
670. Kalia N, Singh J, Kaur M. Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. Annals of clinical microbiology and antimicrobials. 2020;19(1):1-19.
671. Tortelli BA, Lewis WG, Allsworth JE, Member-Meneh N, Foster LR, Reno HE, et al. Associations between the vaginal microbiome and Candida colonization in women of reproductive age. American journal of obstetrics and gynecology. 2020;222(5):471. e1-. e9.
672. Zhou X, Westman R, Hickey R, Hansmann MA, Kennedy C, Osborn TW, et al. Vaginal microbiota of women with frequent vulvovaginal candidiasis. Infection and immunity. 2009;77(9):4130-5.
673. Larsen B, Galask R. Influence of estrogen and normal flora on vaginal candidiasis in the rat. The Journal of reproductive medicine. 1984;29(12):863-8.
674. Noverr MC, Huffnagle GB. Regulation of Candida albicans morphogenesis by fatty acid metabolites. Infection and immunity. 2004;72(11):6206-10.
675. Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC, 2010. Department of Health and Human Services, Centers for Disease Control and …; 2010.
676. Marziali G, Foschi C, Parolin C, Vitali B, Marangoni A. In-vitro effect of vaginal lactobacilli against group B Streptococcus. Microbial pathogenesis. 2019;136:103692.
677. Zárate G, Nader-Macias M. Influence of probiotic vaginal lactobacilli on in vitro adhesion of urogenital pathogens to vaginal epithelial cells. Letters in Applied Microbiology. 2006;43(2):174-80.
678. Sharpe M, Shah V, Freire-Lizama T, Cates EC, McGrath K, David I, et al. Effectiveness of oral intake of Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 on Group B Streptococcus colonization during pregnancy: a midwifery-led double-blind randomized controlled pilot trial. The Journal of Maternal-Fetal & Neonatal Medicine. 2021;34(11):1814-21.
679. Olsen P, Williamson M, Traynor V, Georgiou C. The impact of oral probiotics on vaginal Group B Streptococcal colonisation rates in pregnant women: A pilot randomised control study. Women and Birth. 2018;31(1):31-7.
680. Di Pierro F, Parolari A, Brundu B, Nigro R. Positive clinical outcomes derived from using a proprietary mixture of selected strains during pregnancy. Acta Bio Medica: Atenei Parmensis. 2016;87(3):259.
681. O'Hanlon DE, Gajer P, Brotman RM, Ravel J. Asymptomatic bacterial vaginosis is associated with depletion of mature superficial cells shed from the vaginal epithelium. Frontiers in cellular and infection microbiology. 2020;10:106.

682. Muenzner P, Bachmann V, Zimmermann W, Hentschel J, Hauck CR. Human-restricted bacterial pathogens block shedding of epithelial cells by stimulating integrin activation. Science. 2010;329(5996):1197-201.
683. Vornhagen J, Armistead B, Santana-Ufret V, Gendrin C, Merillat S, Coleman M, et al. Group B streptococcus exploits vaginal epithelial exfoliation for ascending infection. The Journal of clinical investigation. 2018;128(5):1985-99.
684. Amegashie CP, Gilbert NM, Peipert JF, Allsworth JE, Lewis WG, Lewis AL. Relationship between nugent score and vaginal epithelial exfoliation. PLoS One. 2017;12(5):e0177797.
685. Machado D, Castro J, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Cerca N. Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. Frontiers in microbiology. 2016;6:172812.
686. Hemmerling A, Harrison WG, Brown JM, Moscicki B, Oziemkowska M, Bukusi EA, et al. Trypan blue staining to determine vaginal exposure in two types of plastic vaginal applicators containing two different microbicide formulations. Sexually transmitted diseases. 2012;39(9):710-2.
687. Stapleton AE. The vaginal microbiota and urinary tract infection. Microbiology spectrum. 2016;4(6):10.1128/microbiolspec. uti-0025-2016.
688. Hooton TM, Fennell CL, Clark AM, Stamm WE. Nonoxynol-9: differential antibacterial activity and enhancement of bacterial adherence to vaginal epithelial cells. Journal of Infectious Diseases. 1991;164(6):1216-9.
689. Schwebke JR, Richey CM, Weiss HL. Correlation of behaviors with microbiological changes in vaginal flora. The Journal of infectious diseases. 1999;180(5):1632-6.
690. Vallor AC, Antonio MA, Hawes SE, Hillier SL. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. The Journal of infectious diseases. 2001;184(11):1431-6.
691. Brotman RM, He X, Gajer P, Fadrosh D, Sharma E, Mongodin EF, et al. Association between cigarette smoking and the vaginal microbiota: a pilot study. BMC infectious diseases. 2014;14(1):1-11.
692. Nelson TM, Borgogna J, Michalek R, Roberts DW, Rath J, Glover E, et al. Cigarette smoking is associated with an altered vaginal tract metabolomic profile. Scientific reports. 2018;8(1):852.
693. Chopyk J, Bojanowski CM, Shin J, Moshensky A, Fuentes AL, Bonde SS, et al. Compositional differences in the oral microbiome of e-cigarette users. Frontiers in Microbiology. 2021;12:599664.
694. Caldwell J, Matson A, Mosha M, Hagadorn JI, Moore J, Brownell E. Maternal H-antigen secretor status is an early biomarker for potential preterm delivery. Journal of Perinatology. 2021;41(9):2147-55.
695. Blackwell C, Jonsdottir K, Hanson M, Todd W, Chaudhuri A, Mathew B, et al. Non-secretion of ABO antigens predisposing to infection by Neisseria meningitidis and Streptococcus pneumoniae. The Lancet. 1986;328(8501):284-5.
696. Kundu S, Lee Y, Sykes L, Chan D, Lewis H, Brown R, et al. The effect of secretor status and the vaginal microbiome on birth outcome. medRxiv. 2021:2021.11. 24.21266804.
697. Mountain K, MacIntyre D, Chan D, Hyde A, Pasint-Magyar J, Lee Y, et al. ABO blood group antigens and preterm birth risk. Journal of Reproductive Immunology. 2023;159:104083.
698. Squadrito FJ, del Portal D. Nitrofurantoin. StatPearls [Internet]: StatPearls Publishing; 2022.

699. Akhavan BJ, Khanna NR, Vijhani P. Amoxicillin. 2018.
700. Danielsen M, Wind A. Susceptibility of Lactobacillus spp. to antimicrobial agents. International journal of food microbiology. 2003;82(1):1-11.
701. Sorbara MT, Pamer EG. Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. Mucosal immunology. 2019;12(1):1-9.
702. Sonnenburg ED, Zheng H, Joglekar P, Higginbottom SK, Firbank SJ, Bolam DN, et al. Specificity of polysaccharide use in intestinal bacteroides species determines diet-induced microbiota alterations. Cell. 2010;141(7):1241-52.
703. Sonnenburg ED, Smits SA, Tikhonov M, Higginbottom SK, Wingreen NS, Sonnenburg JL. Diet-induced extinctions in the gut microbiota compound over generations. Nature. 2016;529(7585):212-5.
704. Bookstaver PB, Bland CM, Griffin B, Stover KR, Eiland LS, McLaughlin M. A review of antibiotic use in pregnancy. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2015;35(11):1052-62.
705. Romero R, Chaiworapongsa T, Kuivaniemi H, Tromp G. Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic epidemiology in the prevention of preterm birth. American Journal of Obstetrics & Gynecology. 2004;190(6):1509-19.
706. Daskalakis GJ, Karambelas AK. Vaginal probiotic administration in the management of preterm premature rupture of membranes. Fetal Diagnosis and Therapy. 2017;42(2):92-8.
707. Spiegel C. Lactobacillus dose required to restore and maintain a normal vaginal flora. Am J Obstet Gynecol. 1989;27(3 Pt 52):212-91.
708. Reid G, Bruce AW, Fraser N, Heinemann C, Owen J, Henning B. Oral probiotics can resolve urogenital infections. FEMS Immunology & Medical Microbiology. 2001;30(1):49-52.
709. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. Journal of Infectious Diseases. 1999;180(6):1950-6.
710. Committee EFSAS. Scientific opinion: The maintenance of the list of QPS microorganisms intentionally added to food or feed. Scientific Opinion of the Panel on Biological Hazards (Question No EFSA-Q-2008-006). The EFSA Journal. 2008;923:1-48.
711. Vesty A, Gear K, Biswas K, Mackenzie BW, Taylor MW, Douglas RG. Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer. Supportive Care in Cancer. 2020;28:2683-91.
712. Leiby JS, McCormick K, Sherrill-Mix S, Clarke EL, Kessler LR, Taylor LJ, et al. Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. Microbiome. 2018;6:1-11.
713. Kuperman A, Zimmerman A, Hamadia S, Ziv O, Gurevich V, Fichtman B, et al. Deep microbial analysis of multiple placentas shows no evidence for a placental microbiome. BJOG: An International Journal of Obstetrics & Gynaecology. 2020;127(2):159-69.
714. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC biology. 2014;12:1-12.
715. de Goffau MC, Lager S, Salter SJ, Wagner J, Kronbichler A, Charnock-Jones DS, et al. Recognizing the reagent microbiome. Nature microbiology. 2018;3(8):851-3.
716. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics. 2010;26(6):715-21.

717. Madan JC, Salari RC, Saxena D, Davidson L, O'Toole GA, Moore JH, et al. Gut microbial colonisation in premature neonates predicts neonatal sepsis. Archives of Disease in Childhood-Fetal and Neonatal Edition. 2012.
718. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? Research in microbiology. 2008;159(3):187-93.
719. Collado MC, Rautava S, Aakko J, Isolauri E, Salminen S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Scientific reports. 2016;6(1):1-13.
720. Matsumiya Y, Kato N, Watanabe K, Kato H. Molecular epidemiological study of vertical transmission of vaginal Lactobacillus species from mothers to newborn infants in Japanese, by arbitrarily primed polymerase chain reaction. Journal of infection and chemotherapy. 2002;8(1):43-9.
721. Amabebe E, Anumba DO. Female gut and genital tract microbiota-induced crosstalk and differential effects of short-chain fatty acids on immune sequelae. Frontiers in Immunology. 2020;11:553047.
722. Gritz EC, Bhandari V. The human neonatal gut microbiome: a brief review. Frontiers in pediatrics. 2015;3:17.
723. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences. 2010;107(26):11971-5.
724. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature. 2018;562(7728):583-8.
725. Jost T, Lacroix C, Braegger CP, Chassard C. New insights in gut microbiota establishment in healthy breast fed neonates. 2012.
726. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. Early human development. 2010;86(1):13-5.
727. Marques TM, Wall R, Ross RP, Fitzgerald GF, Ryan CA, Stanton C. Programming infant gut microbiota: influence of dietary and environmental factors. Current opinion in biotechnology. 2010;21(2):149-56.
728. Pflughoeft KJ, Versalovic J. Human microbiome in health and disease. Annual Review of Pathology: Mechanisms of Disease. 2012;7:99-122.
729. Kuppala VS, Meinzen-Derr J, Morrow AL, Schibler KR. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. The Journal of pediatrics. 2011;159(5):720-5.
730. Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O'leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. Nature medicine. 2014;20(5):524-30.
731. Odendaal J, Black N, Bennett PR, Brosens J, Quenby S, MacIntyre DA. The endometrial microbiota and early pregnancy loss. Human Reproduction. 2024:dead274.
732. Bradford LL, Ravel J. The vaginal mycobiome: A contemporary perspective on fungi in women's health and diseases. Virulence. 2017;8(3):342-51.
733. Cardenas I, Mor G, Aldo P, Lang SM, Stabach P, Sharp A, et al. Placental viral infection sensitizes to endotoxin-induced pre-term labor: a double hit hypothesis. American journal of reproductive immunology. 2011;65(2):110-7.

734. Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. Biochemical and biophysical research communications. 2016;469(4):967-77.
735. Stafford GP, Parker JL, Amabebe E, Paley M, Anumba DO. Spontaneous preterm birth is associated with differential expression of vaginal metabolites by lactobacilli-dominated microflora. Frontiers in physiology. 2017;8:282576.
736. Pruski P, Correia GD, Lewis HV, Capuccini K, Inglese P, Chan D, et al. Direct on-swab metabolic profiling of vaginal microbiome host interactions during pregnancy and preterm birth. Nature Communications. 2021;12(1):5967.
737. Saito S. Cytokine network at the feto-maternal interface. Journal of reproductive immunology. 2000;47(2):87-103.
738. Schmid M, Haslinger P, Stary S, Leipold H, Egarter C, Grimm C. Interleukin-1 beta gene polymorphisms and preterm birth. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2012;165(1):33-6.
739. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. Nutrition reviews. 2007;65(suppl_3):S194-S202.
740. Vilotić A, Nacka-Aleksić M, Pirković A, Bojić-Trbojević Ž, Dekanski D, Jovanović Krivokuća M. IL-6 and IL-8: An overview of their roles in healthy and pathological pregnancies. International Journal of Molecular Sciences. 2022;23(23):14574.
741. Lockwood CJ, Arcuri F, Toti P, De Felice C, Krikun G, Guller S, et al. Tumor necrosis factor-α and interleukin-1β regulate interleukin-8 expression in third trimester decidual cells: implications for the genesis of chorioamnionitis. The American journal of pathology. 2006;169(4):1294-302.
742. Romero R, Sepulveda W, Mazor M, Brandt F, Cotton DB, Dinarello CA, et al. The natural interleukin-1 receptor antagonist in tem and preterm parturition. American journal of obstetrics and gynecology. 1992;167(4):863-72.
743. Amabebe E, Chapman DR, Stern VL, Stafford G, Anumba DO. 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. 2018;126:1-10.
744. Matsushima K, Yang D, Oppenheim JJ. Interleukin-8: An evolving chemokine. Cytokine. 2022;153:155828.
745. Chan L-P, Liu C, Chiang F-Y, Wang L-F, Lee K-W, Chen W-T, et al. IL-8 promotes inflammatory mediators and stimulates activation of p38 MAPK/ERK-NF-κB pathway and reduction of JNK in HNSCC. Oncotarget. 2017;8(34):56375.
746. Wu D, LaRosa GJ, Simon M. G protein-coupled signal transduction pathways for interleukin-8. Science. 1993;261(5117):101-3.
747. Baggiolini M, Walz A, Kunkel S. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. The Journal of clinical investigation. 1989;84(4):1045-9.
748. Jones SA, Jenkins BJ. Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. Nature reviews immunology. 2018;18(12):773-89.
749. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochemical journal. 2003;374(1):1-20.

750. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood, The Journal of the American Society of Hematology. 2010;115(11):2167-76.
751. Tang M-X, Hu X-H, Liu Z-Z, Kwak-Kim J, Liao A-H. What are the roles of macrophages and monocytes in human pregnancy? Journal of reproductive immunology. 2015;112:73-80.
752. Pandey M, Awasthi S, Singh U, Mahdi AA. Association of IL-10 gene polymorphism (− 819C> T,− 592C> A and− 1082G> A) with preterm birth. The Indian Journal of Pediatrics. 2018;85:93-101.
753. Belousova V, Svitich O, Timokhina E, Strizhakov A, Bogomazova I. Polymorphism of the IL-1β, TNF, IL-1RA and IL-4 cytokine genes significantly increases the risk of preterm birth. Biochemistry (Moscow). 2019;84:1040-6.
754. Ruiz R, Jallo N, Murphey C, Marti C, Godbold E, Pickler R. Second trimester maternal plasma levels of cytokines IL-1Ra, Il-6 and IL-10 and preterm birth. Journal of Perinatology. 2012;32(7):483-90.
755. Nguyen PV, Kafka JK, Ferreira VH, Roth K, Kaushic C. Innate and adaptive immune responses in male and female reproductive tracts in homeostasis and following HIV infection. Cellular & molecular immunology. 2014;11(5):410-27.
756. Takeda K, Akira S. Toll-like receptors in innate immunity. International immunology. 2005;17(1):1-14.
757. Anton L, Sierra L-J. Common cervicovaginal microbial supernatants alter cervical epithelial function: mechanisms by which Lactobacillus crispatus contributes to cervical health. Frontiers in microbiology. 2018;9:395751.
758. Trifonova R, Doncel G, Fichorova R. Polyanionic microbicides modify Toll-like receptor-mediated cervicovaginal immune responses. Antimicrobial agents and chemotherapy. 2009;53(4):1490-500.
759. Fisette PL, Ram S, Andersen JM, Guo W, Ingalls RR. The Lip lipoprotein from Neisseria gonorrhoeae stimulates cytokine release and NF-κB activation in epithelial cells in a Toll-like receptor 2-dependent manner. Journal of Biological Chemistry. 2003;278(47):46252-60.
760. Doerflinger SY, Throop AL, Herbst-Kralovetz MM. Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. The Journal of infectious diseases. 2014;209(12):1989-99.
761. Bisanz JE, Seney S, McMillan A, Vongsa R, Koenig D, Wong L, et al. A systems biology approach investigating the effect of probiotics on the vaginal microbiome and host responses in a double blind, placebo-controlled clinical trial of post-menopausal women. PloS one. 2014;9(8):e104511.
762. Armstrong E, Hemmerling A, Miller S, Burke KE, Newmann SJ, Morris SR, et al. Sustained effect of LACTIN-V (Lactobacillus crispatus CTV-05) on genital immunology following standard bacterial vaginosis treatment: results from a randomised, placebo-controlled trial. The Lancet Microbe. 2022;3(6):e435-e42.
763. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72(1-2):248-54.
764. Rizzo A, Fiorentino M, Buommino E, Donnarumma G, Losacco A, Bevilacqua N. Lactobacillus crispatus mediates anti-inflammatory cytokine interleukin-10 induction in response to Chlamydia trachomatis infection in vitro. International Journal of Medical Microbiology. 2015;305(8):815-27.

765. Joo H-M, Hyun Y-J, Myoung K-S, Ahn Y-T, Lee J-H, Huh C-S, et al. Lactobacillus johnsonii HY7042 ameliorates Gardnerella vaginalis-induced vaginosis by killing Gardnerella vaginalis and inhibiting NF-κB activation. International immunopharmacology. 2011;11(11):1758-65.
766. Yang S, Li W, Challis JR, Reid G, Kim SO, Bocking AD. Probiotic Lactobacillus rhamnosus GR-1 supernatant prevents lipopolysaccharide-induced preterm birth and reduces inflammation in pregnant CD-1 mice. American Journal of Obstetrics and Gynecology. 2014;211(1):44. e1-. e12.
767. Happel A-U, Singh R, Mitchev N, Mlisana K, Jaspan HB, Barnabas SL, et al. Testing the regulatory framework in South Africa–a single-blind randomized pilot trial of commercial probiotic supplementation to standard therapy in women with bacterial vaginosis. BMC infectious diseases. 2020;20:1-13.
768. Vitali B, Cruciani F, Baldassarre ME, Capursi T, Spisni E, Valerii MC, et al. Dietary supplementation with probiotics during late pregnancy: outcome on vaginal microbiota and cytokine secretion. BMC microbiology. 2012;12:1-14.
769. Chetwin E, Manhanzva MT, Abrahams AG, Froissart R, Gamieldien H, Jaspan H, et al. Antimicrobial and inflammatory properties of South African clinical Lactobacillus isolates and vaginal probiotics. Scientific reports. 2019;9(1):1917.
770. Hemalatha R, Mastromarino P, Ramalaxmi B, Balakrishna N, Sesikeran B. Effectiveness of vaginal tablets containing lactobacilli versus pH tablets on vaginal health and inflammatory cytokines: a randomized, double-blind study. European journal of clinical microbiology & infectious diseases. 2012;31:3097-105.
771. Nagarajan UM, Sikes JD, Yeruva L, Prantner D. Significant role of IL-1 signaling, but limited role of inflammasome activation, in oviduct pathology during Chlamydia muridarum genital infection. The Journal of Immunology. 2012;188(6):2866-75.
772. Butler DS, Silvestroni A, Stapleton AE. Cytoprotective effect of Lactobacillus crispatus CTV-05 against uropathogenic E. coli. MDPI; 2016.
773. Anton L, Ferguson B, Friedman ES, Gerson KD, Brown AG, Elovitz MA. Gardnerella vaginalis alters cervicovaginal epithelial cell function through microbe-specific immune responses. Microbiome. 2022;10(1):119.
774. Agace W, Hedges S, Andersson U, Andersson J, Ceska M, Svanborg C. Selective cytokine production by epithelial cells following exposure to Escherichia coli. Infection and immunity. 1993;61(2):602-9.
775. Winkler M, Fischer D-C, Ruck P, Marx T, Kaiserling E, Oberpichler A, et al. Parturition at term: parallel increases in interleukin-8 and proteinase concentrations and neutrophil count in the lower uterine segment. Human Reproduction. 1999;14(4):1096-100.
776. Florova V, Romero R, Tarca AL, Galaz J, Motomura K, Ahmad MM, et al. Vaginal host immune-microbiome interactions in a cohort of primarily African-American women who ultimately underwent spontaneous preterm birth or delivered at term. Cytokine. 2021;137:155316.
777. Imlay JA. Diagnosing oxidative stress in bacteria: not as easy as you might think. Current opinion in microbiology. 2015;24:124-31.
778. Nunn KL, Wang Y-Y, Harit D, Humphrys MS, Ma B, Cone R, et al. Enhanced trapping of HIV-1 by human cervicovaginal mucus is associated with Lactobacillus crispatus-dominant microbiota. MBio. 2015;6(5):10.1128/mbio. 01084-15.

779. Yoon BH, Romero R, Park JS, Kim CJ, Kim SH, Choi J-H, et al. Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. American journal of obstetrics and gynecology. 2000;182(3):675-81.
780. Romero R, Yoon BH, Mazor M, Gomez R, Gonzalez R, Diamond MP, et al. A comparative study of the diagnostic performance of amniotic fluid glucose, white blood cell count, interleukin-6, and gram stain in the detection of microbial invasion in patients with preterm premature rupture of membranes. American journal of obstetrics and gynecology. 1993;169(4):839-51.
781. Vousden N, Chandiramani M, Seed P, Shennan A. Interleukin-6 bedside testing in women at high risk of preterm birth. The Journal of Maternal-Fetal & Neonatal Medicine. 2011;24(10):1301-4.
782. El-Ghazaly TE, Abdelazim IA, Elshabrawy A. Interleukin-6 bedside test in detecting chorioamnionitis in women with preterm premature rupture of fetal membranes. Ginekologia Polska. 2022;93(10):835-41.
783. Buxton MA, Meraz-Cruz N, Sanchez BN, Foxman B, Castillo-Castrejon M, O'Neill MS, et al. Timing of Cervico-Vaginal Cytokine Collection during pregnancy and Preterm Birth: a comparative analysis in the PRINCESA Cohort. International Journal of Environmental Research and Public Health. 2021;18(7):3436.
784. Köck K, Köck F, Klein K, Bancher-Todesca D, Helmer H. Diabetes mellitus and the risk of preterm birth with regard to the risk of spontaneous preterm birth. The Journal of Maternal-Fetal & Neonatal Medicine. 2010;23(9):1004-8.
785. Liu L, Wang L, Yang W, Ni W, Jin L, Liu J, et al. Gestational hypertension and pre-eclampsia and risk of spontaneous premature rupture of membranes: A population-based cohort study. International Journal of Gynecology & Obstetrics. 2019;147(2):195-201.
786. Dutt R, Raker C, Anderson BL. Ethnic variations in cervical cytokine concentrations and vaginal flora during pregnancy. American Journal of Reproductive Immunology. 2015;73(2):141-50.
787. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P, et al. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. The ISME journal. 2007;1(2):121-33.
788. Yavari Kia P, Baradaran B, Shahnazi M, Asghari Jafarabadi M, Khaze V, Pourasad Shahrak S. Maternal serum and cervicovaginal IL-6 in patients with symptoms of preterm labor. Iranian Journal of Immunology. 2016;13(3):229-36.
789. Wilczyński JR, Tchorzewski H, Głowacka E, Banasik M, Lewkowicz P, Szpakowski M, et al. Cytokine secretion by decidual lymphocytes in transient hypertension of pregnancy and pre-eclampsia. Mediators of Inflammation. 2002;11:105-11.
790. Spear GT, Kendrick SR, Chen HY, Thomas TT, Bahk M, Balderas R, et al. Multiplex immunoassay of lower genital tract mucosal fluid from women attending an urban STD clinic shows broadly increased IL1ß and lactoferrin. PloS one. 2011;6(5):e19560.
791. Agrawal T, Vats V, Wallace PK, Salhan S, Mittal A. Cervical cytokine responses in women with primary or recurrent chlamydial infection. Journal of Interferon & Cytokine Research. 2007;27(3):221-6.
792. Simhan HN, Caritis SN, Hillier SL, Krohn MA. Cervical anti-inflammatory cytokine concentrations among first-trimester pregnant smokers. American journal of obstetrics and gynecology. 2005;193(6):1999-2003.

793. Rametse CL, Adefuye AO, Olivier AJ, Curry L, Gamieldien H, Burgers WA, et al. Inflammatory cytokine profiles of semen influence cytokine responses of cervicovaginal epithelial cells. Frontiers in immunology. 2018;9:2721.
794. Ryckman KK, Williams SM, Kalinka J. Correlations of selected vaginal cytokine levels with pregnancy-related traits in women with bacterial vaginosis and mycoplasmas. Journal of reproductive immunology. 2008;78(2):172-80.
795. Xiao M, Lu B, Ding R, Liu X, Wu X, Li Y, et al. Metatranscriptomic analysis of host response and vaginal microbiome of patients with severe COVID-19. Science China Life Sciences. 2022;65(7):1473-6.
796. Buxton MA, Meraz-Cruz N, Sanchez BN, Gronlund CJ, Foxman B, Vadillo-Ortega F, et al. Air pollution and inflammation: Findings from concurrent repeated measures of systemic and reproductive tract cytokines during term pregnancy in Mexico City. Science of the total environment. 2019;681:235-41.
797. NHS. NHS Long Term Plan https://www.longtermplan.nhs.uk/, accessed Jan 2023.
798. Ouwehand AC, Invernici MM, Furlaneto FA, Messora MR. Effectiveness of multi-strain versus single-strain probiotics: current status and recommendations for the future. Journal of clinical gastroenterology. 2018;52:S35-S40.
799. Chaemsaithong P, Romero R, Korzeniewski SJ, Martinez-Varea A, Dong Z, Yoon BH, et al. A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. The Journal of Maternal-Fetal & Neonatal Medicine. 2016;29(3):349-59.
800. Gottschick C, Szafranski SP, Kunze B, Sztajer H, Masur C, Abels C, et al. Screening of compounds against Gardnerella vaginalis biofilms. PloS one. 2016;11(4):e0154086.
801. Gottschick C, Deng Z-L, Vital M, Masur C, Abels C, Pieper DH, et al. Treatment of biofilms in bacterial vaginosis by an amphoteric tenside pessary-clinical study and microbiota analysis. Microbiome. 2017;5:1-15.
802. Sousa LG, Pereira SA, Cerca N. Fighting polymicrobial biofilms in bacterial vaginosis. Microbial Biotechnology. 2023;16(7):1423-37.
803. Haahr T, Zacho J, Bräuner M, Shathmigha K, Skov Jensen J, Humaidan P. Reproductive outcome of patients undergoing in vitro fertilisation treatment and diagnosed with bacterial vaginosis or abnormal vaginal microbiota: a systematic PRISMA review and meta-analysis. BJOG: An International Journal of Obstetrics & Gynaecology. 2019;126(2):200-7.
804. Grewal K, Lee YS, Smith A, Brosens JJ, Bourne T, Al-Memar M, et al. Chromosomally normal miscarriage is associated with vaginal dysbiosis and local inflammation. BMC medicine. 2022;20(1):38.
805. Lev-Sagie A, Goldman-Wohl D, Cohen Y, Dori-Bachash M, Leshem A, Mor U, et al. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. Nature medicine. 2019;25(10):1500-4.
806. Wrønding T, Vomstein K, Bosma EF, Mortensen B, Westh H, Heintz JE, et al. Antibiotic-free vaginal microbiota transplant with donor engraftment, dysbiosis resolution and live birth after recurrent pregnancy loss: a proof of concept case study. EClinicalMedicine. 2023;61.
807. Deshpande G, Jape G, Rao S, Patole S. Benefits of probiotics in preterm neonates in low-income and medium-income countries: a systematic review of randomised controlled trials. BMJ open. 2017;7(12):e017638.

808. Ali SA, Ali SA, Suhail N. Importance of storing medicines on required temperature in pharmacies and role of community pharmacies in rural areas: literature review. i-Manager's Journal on Nursing. 2016;6(2):32.
809. HUGHES VL, HILLIER SL. Microbiologic characteristics of Lactobacillus products used for colonization of the vagina. Obstetrics & Gynecology. 1990;75(2):244-8.
810. Anisimova E, Gorokhova I, Karimullina G, Yarullina D. Alarming antibiotic resistance of lactobacilli isolated from probiotic preparations and dietary supplements. Antibiotics. 2022;11(11):1557.
811. Jackson SA, Schoeni JL, Vegge C, Pane M, Stahl B, Bradley M, et al. Improving end-user trust in the quality of commercial probiotic products. Frontiers in microbiology. 2019;10:739.

8 Appendix

8.1 Chapter 3 Appendices

Imperial College Healthcare NHS
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We are Interested in Your Opinion…

Who we are

We are a group of scientists and doctors interested in finding out why women sometimes deliver their baby prematurely and whether changing the type of bacteria in the vagina could reduce the risk of early birth. We are planning to start a new research study involving patients from the prematurity clinic and your opinion matters.

What is the purpose of the study?

Every woman has bacteria in her vagina. In the great majority of cases there is only one main species bacteria present, called *Lactobacillus*. *Lactobacillus* is the same type of organism found in yoghurts, milk-based health drinks and probiotics. We have discovered that a particular strain of *Lactobacillus*, called *Lactobacillus crispatus* helps to reduce the risk of preterm birth. As part of this study, research women will be offered a vaginal applicator containing *Lactobacillus crispatus*.

Questions for you…

1. Do you/have you ever taken probiotics? Yes □ No □

2. On a scale of 0-5, how likely would you be to take a probiotic during pregnancy to help reduce the risk of premature labour? (0 = very unlikely, 5 = very likely)

0 1 2 3 4 5

3. On a scale of 0-5, how likely would you be to take a vaginal probiotic (as opposed to oral) to help reduce the risk of premature labour? (0 = very unlikely, 5 = very likely)

0 1 2 3 4 5

4. On a scale of 0-5, how likely would you be to take a vaginal probiotic (as opposed to oral) as part of a research study during your pregnancy? (0 = very unlikely, 5 = very likely)

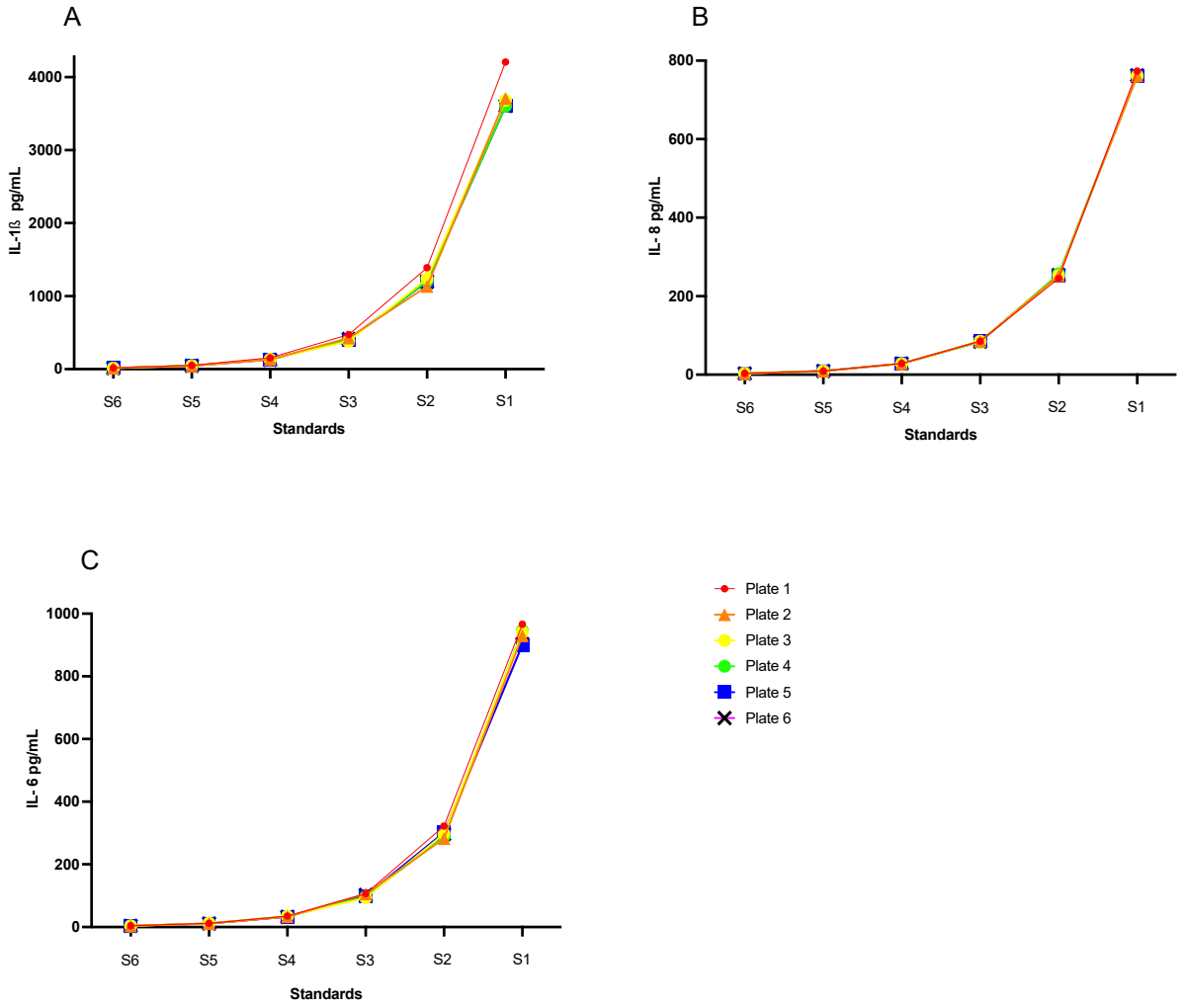
0 1 2 3 4 5

Appendix 3.1 – Pre-study questionnaire. A pre-study questionnaire was given to women in the PTB prevention clinic to gauge acceptability and willingness to participate in clinical research.

Commercial Probiotic	Mode of administration	Preparation	Bacteria
Biocare Intrafresh®	Vaginal	Wax pessary	*L. acidophilus* *L. gasseri*
Optibac (women)	Oral	Capsule	*L. paracasei* F-19® *L. reuteri* RC-14® *L. rhamnosus* GR-1®
Daye Proviotic	Oral or vaginal	Capsule	*L. plantarum* GLP3
Canesflor	Vaginal	Capsule	*L. plantarum* P17630 VI
Vacramal	Oral	Capsule	*L. acidophilus* *L. rhamnosus* *L. casei* *L. crispatus* *L. fermentum* *L. gasseri* *L. paracasei* *L. plantarum*
Vitaflora Wellgard	Oral	Capsule	*L. plantarum* *L. rhamnosus* *L. animalis*
Pro-ven Probiotics	Not specified	Capsule	*L. salivarius* (CUL61) *L. paracasei* (CUL08) *B. animalis subsp. Lactis* (CUL34) *B. bifidum* (CUL20)
Physioflor	Vaginal	Capsule	*L. crispatus* (IP174178)
Probioscience premium	Not specified	Capsule	*L. rhamnosus* *L. reuteri* *L. crispatus*
Orogyn	Oral	Capsule	*L. rhamnosus* Rosell®-11 *L. helveticus* Rosell®-52 *L. helveticus* Lafti® L10 *L. brevis* HA-112
Femibion	Oral	Capsule	*L. rhamnosus* GR-1 *L. reuteri* RC-14
Gynophilus	Vaginal	Tablet	*L. rhamnosus*
Hydralin flora	Vaginal	Capsule	*L. plantarum* P17630
Medigyn	Not specified	Capsule	*L. rhamnosus* (PB01)™ *L. gasseri* (EB01)™
Bactigyn	Vaginal	Capsule	*B. bifidum* W28 *L. acidophilus* W70 *L. brevis* W63 *L. helveticus* W74 *L. plantarum* W21 *L. salivarius* W24
Ecovag	Vaginal	Capsule	*L. rhamnosus* PB01™ *L. gasseri* EB01™
Ergyphilus	Oral	Capsule	*L. rhamnosus* GG ATCC 53103 *L. acidophilus* DSM 21717 *L. plantarum* LMG P-21021
Optibac (pregnancy)	Oral	Capsule	*L. rhamnosus* HN001 *L. acidophilus* La-14® *B. lactis* HN019

Appendix 3.2 – Commercially available probiotics. Oral and vaginal probiotics specifically marketed at improving 'vaginal health' were purchased. Products were stored according to manufacturer guidance. Bacterial species were recorded and if specified, strain recorded.

8.2 Chapter 5 Appendices



Appendix 5.1 – Merged plotting of standard curves across six plates for cytokine analytes (A) IL-1ß **(B)** IL-8 **(C)** IL-6