Characteristics of the Cervical Microbiome in Women with Cervical Insufficiency (CI) and its Role in Predicting the Successful Cerclage / Pessary Intervention

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Do not conform to the pattern of this world, but be transformed by the renewing of your mind. Then you will be able to test and approve what God's will is-his good, pleasing and perfect will.

Romans 12:2

格物致知，誠意正心，知行合一。

《大學》

Declaration of originality

The work contained in this thesis is the original research performed by the author in the Department of Obstetrics and Gynaecology, the Chinese University of Hong Kong. No part of the work described in this thesis has been, already been or is being currently submitted for any such degree, diploma or other qualifications.

Abstract of thesis entitled: Characteristics of the Cervical Microbiome in Women with Cervical Insufficiency (CI) and its Role in Predicting the Successful Cerclage / Pessary Intervention

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Abstract

Cervical insufficiency (CI) as asymptomatic cervical shortening or dilation in the second trimester of pregnancy presents in up to 1% of obstetric populations globally. Approximately 40% of pregnancies with CI might result in preterm birth. The current intervention, such as cerclage and pessary could mechanically strengthen cervix and prolong pregnancy to term birth with risk ratio (RR) 0.80 (95% Confidence Interval 0.69-0.95). However, around 30% of pregnancies with CI could not benefit from cervical intervention. This structural weakness of cervical tissue is also associated with the increased risk of ascending infection. Therefore, it was hypothesized that some microbiome colonization might exist before intervention in female lower genital tract and lead to intervention failure. It was proposed to assess the alteration of cervical microbiome before intervention in CI patients by applying 16S rRNA gene pyrosequencing and evaluating the possibility of cervical microbial signature as the predictor of successful intervention in CI population.

Firstly, the alteration of cervical microbiome in pregnant women with cervical insufficiency was explored. The cervical swab samples were collected in mid-

trimester (gestational age from 13^{+4} weeks to 25^{+3} weeks) before the intervention from a group of CI patients. Their outcomes were then followed up and they were divided into two groups according to the outcomes: i) CI patients with spontaneous preterm birth (CI_sPTB group, n = 10); ii) CI patients with term birth (CI_TB group, n = 15). Cervical swab samples were also collected prospectively at mid-trimester from a control group who had normal cervical length and delivered in term (Nm_TB group, n = 13). DNA was extracted from each sample of these three groups. 16S rRNA gene was PCR amplified and sequenced utilizing pyrosequencing. The abundances of over 9,600 culturable and non-culturable bacterial taxa were analyzed in each sample. The microbial biodiversity within-sample (alpha-diversity) and between-samples (beta-diversity) were also estimated. The community state type (CST) IV, which represents the pathogenic dysbiosis of microbial status, was found most frequent in the CI_sPTB group (50%, 5/10), rather than in the CI_TB group (6.7%, 1/15) and the Nm_TB group (7.7%, 1/13) (Kruskal-Wallis, $p = 0.028$). A correlation between the group types and the CST condition was also noticed (Spearman coefficient = - 0.330, $p = 0.043$). It implied that the dysbiosis existed in the CI_sPTB group rather than the CI_TB group. Both the cervical condition and CST status are associated with spontaneous preterm birth (sPTB) (Chi-square test, $p = 0.022$).

Secondly, specific cervical microbial signature as the predictor of successful intervention in CI population was evaluated. The relative abundance between the CI_sPTB group and the CI_TB group was compared. Seven bacterial taxa were identified significantly increased in the CI_sPTB group than the CI_TB group (Mann-Whitney, $p < 0.01$; False Discovery Rate < 5%). The potential clinical application of these differentially abundant taxa in predicting sPTB after cerclage /

pessary intervention was also discussed. We calculated an LA7 score, the total abundance of the seven selected taxa, for each patient. Using LA7 > 2.26 to define a positive result, we correctly identified the CI patients who resulted in “sPTB after intervention”, with sensitivity of 90% (9/10) and specificity of 93% (14/15). Compared with LA7-negative patients, the LA7-positive patients were found a shorter interval between intervention and delivery [median days, 10 days vs. 129 days; Log-rank test, $p < 0.0001$; Hazard Ratio, 5.74; 95% Confidence Interval, 1.59-20.7].

In summary, compared to those CI patients delivered term and control cases, the alteration of cervical microbiome was noticed in CI patients delivered spontaneous preterm birth. The cervical microbiome signature was identified as the predictor of successful intervention in pregnant women with CI, which might help screen patients and predict the prognosis of benefiting from cervical intervention.

摘　要

宮頸機能不全（CI）是孕中期無癥狀性的宮頸長度縮短或宮口擴張，見於高達 1%的產科人群。合併 CI 的妊娠有約 40%的風險發生早產。當前的干預手段，如宮頸環紮或宮頸托可機械性加固宮頸，延長妊娠直至足月（RR 0.80，95% Confidence Interval 0.69-0.95）。但是仍有約 30%的 CI 患者不能從中受益，仍然發生了早產。宮頸組織薄弱也與上行性感染風險升高相關。因此。本研究中我們假設系干預前已存在於下生殖道中的不明感染導致了干預失敗。我們擬行 16S rRNA 基因大規模並行焦磷酸測序技術，評估 CI 患者宮頸微生物群的改變，並評價利用宮頸特徵性微生物預測 CI 治療成功的可能性。

首先，研究探討了 CI 孕婦宮頸微生物群的改變。我們收集 CI 孕婦孕中期干預前（孕 13+4 – 25+3 周）的宮頸拭子樣本，隨訪並根據妊娠結局分為 1）CI 經干預後自發早產組（CI_sPTB, n = 10）、CI 經干預後足月分娩組（CI_TB, n = 15）。同時，研究亦納入孕中期宮頸長度正常且足月分娩的壹組孕婦作為對照組（Nm_TB, n = 13）並收集該組孕婦的孕中期宮頸拭子樣本。樣本抽提 DNA 後，對 16S rRNA 基因行 PCR 擴增，並行大規模並行測序（MPS）。該方法可分析超過 9600 種細菌的種屬和相對豐度，其中大部分系無法經傳統培養手段獲得的。我們比較了微生物群的樣本內生物多樣性（α-多樣性）和樣本間生物多樣性（β-多樣性），發現三個組中 CI_sPTB 組具有最大的生物多樣性，而 CI_TB 組和 Nm_TB 組的多樣性相當，這提示與 CI_TB 組相比，生物失調更見於 CI_sPTB 組。

其次，我們也評估了宮頸特徵性微生物用於 CI 干預效果預測的可能。經過比較 CI_sPTB 組和 CI_TB 組細菌的相對豐度，我們發現 CI_sPTB 組中有七種細菌種屬的相對豐度明顯升高 (Mann-Whitney, $p < 0.01$; FDR < 5%)，在此基礎上，建立 LA7 這壹指標用於干預效果的預測。結果發現當使用 LA7 > 2.26 作為陽性閾值時，檢出 CI_sPTB 的敏感性為 90%，特異性為 93%。與 LA7 陰性的 CI 患者相比，LA7 陽性患者的干預-分娩時間間隔更短 [中位天數, 10 天 vs. 129 天; Log-rank test, $p < 0.0001$; Hazard Ratio, 5.74; 95% Confidence Interval, 1.59-20.7]. 討論了干預後結局預測的潛在臨床應用可能。

總之，在經治療後仍早產的 CI 患者宮頸中存在微生物的改變。宮頸特徵性微生物可作為預測干預效果的預測因子，可有助於篩選真正受益於宮頸干預的 CI 患者並預測其預後。

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Abbreviations

Nucleotides:

A Adenine

T Thymine

C Cytosine

G Guanine

17-OHPC	17-hydroxyprogesterone caproate
AF	Amniotic fluid
ART	Assisted reproductive technology
AV	Aerobic vaginitis
AVF	Abnormal vaginal flora
BV	Bacterial vaginosis
BV-AA	Bacterial vaginosis-associated agents
CI	Cervical insufficiency
HIV	Human immunodeficiency virus
HMP	Human Microbiome Project
HPV	Human papillomavirus
IAI	Intrauterine infection
IL	Interleukin
JSD	Jensen-Shannon divergence
KEGG	Kyoto Encyclopedia of Genes and Genomes database
KO	KEGG orthologues
MIAC	Microbial invasion of the amniotic cavity
MDS	Multi-dimensional scaling
MID	Multiplex identifier
MPS	Massive Parallel Sequencing
RCT	Randomized Clinical Trial

RR	Risk ratio
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PTB	Preterm birth
PTL	Preterm labor
PROM	Premature rupture of membrane
PPROM	preterm premature rupture of membrane
sPTB	Spontaneous preterm birth
TB	Term birth
TVS	Transvaginal ultrasonography
VMF	Vaginal microflora
ZIG	Zero-inflated Gaussian model

Publications

1. **Meng M**, Leung TY, Cheung ACY, Cheung KWC, Hui A, Cheng YK, Law LW, Son G, Sung TJ, Lee KY, Chim SSC. Cervical microbiome for identification of cervical insufficiency after cerclage / pessary treatment. The 14th World Congress in Fetal Medicine, Crete, Greece, 24 Jun 2015. (Oral presentation)

2. **Meng M**, Leung TY, Cheung ACY, Cheung KWC, Hui A, Cheng YK, Law LW, Son G, Sung TJ, Lee KY, Chim SSC. Cervical microbiome signature for identifying patients at risk of spontaneous preterm birth after cerclage / pessary intervention. The 11th Asia Pacific Congress in Maternal Fetal Medicine, Taipei, Taiwan, 29 Nov 2015. (Oral presentation)

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Section I: Introduction

Chapter 1 Overview of cervical insufficiency

1.1 Definition and epidemiology

Cervical insufficiency (CI), as the premature asymptomatic cervical shortening or dilation, is officially defined as "the inability of the uterine cervix to retain a pregnancy in the absence of the signs and symptoms of clinical contractions, or labor, or both in the second trimester" (*1*). Nevertheless, due to the lack of consensus criteria for clinical diagnosis, the incidence of CI could not be estimated accurately (*2*).

Epidemiology surveys based on the large scale registry studies have suggested that CI presents in up to 1% of obstetric populations (Table 1-1) (*3-5*). CI has been estimated to account for 8% of recurrent mid-trimester loss (*6*), and 10-25% of mid-trimester miscarriage (*7, 8*).

CI can increase the risk of preterm birth (PTB), which affects 5 to 18% of pregnancies (*7*) and remains the leading cause for neonatal mortality as well as antenatal hospitalization (*9*) The subgroup analysis has implied that pregnant women with black race, advanced maternal age, cervical conization, higher order multiple gestations, assisted reproductive technology (ART) for maternal factor infertility or increased number of previous pregnancy terminations are more likely to suffer from CI (*3-5*). The risk factors for CI are summarized in Table 1-2.

1.2 Clinical manifestation

Mid-trimester short cervical length (CL) with prior mid-trimester spontaneous preterm birth (sPTB) / miscarriage, or the mid-trimester cervical dilatation without labor was considered as clues for diagnosis. However, women with prior sPTB only accounted for 10% of PTB before 34 weeks of gestation (*10, 11*). In primiparas, the mid-trimester asymptomatic cervical shortening or dilatation could be the only presentation. Short CL, funneling (dilatation of the internal cervical canal) and sludge (debris in amniotic fluid (AF)) would be detected via transvaginal ultrasonography (TVS) (*12*), while dilatation was usually noted by physical examination.

Currently, mid-trimester CL measured by TVS has been considered to be the best predictor of sPTB (*13*). Table 1-3 shows more detail information. The risk of sPTB has been proven to be inversely proportional to CL and those with the shortest CL have the highest risk of prematurity (*14*). The PTB risk associated with a CL at or below the 3rd percentile (15 mm) was 50% (*15*). A cervical length below the 10th centile (25 mm) for gestational age is considered to be short. Depending on the population under study and the gestational age of assessment, the chosen threshold is usually 25 - 30 mm with 25 - 30% of PTB risk (*16*). In Chinese population, around 4.6% of Chinese pregnancies (203/4438) have a mid-trimester CL below 25 mm (*17*). Table 1-4 shows the prevalence of short CL measured by TVS.

Funneling is a dilatation of internal os under the condition of short CL (< 25mm). According to the percent funneling which is defined as funnel length divided by total CL (the sum of funnel length and functional length), the funneling could be classified

into mild (< 25%, “T” or “Y” shape), moderate (25% - 50%, “V” shape) and severe (> 50%, “U” shape) funneling (Figure 1-1). The “V” and “U” shape funneling are both strongly associated with PTB (*18-20*). Amniotic fluid sludge is defined as the free-floating matter in AF close to the cervix. Sludge is considered as the marker of microbial invasion of amniotic cavity (MIAC), chorioamnionitis and funisitis which would increase the risk of PTB (*20, 21*).

Cervical dilatation is usually considered as the acute cervical insufficiency which could be late clinical presentation following cervical shortening. It is characterized by advanced dilation and effacement, spotting, unprovoked prolapsed membranes or ruptured membranes, or contractions that seem inadequate to explain the advanced effacement and dilation (*22*).

1.3 Cervix during pregnancy

1.3.1 Normal cervix

Normal cervix during pregnancy is long and closed (Figure 1-2). Three cervical structures are related to maintain pregnancy: i) the mucus-secreting simple columnar epithelium which constitutes the surface of endocervical canal; ii) the cervical stroma consisting of 85% of fibrous connective tissue and 15% of smooth muscle (*23, 24*); iii) the cervical glands regulated by progesterone which secrets mucus during pregnancy period.

The increased progesterone would thicken the mucus with high viscoelasticity mainly due to the large glycoproteins inside. The mucus fills the endocervical canal, acts as a physical and immunological barrier to inhibit the viral replication, the

diffusion of larger molecules or bacteria in vagina, and arrest the ascending infection by stimulating the inflammatory response (*24, 25*).

Constituted of collagen, proteoglycans, hyaluronan, elastin and water (*26*), the fibrous extracellular matrix (ECM) in stromal wall is crucial for cervical sustain capability. The cervical ripening could be partially explained as collagen remodeling, changing the cervical morphology and biomechanical function, as well as involving cervical shortening, softening and advanced dilation with decreased strength and increased extensibility. With a relatively higher extent of cervical remodeling, the multiparous cervix could help women to deliver a baby in a shorter duration. Nevertheless, the protective role of cervicovaginal hyaluronan against infection-mediated PTB (*27*) has been proven. Higher concentrations of collagen and hyaluronan are noticed in primiparas with longer cervical dilatation time during established labor (*28*).

1.3.2 Cervix with CI

Short cervix is the most common presentation in CI cervix, especially in primiparous women (Figure 1-2). The histological structure changes of CI cervix include: i) the increased amount of smooth muscle (*29*); ii) the increased collagen solubility (*26, 30*); iii) higher collagen extractabilities and collagenolytic activities (*28*), and; iv) the decreased elastin concentration and elastic fibers (*31*). The histological changes indicate a high proportion of newly synthesized collagen with low biomechanical strength, which is too weak to sustain the fetus to term.

Besides, the shortened or dilated cervix (especially the dilated condition) would lead to a concomitant loss of the mucus plug which primarily acts as an effective mechanical and immunological barrier, owing to its rich contents of antimicrobial peptides, immunoglobulins and phagocytes (*32*).

As a result, the fetal membrane would be exposed to bacteria in vagina, resulting in subclinical ascending infection and the increased risk of preterm birth (*15, 32, 33*).

1.4 Cervical intervention for CI

1.4.1 Cerclage

Cervical cerclage could effectively decrease PTB and prolong pregnancy for pregnancies with high risk of PTB which mainly involves acute cervical dilatation and short CL with previous miscarriage or PTB (*1, 34*). It is the surgical procedure to mechanically strengthen the CI cervix with sutures. Two types of transvaginal cerclage have been widely used: i) Shirodkar cerclage placed close to internal os which needs to expose the internal os by surgical separation (*35*); ii) McDonald cerclage placed around the entire cervix with a purse-string suture without surgical dissection (*36*) (Figure 1-3a). McDonald is more preferred as its simplicity (*37*). In the condition of technical difficulty or recurrent failure of transvaginal cerclage, transabdominal cerclage could be considered (*38, 39*).

Cerclage could not only reduce PTB but also prolong pregnancy. The suture could support cervical structure, maintain CL, close the cervical os, and help retain the mucus plug to prevent against infections. It is performed for three clinical indications: i) history-indicated cerclage for women with a history-based diagnosis CI at 12 to 14

weeks; ii) ultrasound-indicated cerclage offered for women with mid-trimester short cervix and prior PTB/miscarriage; and iii) physical-indicated cerclage (emergency/rescue cerclage) provided to women with dilated cervix.

Current evidences show that emergency cerclage might prolong pregnancy by 4-5 weeks, with a two-fold reduction of PTB before 34 weeks (*39*). A Cochrane review published in 2012 summarizes the cerclage intervention in singleton pregnancies with high risk of preterm birth (9 trials, n = 2,898). It has been found that cerclage could reduce PTB from 39% to 31% with an average relative risk (RR) 0.80 (95% Confidence Interval 0.71-0.89) (Figure 1-4) (*34*). However, due to the invasiveness, cerclage could also cause bleeding complications, infection and miscarriage. The same study found that the cerclage was associated with the increased vaginal discharge/bleeding, fever (average RR 2.25; 95% Confidence Interval 0.89 to 5.69; three trials, 953 women), and higher caesarean section rate (RR 1.19; 95% Confidence Interval 1.01 to 1.40; 8 trials, 2817 women). Furthermore, in the cerclage group, nearly one third of pregnancies resulted in PTB, which indicated that cerclage as the cervical intervention is not always successful, and implied the necessity to choose the candidates who could truly benefit from the operation (*34*).

1.4.2 Pessary

Cervical pessary is a silicone ring with larger outer border (65 - 70 mm) for pelvic floor supporting, and the smaller inner border (32 - 35 mm) for cervical closure (*40*) (Figure 1-3b). The pessary would direct the inclination of cervical canal from central alignment towards posterior to reduce the weight of pregnancy on cervix (*40*) and help retain mucus plug for immunological barrier (*41*). As it is non-invasive and

operator-independent, it could be applied to pregnancies in outpatient clinics, especially for those primiparas with a short cervix or those with difficulties in clinical decision for cerclage placement (*42-45*).

However, the intervention efficiency still remains controversial (*46*). Up to now, only three well-designed randomized clinical trials (RCT) in singleton pregnancies with CL below 25 mm have been reported (Table 1-5). The Pesario Cervical Para Evitar Prematurida (PECEP) study conducted in Spain (2007 - 2010) concludes that pessary could significantly reduce PTB, lower birth weight, reduce neonatal morbidity and composite adverse neonatal outcomes in singleton pregnancies with short CL (*41*); while the latter two studies conducted in Hong Kong and UK respectively show that no significant difference exist between the pessary group and expectant group (*17, 47*). Besides, a study published in 2013 compared the prevention effect of progesterone (n = 59), cerclage (n = 142) or pessary (n = 42) in pregnancies with a prior history of PTB and short CL. It implies similar effectiveness of the progesterone, cerclage and pessary in high risk pregnancies (*48*). The inconsistent findings suggest more powerful RCT studies as evidences (*49*).

1.4.3 Vaginal progesterone

Vaginal progesterone or 17-hydroxyprogesterone caproate (17-OHPC) administration could decrease over 40% of PTB in singleton pregnancies with a short cervix and with/without prior sPTB, especially in those with a shorter CL (≤ 15mm) (*50-52*). Progesterone could mediate the uterine progesterone receptors to enhance the uterine quiescence by reducing the expression of contraction-associated proteins (*53*) and inflammatory cytokines/chemokines (*54*), as well as protect the fetal

membranes from preterm premature rupture (PPROM) through prevention of its apoptosis (*55*).

The current opinion on PTB prevention is to identify the short cervix through universal CL screening, and offer the vaginal progesterone to pregnancies with short CL (*56*). Based on the progesterone supplementary, further cervical intervention such as cerclage and pessary could be further considered.

In summary, cervical cerclage is suggested to be applied to pregnancies with dilated cervix (emergency cerclage) or pregnancies with mid-trimester short CL and prior miscarriage/PTB (elective cerclage), which could reduce 20% of PTB risk. The cervical pessary and progesterone could be offered to pregnancies with mid-trimester short CL. Vaginal progesterone would lower over 40% of PTB risk. Nonetheless, the efficiency of pessary remains controversial based on the current clinical evidences and demands more researches in this area.

1.5 Cervical intervention failure

Though cervical interventions can reduce PTB, they may not always be effective. The technical success in the placement of a cerclage or pessary does not equate to term delivery (TB), and at least one third of cases still deliver at preterm (*34*). Compared to the ultrasound-indicated cerclage, emergency cerclage has a higher failure rate (*57*). The exposed membrane to vagina prominently increases the risk of ascending infection. The loss of mucus plug severely damages the cervical function as the immunological barrier, which results in the increased risk of intrauterine infection (IAI). A recent publication has shown that the membrane prolapse in vagina

or post-cerclage pre-labor PROM strongly predict the intervention failure (*58*). According to the published data, the subclinical intra-amniotic inflammation exists widely in CI pregnancies with dilated cervix (38.9-64.5%) (*59-61*) or short cervix (9-25.5%) (*62-65*). Exploration of IAI by culture method in CI patients with a dilated cervix shows a highly prevalent IAI (51.5%) before cervical intervention and the 2.5-fold higher PTB rate in CI patients with IAI than those without IAI after intervention. It suggests a crucial role of pre-intervention IAI in PTB pathogenesis (*65*).

After the exclusion of IAI, the prognosis of intervention in CI would be good (*60*). Judging from another point of view, the premature cervical shortening and dilatation could be the result from IAI or genital tract infection. If it is true, the cervical intervention would not alter the clinical outcome, as the PTB process has already started up. Thus, to rule out IAI, especially the subclinical IAI, a pre-intervention amniocentesis has been widely suggested (*66, 67*). Culture technique (the aerobic and anaerobic bacteria, ureaplasma and mycoplasma) are utilized to detect intrauterine infection, while the elevated cytokines (e.g. matrix metallopeptidase 8 (MMP-8) or interleukin 6 (IL-6)) in AF is considered as the predictor of intrauterine inflammation (*68, 69*). Interestingly, compared to the intrauterine infection, the intrauterine inflammation is more prevalent and consistent with the clinical outcome, which could be explained by the possibility that some other uncultivable pathogens in AF play an important role in the stimulation of inflammation. Unfortunately, due to the disadvantage of invasiveness, the 0.5% risk of miscarriage and potential risk of infection, the pre-intervention amniocentesis has not been implemented in routine clinical practice yet.

IAI usually results from the migration of cervicovaginal flora through the cervical canal to infect the fetal membranes, placenta, AF, and fetus (*70*). In normal pregnancy, the cervical mucus plug, placenta, and membranes can be a barrier to the infection of AF and fetus, while Lactobacilli in the vagina could inhibit the virulence of pathogenic organisms. In CI cervix, the cervical histological changes might lead to the alteration of metabolism and immunology (*71*), resulting in the dysbiosis of cervicovaginal bacterial community, that is, vaginal infection. Donders *et al* has shown the association existing between abnormal vaginal flora (flora disturbance with anaerobic or aerobic overgrowth) and cervical length, and suggested to utilize both as the risk factors for PTB (*72*). As the cervix is too weak to act as the barrier for ascending infection, vaginal infection could easily go through the cervical canal, and cause IAI (*73, 74*) (Figure 1-5). **Thus, the microbial composition and status in the endocervical canal, the path for ascending infection, is crucially important to understand the mechanism of PTB in CI condition.**

1.6 Update in cervicovaginal microbiome research

The traditional technique in microbiome research is culture-dependent. It could only detect less than 100 bacterial species with low sensitivity, and is unable to give quantitative information for each species within the bacterial community. Such limitation leads to the high false negative results in the detection of infection. For example, CI patients usually have a high positive rate of intrauterine inflammation rather than the infection which can be traditionally detected by culture (*69*). The molecular methods are culture-independent, and could identify the nonviable and uncultivable bacteria quickly and sensitively. The 16S rRNA gene sequence-based

methods could offer relative abundance for over 9600 bacterial species, which helps understand the bacterial community status thoroughly (*75*).

Community state type (CST) is the cluster of community states (the species composition and abundance of a vaginal community at a specific point in time) which are similar in terms of the kinds and relative abundances of the observed phylotypes (*76*). So far, six CSTs have been described for vaginal microbiomes, including CST I, *Lactobacillus crispatus*-dominant; CST II, *Lactobacillus gasseri*-dominant; CST III, *Lactobacillus iners*-dominant; CST IV-A, diverse community characterized by various species of anaerobic bacteria belonging to the genera *Anaerococcus*, *Peptoniphilus*, *Prevotella* and *Streptococcus*; CST IV-B, diverse community characterized by higher proportions of the genera *Atopobium* and *Megasphaera* among others; as well as CST V, *Lactobacillus jensenii*-dominant (*76-79*).

1.6.1 Lactobacilli dominated vaginal microbiome

The *Lactobacilli* species dominated in CST I, II, III and V are taxonomically similar to each other. However, specific *Lactobacillus* species can produce mildly different antimicrobial compounds from strain to strain. Compared to non-pregnant women, pregnant women have the vaginal microbiome with higher stability and lower microbial diversity, which means higher abundance of *Lactobacilli* (*L. crispatus*, *L. gasseri* and *L. jensenii*) and lower abundance of non-*Lactobacilli* species (*80*).

The cervicovaginal microbiome is not unique. The compositional changes exist throughout the life (*81*). The hormonal changes, nutrient alteration, or the vaginal pH

fluctuation would affect the microbial composition and induce the CST shifting between each other. Different CST might stand for different status of vaginal flora. For instance, CST I, dominated by *L. crispatus* which is most stable and protective is more frequent in normal vaginal flora; on the other hand, CST III, dominated by *L. iners*, is more likely to equal to a transition status between the normal vaginal flora and abnormal vaginal flora (AVF) (*82-84*). The observation on the vaginal flora dominated by early-trimester *Lactobacilli* shows that *L. iners* dominates in 85% of cases, resulting in PTB, versus 16% in TB ($p < 0.001$); furthermore, it also seems that the TB cases shares more kinds of *Lactobacillus* species at the same time (*85*). The molecular method also implies that *L. crispatus* is more beneficial than *L. iners*. **Longitudinal studies show that a *L. crispatus*-dominated CST is more likely to shift to a *L. iners*-dominated or mixed lactobacilli vaginal microbiome than to full dysbiosis** (*86*).

1.6.2 Non-lactobacilli dominated vaginal microbiome

CST IV A/B, the highly diverse community, is the AVF with the microbial dysbiosis, and could also be defined as the lack of *Lactobacilli* and the overgrowth of anaerobic bacteria or aerobic bacteria. CST IV A/B during pregnancy is associated with an increased risk of PTB (*87, 88*).

Bacterial vaginosis (BV) is characterized by the foul-smelling discharge with no obvious inflammation. Hay or Nugent criteria based on the Gram stain diagnoses BV by the decreased *Lactobacilli* and the increased anaerobic bacteria, while Amsel's criteria, based on the clinical manifestation, diagnoses clinically. Culture is useless due to the common pathogens in BV. For instance, *Gardnerella vaginalis* and

mycoplasmas are also colonized in 30-50% of healthy women as part of their normal vaginal flora (*89*).

BV is an independent risk factor for morbidity. Pregnancies with BV would have the increased risk of PTB (*90, 91*), IAI (*92*), chorioamnionitis (*73*) and low birth weight (*93*). Therefore, the diagnosis of BV is essential, especially in pregnant females, as early as possible to prevent complications (*94*).

The molecular-based microbial explorations of vaginal flora in BV suggest that it is the poly-bacterial dysbiosis with greater species richness and diversity. No specific species has been found to present universally in all the BV cases. Instead, various bacterial species are found to be associated with varied BV-related symptoms. As a result, the PCR-based package named bacterial vaginosis-associated agents (BV-AA), which involves thirteen bacterial species, has been developed for molecular diagnosis of BV (*95*). Besides, the observation among women infected with human immunodeficiency virus (HIV) and human papillomavirus (HPV) found that the dysbiosis is consistently associated with infection, which indicated the possibility that immunology deficiency would induce the dysbiosis in vaginal microbiome (*86, 96*). Considering the immunology deficiency in CI cervix, it is hypothesized that dysbiosis might be consistently associated with the severity of cervical insufficiency.

Aerobic vaginitis (AV), a recently proposed term for genital tract infection in women, is usually associated with aerobic micro-organisms, mainly *Group B streptococci* and *Escherichia coli*. Different from BV which is usually without prominent immune response, AV is characterized by an abnormal vaginal microbiome accompanied by an increased inflammatory reaction, with higher levels of interleukin (IL)-1, IL-6 and

IL-8 (*97*), thus is associated with an increased risk of preterm delivery, chorioamnionitis and fetal funisitis. The broader spectrum drug like clindamycin is commonly applied for pregnancy with AV to prevent the infection-related preterm birth (*98*).

In southwest China, the AV prevalence based on the performed enzymes is 15.40%, and is characterized by sexually active age and combined infection predominated by the AV and BV type (61.33%) (*99*). No sequence-based exploration of AV has been reported yet.

Table 1-1 The incidence of CI

Study	Incidence	Races / Ethnics	Sample size (n)
Danish National Patient Register (NPR) 1980-1990 (*3*)	4.6/1000 births	Danish	53,989
Massachusetts live birth-infant death records data for resident births in 1997–1998 (*4*)	4.1/1000 births	over 90% non-Hispanic white	157,066
US 2005 Natality data file (*5*)	2.4/1000 births	76.4% white, 15% black, 7.4% Asian, 0.2% American Native	1,115,541

Table 1-2 Risk factors contributing CI

Congenital cervical abnormalities
genetic disorders affecting collagen (e.g., Ehlers-Danlos syndrome) (*100-102*)
uterine anomalies (*103, 104*)
in utero diethylstilbestrol (DES) exposure (*105*)
biologic variation
Acquired cervical abnormalities (cervical trauma)
acquired during delivery (spontaneous, forceps- or vacuum-assisted, cesarean) (*106*)
rapid mechanical cervical dilation before a gynecologic procedure (*107, 108*)
treatment of cervical intraepithelial neoplasia
Obstetric history
history of previous second trimester loss
history of precipitous labor or advanced dilation before labor onset
history of recurrent spontaneous and therapeutic abortions

Table 1-3 CL distribution and PTB risk *

Cervical length (mm)	Prevalence	Risk
1-10	0.6%	44%
11-15	0.5%	23%
16-25	7.1%	3.6%
26-30	16.6%	1.3%
31-35	27.4%	0.8%
36-40	26.9%	0.6%
>40	21.0%	0.4%

*This table was cited from (*47*).

Table 1-4 Prevalence of short CL measured by TVS

CL screening studies	Criteria	Races / Ethnics	Incidence	Sources
Singleton pregnancies at 20 to 24 weeks (n = 4,438)	CL < 25 mm	Asian	4.6% (203/4,438)	(*17*)
Pregnancies at 20 to 23 weeks (n = 11,875)	CL ≤ 25 mm	58% White / 29% Latin American	6.1% (726/11,875)	(*41*)
Singleton pregnancies at 20 to 24 weeks (Database with n > 50,000)	CL ≤ 25 mm	65% White /27% Black	8.2%	(*47*)

TVS, transvaginal ultrasonography

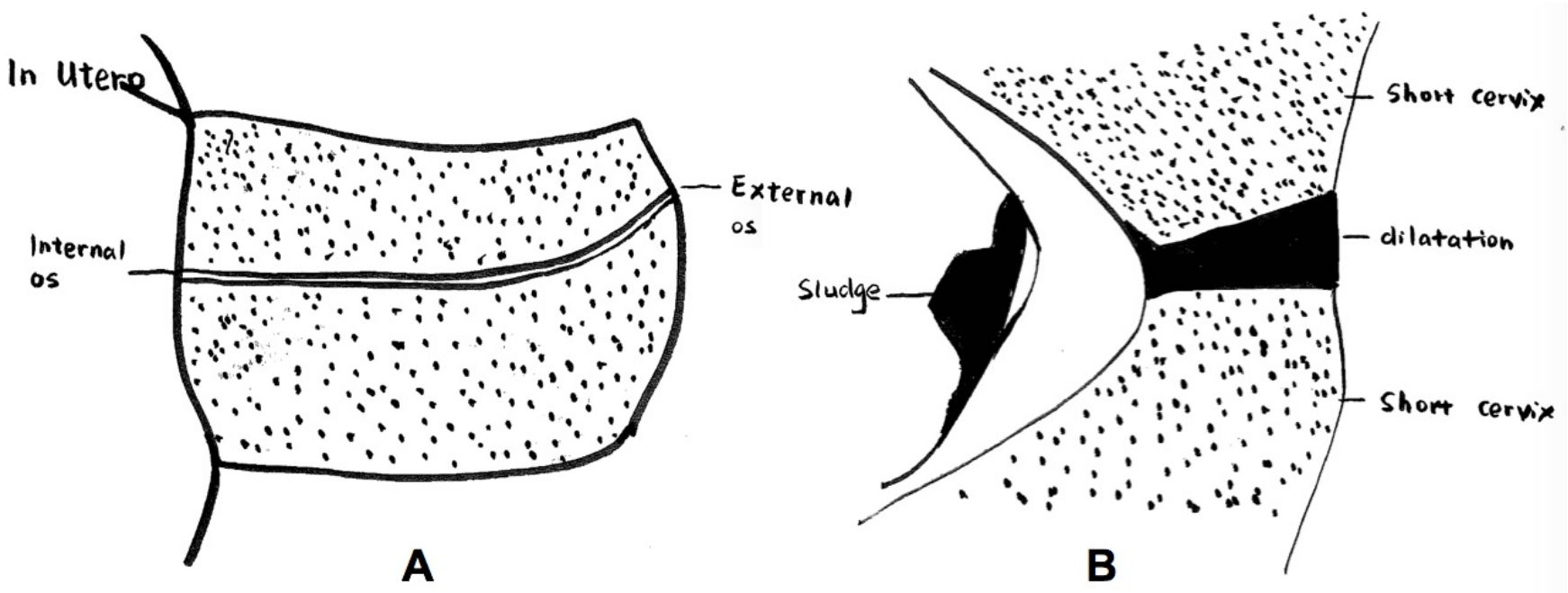


Figure 1-1 Ultrasound evaluation of the cervix.

(A) Normal cervix; **(B)** "U" shape funneling and sludge (*109*).

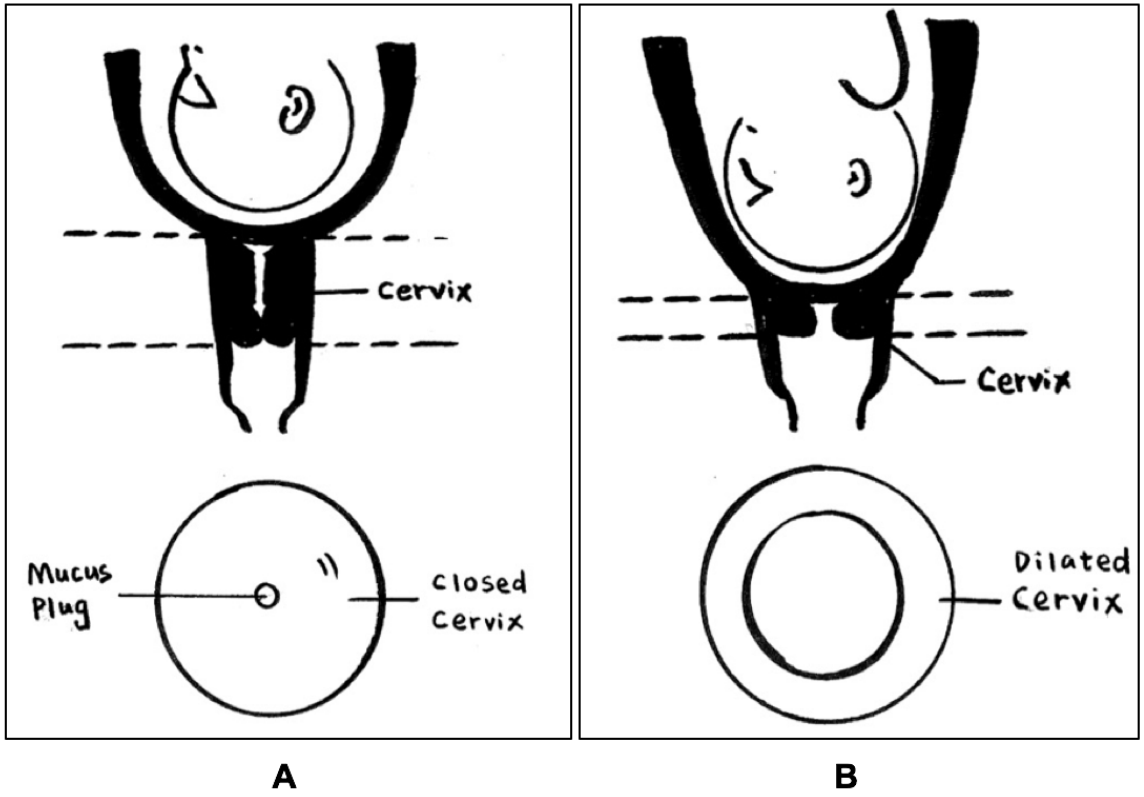


Figure 1-2 Normal cervix and cervix with cervical insufficiency (CI).
(A) Normal cervix: normal cervical length and closed os, mucus plug exists as infection barrier;
(B) CI cervix: short cervical length with / without dilatation, mucus plug decreases.

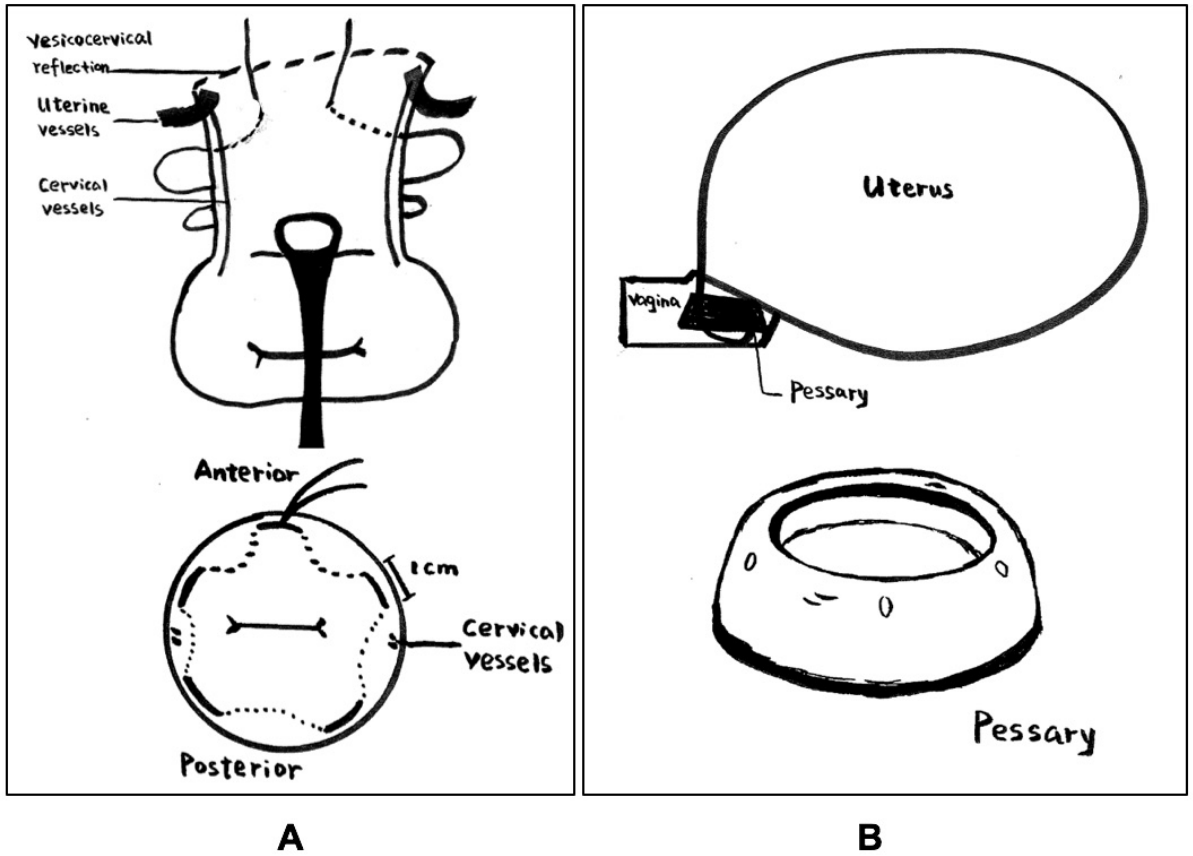


Figure 1-3 Cerclage and pessary.

(A) McDonald Cerclage (*110*); **(B)** Cerclage pessary (*111*).

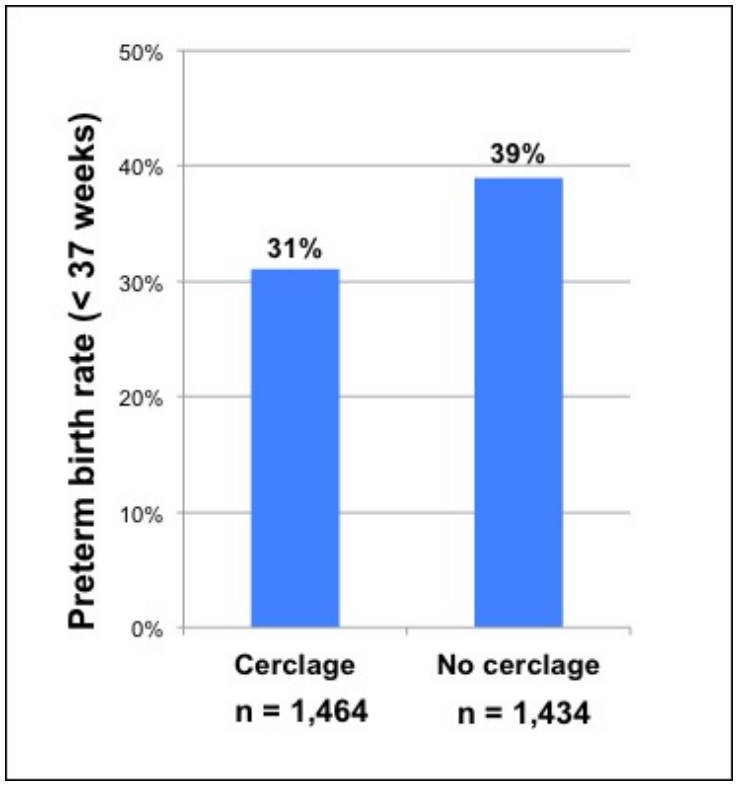


Figure 1-4 Cerclage intervention in singleton pregnancies with high risk of PTB.

Compared with no treatment, cerclage was associated with significant reduction in preterm births before 37 weeks (average RR 0.80; 95% Confidence Interval 0.69 to 0.95; nine trials, 2898 women) (Chi-square test, p = 0.0082).

Table 1-5 Results comparison among three pessary RCTs

Study	**Sample size (Pessary vs. Control)**	**Recruitment criteria**	**Races / Ethnics**	**PTB (< 34 weeks) rate (Pessary vs. Control)**	**OR / RR (95%** Confidence Interval**)**	**p value**
Spain (*41*)	192 vs. 193	CL ≤ 25mm between 18 to 22 weeks	58% White, 29% Latin American	6% vs. 27%	OR 0.18 (0.08-0.37)	< 0.0001
HK (*17*)	53 vs. 55	Singletons with CL < 25mm between 20 to 24 weeks	Asian	9.4% vs. 5.5%	RR 1.04 (0.94-1.12)	0.46
UK (*47*)	465 vs. 467	Singletons with CL ≤ 25mm between 20 to 24^{+6} weeks	65% White, 27% Black	12.0% vs. 10.8%	OR 1.36 (0.86-2.15)	0.47

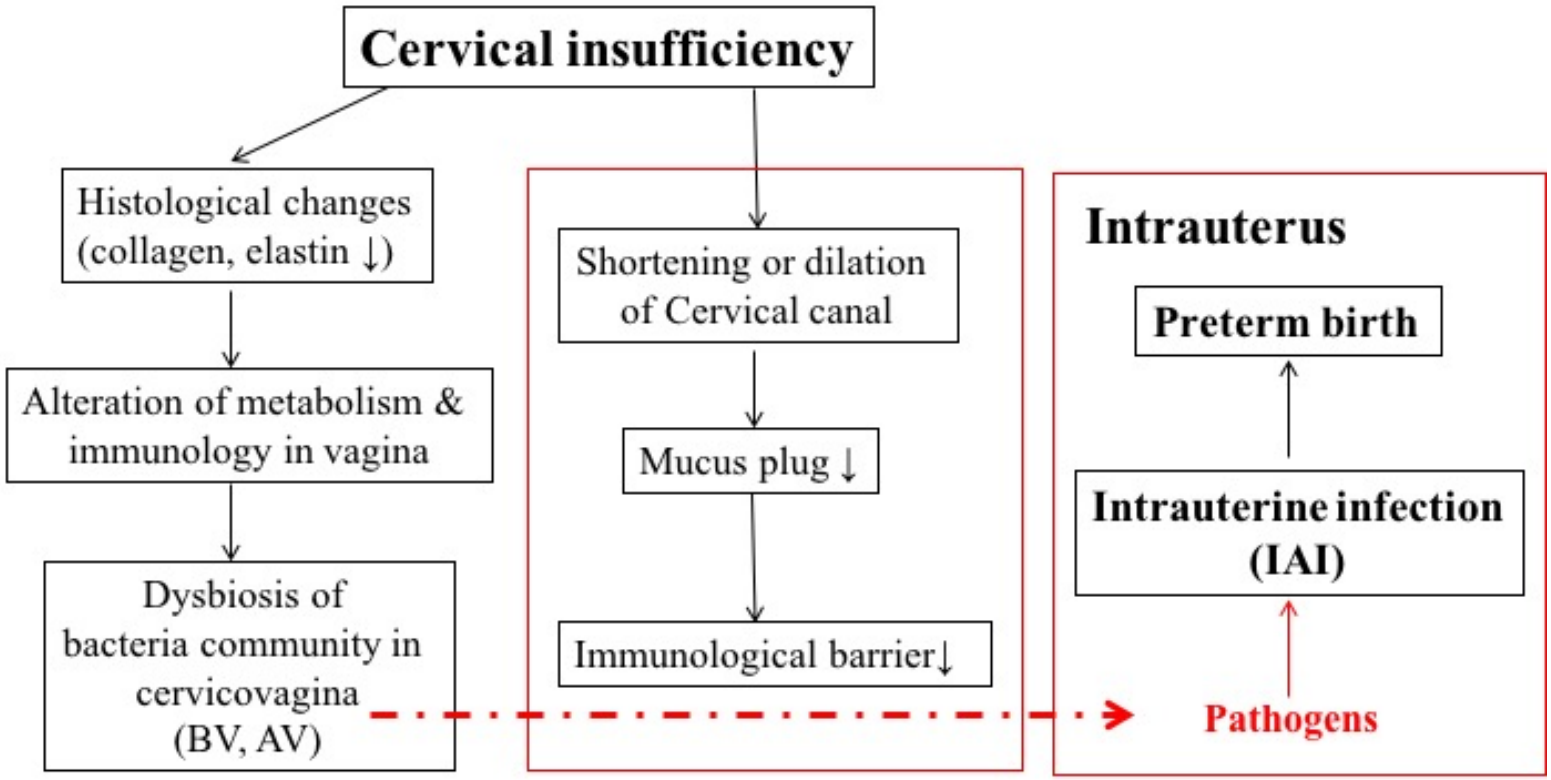


Figure 1-5 The possible mechanism of PTB in CI condition.

BV, bacterial vaginosis; **AV**, aerobic vaginitis; **IAI**, intra-amniotic infection.

Chapter 2 Objectives

Both of the structural and histological changes occur in the cervix with cervical insufficiency, resulting in functional deficiency of cervicovagina as the barrier to prevent ascending infection. The high prevalence of pre-intervention intra-amniotic infection in CI patients with cervical intervention failure implies that the ascending infection might play a significant role in the pathogenesis of PTB. Furthermore, the special condition of CI might complicate with the changes in metabolism and immunology, which would arouse the dysbiosis of cervicovaginal microbiome. **However, the microbial composition and status in the endocervical canal is barely known, which is the one and only path for ascending infection from vagina into uterus.**

Here, hypotheses have been put forward as below:

i) In cervical insufficiency, the pre-intervention cervical microbiome profile might have altered;

ii) The microbial dysbiosis exists in those CI patients, resulting in sPTB;

iii) The microbial alteration might help to indicate the patients at the risk of sPTB even though a cervical intervention has been offered.

The objectives of this study involve:

i) To characterize the cervical microbiome in mid-trimester pregnant women with CI utilizing the sequence-based techniques;

ii) To develop a potential predictor to screen the candidates that would truly benefit from cervical interventions amongst pregnant women with CI.

Section II: Materials and methods

Chapter 3 Materials and Methods

3.1 Patients recruitment and clinical follow-up

This is a case-control study in which CI is defined as "painless dilation and shortening of the cervix in the second trimester of pregnancy, resulting in pregnancy loss or preterm delivery" (*112*).

After ethical approval was obtained from the institutional review board, this study was conducted in both the Department of Obstetrics & Gynaecology of the Hallym University Medical Centre (Seoul, South Korea) and the Department of Obstetrics & Gynaecology of the Chinese University of Hong Kong (Hong Kong). Pregnant women with high risk of preterm birth (e.g. previous history of early PTB/miscarriage, cervical conization, and suspicious short cervix by trans-abdominal sonography) came to the PTB clinic for counseling. All the participants signed the informed consent agreement.

Inclusion criteria: singleton pregnancies in mid-trimester, with CL < 25 mm under TVS, or; cervical dilation, 1 cm to 5 cm by physical examination, and; intact membrane without contractions. Besides, mid-trimester pregnant women with the closed os and CL ≥ 25 mm were also recruited and defined as normal cervix for control. CL measurements were performed and described by the Fetal Medicine Foundation (https://fetalmedicine.org/cervicalassessment).

Exclusion criteria: pregnancies complicated by preeclampsia, growth restriction, fetal distress, iatrogenic preterm birth, fetal structural or chromosomal abnormalities and multiple pregnancies. Women who had sexual activity or vaginal application (e.g. vaginal medication or suppositories, douche) within 48 hours, or antibiotics / antimycotics treatment within 30 days before sampling were also excluded.

All the participants were followed-up to delivery. CI women delivered sPTB were assigned to the CI_sPTB group, while those delivered TB were assigned to the CI_TB group. The control cases with TB were grouped as Nm_TB group. The medical documentations were recorded for reference (Figure 3-1).

3.2 Sample collection and DNA extraction

All procedures for sample collection were aseptic without touching the cervical mucus plug, and processed utilizing a sterile calcium alginate swabs (Puritan, Calgiswab type 3, Hardwood Products Co., Guilford, ME, USA). Samples were immediately collected under direct vision when cervix was exposed by speculum, before any examination or intervention performed. The swab was inserted into the endocervical canal and rotated for 360 degrees once. For the condition of dilated cervix, the swab was placed at the 12' clock position of the cervix, and then rotated. The cotton head of the swab was stored in the 2 mL DNase/RNase-Free Distilled Water (Life technologies, Carlsbad, CA, USA) at -80℃. Subsequently, the Korean samples were shipped from Seoul to Hong Kong on dry ice. To minimize the batch variation, all the samples were extracted on the same day, according to the method described by Verhelst R (*113*). The method was estimated to strike a balance

between DNA yield and fair representation of bacteria communities commonly found in the female reproductive tract (*114*).

3.3 PCR amplification and sequencing of the 16S rRNA gene

Microbiome is the entire collection of bacteria in a sample. The microbiome analysis is usually based on pyrosequencing of 16S rRNA gene, including hypervariable regions for taxonomic classification of all the bacteria. Compared to traditional culture-based technique which could only cover less than 100 bacterial species, the culture-independent microbiome analysis by MPS could quantitatively detect over 9,600 bacterial taxa with high sensitivity and thus could give relative abundance for each bacterium in microbial community. 16S rRNA gene is commonly found in all bacteria. This gene contains: i) highly conserved regions are conserved among all the bacteria and could be used for PCR primer annealing; ii) the hypervariable regions could be utilized for taxonomic classification if pyrosequencing was performed.

PCR was performed utilizing primers targeting the hyper-variable of the 16S rRNA gene (*115*). Sequencing adaptors and sample-specific multiplex identifiers (MIDs) were also added to the 5' ends of the forward primers according to the manufacturer's protocol. PCR reactions were conducted utilizing the FastStart HiFi PCR System dNTPack (Roche). Reactions with 50 μL reaction volume were run in a PTC-100 thermal controller (MJ Research, BIO-Rad) using the cycling parameters as below: 95 ℃ for initial 2min of denaturation followed by 33 cycles of 30s at 95 ℃, 30s at 40 ℃, and 60s at 72 ℃, with a final extension of 5 min at 72 ℃ and another 5 min at 25 ℃. The presence of the single amplicon was confirmed by gel

electrophoresis on a 2% agarose gel stained with GelRed (Biotium, CA, USA), and purified with QIAquick gel extraction kit (QIAGEN, CA, USA). Pyrosequencing of the purified PCR products was performed on the GX-FLX 454 Titanium platform (Roche).

3.4 Cervical microbiome analysis

3.4.1 Analysis pipeline

The cervical microbiome analysis includes three parts (Figure 3-2): i) Species richness and diversity estimates of cervical microbiome, including the 16S rRNA sequencing data processing, taxonomic assignment, biodiversity estimation, and community state type (CST) frequencies comparison, and predictive functional profiling of cervical microbiomes utilizing the 16S rRNA sequencing; ii) Cervical microbiome signature identification for successful cervical intervention in pregnant women with CI. The detailed steps would be described in each part of the results.

3.4.2 Analytical software

Mothur (http://www.mothur.org) is an effective type of bioinformatics software for quality improvement of the 16S rRNA sequencing data (*116*). It integrates multiple algorithms to trim, screen, and align sequences; calculate distances; assign sequences to operational taxonomic units (OTUs); as well as describe alpha and beta diversity for microbiome samples. The workflow is mainly established on the online 454 SOP (http://www.mothur.org/wiki/454_SOP). In this study, the process was performed on mothur v.1.33.0. Generally, several steps were implemented in this study:

i) To reduce the sequencing error and to trim the sequence when the average quality score over the window dropped below 35, a 50-bp sliding window was used;

ii) To simplify the dataset, the unique sequences were used only;

iii) To remove the chimeras (the artifacts made during the PCR process) from the unique sequences, the sequences was employed as their own reference;

iv) Removed the "contaminated" sequences which are mainly organelles-related or the unknown sequences that could be identified by alignment to RDP reference dataset;

v) To generate the OTUs, the sequences with the pairwise distance less than 0.03 were clustered; and to get the taxonomy information, each OTU was aligned to RDP and SILVA databases;

vi) To analyze the alpha diversity of samples, the collector's curve of the Chao1 richness estimators and the inverse Simpson diversity index were generated.

MetagenomeSeq

(http://bioconductor.org/packages/release/bioc/html/metagenomeSeq.html) as an R package developed for the analysis of high-throughput sequencing microbial marker-gene survey data (*117*), which provides a normalization technique "cumulative-sum scaling" (CSS) for the highly sparse 16S rRNA pyrosequencing data. This CSS method is based on the distribution mixture model of zero-inflated Gaussian (ZIG)

and could better control the biases in measurement across the taxonomic features like OTUs, which makes the comparison between groups more reliable (*118, 119*). The package also provides the function of statistical testing for group comparisons and graphic visualization for metagenomic sequencing data. The following analytical functions are implemented on the MetagenomeSeq:

i) Normalization of the raw read counts for each OTU based on the ZIG mixture model;

ii) Feature aggregation for each case;

iii) Multi-dimensional scaling (MDS) analysis for beta-diversity estimation between different groups;

Tax4Fun (http://tax4fun.gobics.de/) is an R package developed for the prediction of microbial functional diversity based on the 16S rRNA pyrosequencing data. It provides a good approximation to functional profiles obtained from the metagenomic shotgun sequencing approaches (*120*). The software links the SILVA-labeled OTU abundances to the pre-computed metabolic reference based on the KEGG database, and outputs a KO identifier list with relative abundance for each sample. Based on this, the metabolism level could be further compared among different groups.

Entropy (https://cran.r-project.org/web/packages/entropy/index.html) and *Cluster* (https://cran.r-project.org/web/packages/cluster/index.html) belong to the microbial CST generation. Generally, the distance metric of dissimilarity coefficient Jensen-Shannon divergence (JSD) is first generated for each sample by entropy.

Subsequently, samples are clustered into the CST groups based on relative genus abundances using JSD distance and the ward's linkage clustering algorithm (*121*).

Other software: SPSS is employed for statistical comparison. Herein, the Mann-Whitney test is applied for non-parametric comparison between two groups while the Kruskal-Wallis test is used for non-parametric comparison among three groups. The R package "qvalue"(https://bioconductor.org/packages/release/bioc/html/qvalue.html) is used for multiple test correction and the GraphPrism is applied in graphic visualization.

3.4.3 Analytical databases

SILVA (https://www.arb-silva.de) as an interdisciplinary project provides "comprehensive, quality checked and regularly updated datasets of aligned small (16S/18S, SSU) and large subunit (23S/28S, LSU) ribosomal RNA (rRNA) sequences for all three domains of life (Bacteria, Archaea and Eukarya)" (*122*). It is the most widely used database in microbiome research, and can be tolerated by Mothur and Tax4Fun. In this study, SILVA 115 (https://www.arb-silva.de/documentation/release-115/) has been employed for the steps of unique sequencing filtering, sequencing taxonomic annotation and CST assignment.

Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/index.jsp) is an online tool to provide "quality-controlled, aligned and annotated Bacterial and Archaeal 16S rRNA sequences, and Fungal 28S rRNA sequences, and a suite of analysis tools to the scientific community." It has been widely applied in microbiome research (*123*).

In this study, the RDP reference dataset 2.9 has been applied in Mothur for the "contaminated" sequences remove.

KEGG Orthology (KO) (http://www.genome.jp/kegg/ko.html) is the database for molecular-level functions storing. Genome annotation in KEGG is ortholog annotation, assigning KO identifiers (K numbers) to individual genes in the GENES database. Each KO identifier stands for one specific function which can be allocated into the KEGG pathways maps (*124*). In this study, KO identifier would be generated by Tax4Fun based on the SILVA-labeled OTUs, and further utilized for metabolism profile estimation.

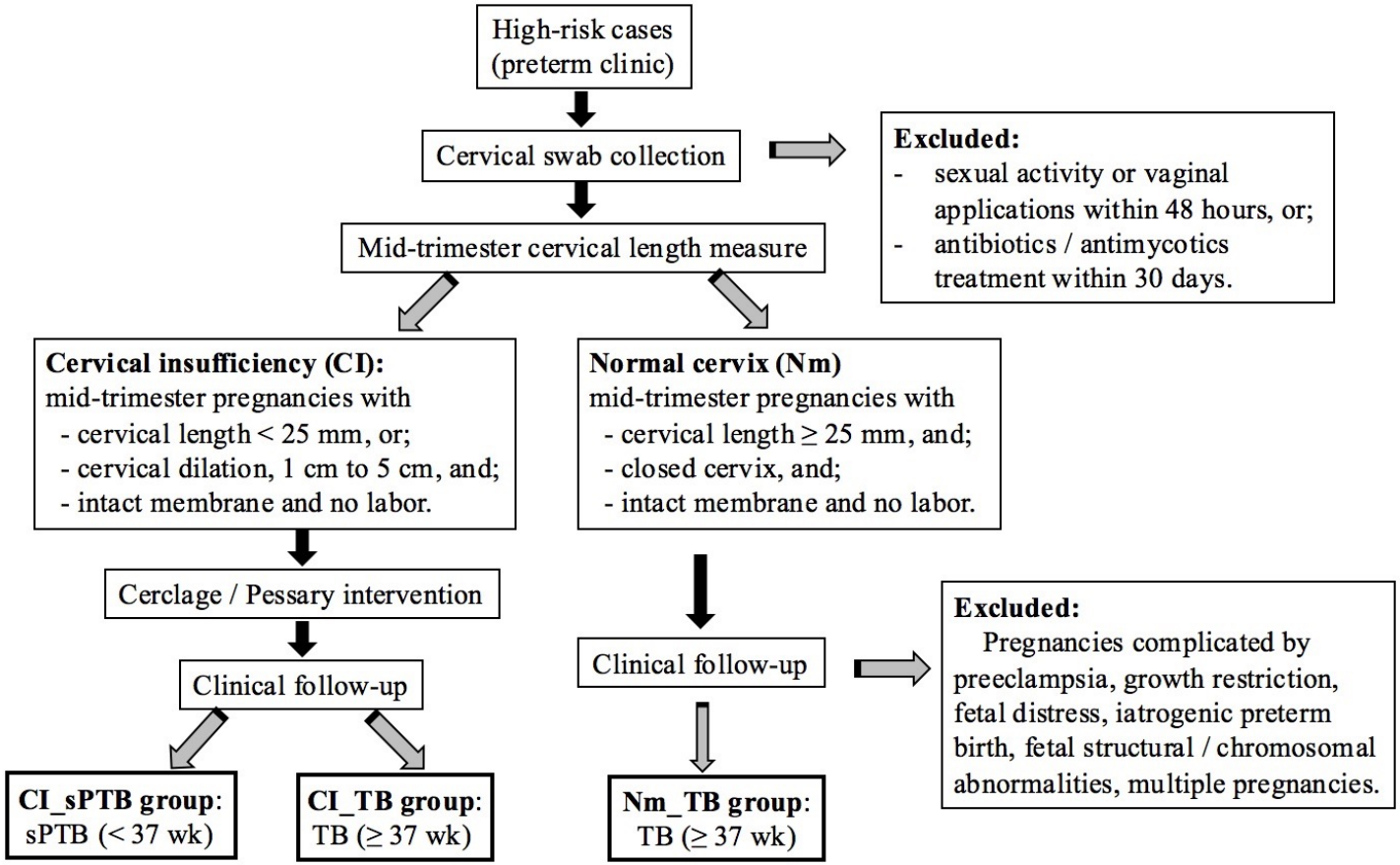


Figure 3-1 Flow diagram for patient recruitment and sample collection.

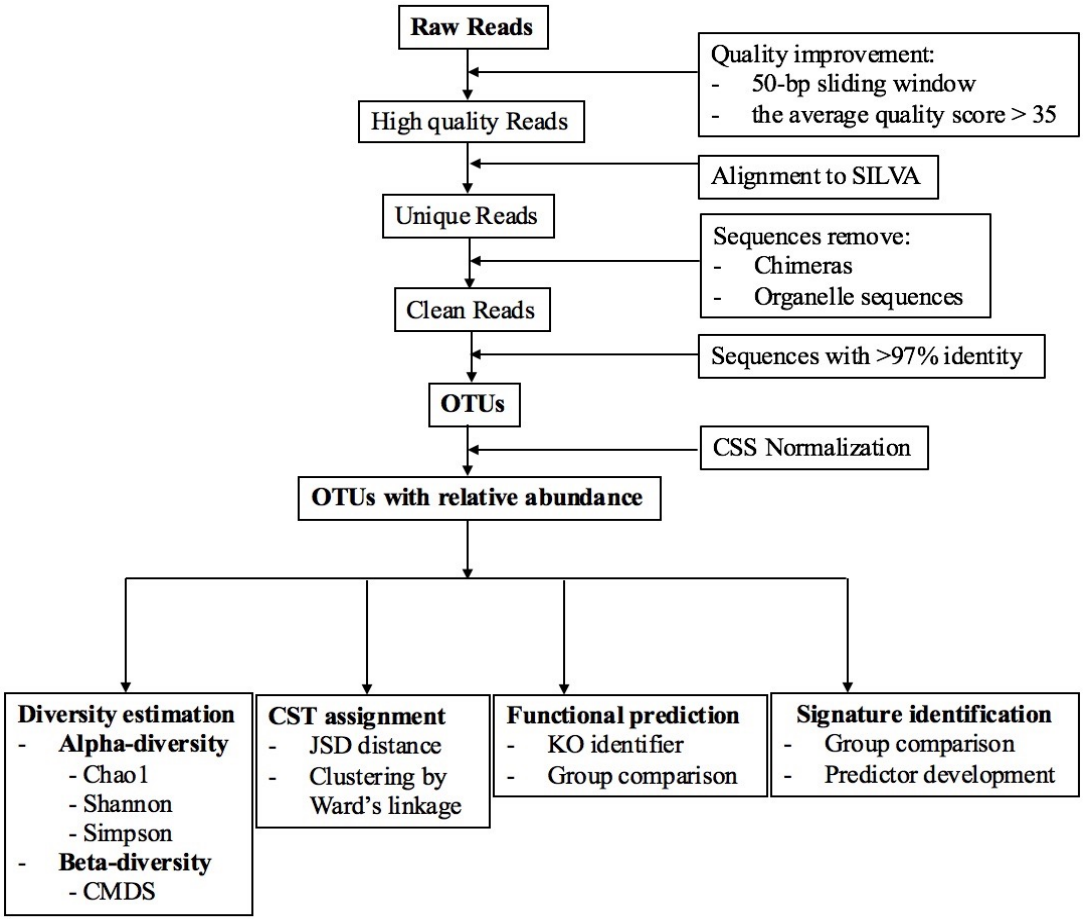


Figure 3-2 Flow diagram for cervical microbiome analysis.

Section III: Results

Chapter 4 Characteristics of Cervical Microbiome in Pregnant Women with Cervical Insufficiency

4.1 Introduction

Preterm birth (PTB) influences 5 to 18% of pregnancies. Spontaneous preterm birth (sPTB) accounts for 55% of PTB (*55*). Up till now, only intra-amniotic infection (IAI) has been causally linked to PTB (*55*). Evidences have shown that microbial invasion of the amniotic cavity (MIAC) and IAI are major causes of preterm labor (PTL) (*125-127*).

Microorganisms isolated from AF are similar to those in cervicovagina, which implies the key role of ascending infection in the pathogenesis of IAI. However, not all women would develop IAI. The observation finding that the dysbiosis in microbiome (e.g. BV) and human immunodeficiency syndrome (e.g. HIV infections) present the increased risk of PTB (*128*) indicates the importance of microbial balance in the lower genital tract for PTB prevention.

CI as the premature cervical ripening is accompanied with micro-environmental alterations in both cervix and vagina, and might easily cause the dysbiosis. This study aimed to explore the composition of endocervical microbiome among pregnant women with short or dilated cervix in mid-trimester, and observe their possible correlation to PTB.

4.2 Methods

4.2.1 Estimation of species richness and biodiversity

After the generation of OTUs, species richness and biodiversity were further estimated on Mothur v.1.33.0 according to the 454 SOP (*116*). Detailed information for each index would be further discussed in the Results part.

4.2.2 CST assignments

The dissimilarity between community states was measured utilizing the Jensen-Shannon (JSD) metric. The clustering of community states was conducted using hierarchical clustering based on the Jensen-Shannon distances between all pairs of community states and the Ward clustering algorithm (*76*). Only OTUs with at least 20 sequences were applied in the clustering process. Both of the JSD metric and clustering were created utilizing the package in R. The number of clusters was determined using the Silhouette measure to enhance the degree of confidence in a particular clustering (*129*). It was applied to the clusters of community states resulted from the Jensen-Shannon hierarchical clustering by cutting the corresponding dendrogram at the level with k leaves (clusters). Herein, k was between 2 and 10 and K value with the maximum of the Silhouette values was chosen for CST clustering. The between-class analysis (BCA) and the principal coordinate analysis (PCoA) were further plotted utilizing the package ade4 in R. Furthermore, the CST distribution was explored amongst the three groups.

4.2.3 Predictive functional profiling of cervical microbiomes

Functional analysis was performed mainly on KEGG Orthologue (KO) markers. Via Tax4Fun (R package), gene family predictions for 16S rRNA pyrosequencing data are made by associating the taxonomic ID of OTUs in the reference database (SILVA115) with pre-computed gene content. The microbial functional profiles, which contained a list of molecular level of functions with KO identifiers and the relative abundances, were further produced by annotation to KEGG Ortholog (KO) database. The non-parametric Kruskal-Wallis test was applied to identify the CI_sPTB related molecular functional markers (KO) by comparison among three groups (CI_sPTB, CI_TB and Nm_TB), multiple test correction was further performed with the false discovery rate (FDR) estimation. The enriched or decreased markers were further annotated to iPath2 (interactive Pathways Explorer 2) (http://pathways.embl.de/iPath2.cgi#), and mapped the CI_sPTB related pathways.

4.3 Results

4.3.1 Characteristics of participants

Overall, 38 patients were recruited into the study (Figure 4-1). Table 4-1 shows the basic characteristic for participants. All the patients are East Asians, among which 10 pregnancies with CI resulted in sPTB (CI_sPTB group), 15 pregnancies with CI led to TB (CI_TB group) and 13 pregnancies without CI resulted in TB (Nm_TB group) (Table 4-1). More detailed clinical information of each case is presented in Table 4-6.

4.3.2 Analysis of the 454 pyrosequencing data

The average raw read counts for the V4-V5 region of the 16S rRNA gene were around 1.05 million. After the steps of flowgram-denoising, quality-filtering (quality-value > 35) and chimera-removing, a dataset consisted of 836,804 high-quality read counts were harvested. The average number of sequences per sample was 22,021 and the coverage percentage was 99.82 - 99.98% (Table 4-3).

550 OTUs were clustered and classified according to the Ribosomal Database Project (RDP, training set version 9). As shown in Figure 4-3, the number of OTUs observed as a function of sampling effort in each group were described via the rarefaction curves (*130*).

The feature aggregating analysis using MetagenomeSeq in R summarized that 18 phyla, 36 classes, 64 orders, 107 families and 186 genera in total were found in our samples. Figure 4-2 presents the phylum-level relative abundance of bacterial in three groups. Histogram of bacterial composition was stacked in all the samples to the phylum level of taxonomic resolution. Firmicutes, Proteobaceria, Bacteroidetes, Actinobacteria, and Fusobacteria were the major bacterial phyla, among which Firmicutes and Actinobacteria were the most abundant phyla with 53.4%, 17.2% in the CI_sPTB samples, 60.7%, 13.5% in the CI_TB groups, and 57.0%, 18.5% in the Nm_TB samples.

4.3.3 Species richness and diversity estimates of the cervical microbiome

Alpha-diversity, the microbial biodiversity within-sample, was assessed by diversity indices *Chao1 index*, *Shannon index* and *Simpson index* which could be computed by Mothur. Chao1 index is the species richness after normalization of subsampling. Shannon index combines estimation of richness (total number of OTUs) and evenness (relative abundance). Higher Shannon index means higher biodiversity per sample. Inverse Simpson index represents the number of uniformly distributed OTUs that were required to have the same diversity as the actual community. Higher Inverse Simpson index indicates higher biodiversity in one sample (*130*). The three indices for each sample are present in Table 4-3. The three groups comparisons for alpha-diversity were also performed using the Kruskal-Wallis test, and no significant difference of the within-sample diversity was found among the three groups, with the p values of Chao1, Shannon, and Inverse Simpson indices over 0.05 (Figure 4-4).

Beta-diversity, the microbial diversity between-samples, was evaluated using *MDS*, in which sites were arranged in two dimensions to best represent the similarity values calculated from species abundance data. Samples that plot nearby have similar composition of species (*131*) (Figure 4-5). The CI_sPTB group revealed the highest sparse biodiversity among the three groups, while the CI_TB group and Nm_TB group shared the relatively similar biodiversity with each other. It implies the dysbiosis existing in CI_sPTB group, rather than the CI_TB group.

4.3.4 CST of the cervical microbiome

After the JSD metric creation, the number of clusters was validated by the Silhouette values, and the maximum of Silhouette values was at k = 6. The corresponding six clusters were depicted with similar bacterial composition and abundance (Figure 4-4) (*76, 81*). Three CSTs were mainly dominated by *L. crispatus* (CST I), *L. gasseri* (CST II) and *L. iners* (CST III). The clustered CST IV-A and CST IV-B were characterized with the deficiency of *Lactobacillus spp.* and highly diverse in taxa composition. CST IV-A had the increase *Sneathia, Prevotella, Megasphaera, Peptoniphilus, Parvimonas, Anaerococcus, Mycoplasma,* and other taxa, while CST IV-B had prominent higher relative abundance of *Atopobium* and *Gardnerella* with a few other taxa. The previously reported CST V was not observed in this study. Utilizing the JSD metric, the between-class correspondence analysis (BCA) was performed for each CST, showing a good discrimination between the CST IV-A and the CST IV- B from other CSTs (Figure 4-7a). Furthermore, PCoA analysis was performed utilizing JSD to determine the consistency of differentiation among CSTs defined by the cluster analysis (Figure 4-7b). In an agreement with cluster analysis, individuals from each CSTs formed a separate plot and could be clearly distinguished from other CSTs.

Figure 4-8 shows the CST distribution in each group. Generally, the distribution was not significantly different among women with three varied pregnancy status and six CST types probably due to the limited sample size of our study. However, some changes could be easily noticed: compared to other groups, the CI_sPTB group had a relatively lower frequency of the most protective CST I (2/10) and higher frequency

of the CST IV-A (3/10) and the CST IV-B (2/10), which is more likely on behalf of the microbial dysbiosis. On the other hand, the CI_TB group had more CST I (9/15) and less CST IV-A (0/15) and CST IV-B (1/15). Further statistical analysis showed that the frequencies of CST I and CST-IV (A, B) were found significantly different between the CI_sPTB group and the CI_TB group (Fisher's Exact test, $p = 0.035$), which might give a hint that the microbial dysbiosis may be partially responsible for the cervical intervention failure (RR 0.22; 95% Confidence Interval 0.06-0.80).

The CSTs distribution and the sPTB rates were also explored in various cervical conditions, including the dilation and short CL (≤ 25 mm) with the closed os and normal cervix (closed os, CL > 25mm) (Figure 4-9). The finding showed that: i) in pregnant women with mid-trimester cervical dilation (n = 11), except 4 women with CST I who retrieved to TB, all others delivered in sPTB, though cervical intervention had been performed; ii) in pregnant women with mid-trimester short CL (n = 14), except three women (each with CST II, CST IV-A and CST IV-B, respectively) who delivered sPTB, the rest women delivered TB. This finding indicated: i) the cervical condition is an important factor which prominently affects the clinical outcome of sPTB; ii) the *L.crispatus* might play a protective role in preventing PTB in women with dilated cervix, according to the observation that two thirds of women with CST I (4/6) delivered TB; iii) for the *L.iners dominated* (CST III) cases, the outcomes varied in women with different cervical conditions: those with dilated cervix resulted in sPTB (n = 2), while those without cervical dilation delivered TB (n = 5 for short cervix, n = 4 for normal cervix), which was consistent with the previous reports about the transitional role of *L.iners*.

4.4 Discussion

Cervical insufficiency (CI) is one of the important risk factors of PTB. However, the definition of cervical insufficiency (CI) still remains inconsistent. The official definition for CI is "The inability of the uterine cervix to retain a pregnancy in the absence of the signs and symptoms of clinical contractions, or labor, or both in the second trimester" (*1*). However, it is unclear how a clinician can objectively use this definition (*61*). In real practice, if a pregnant woman presents painless dilation and/or shortening of cervix in the second trimester of pregnancy, and results in pregnancy loss or preterm delivery, the diagnosis of CI would be considered (*112*). In this study, the mid-trimester pregnant women with asymptomatic cervical dilation and/or short cervix (CL ≤ 25 mm) were defined as CI and recruited for further study.

As described in the previous chapter one, cervical microbiome might play a crucial role in the pathogenesis of ascending infection, including IAI and sPTB for CI pregnancies. However, up till now, the cervicovaginal microbial composition remains unknown. In this part of study, cervical microbiome from dilated and/or short cervix (CL ≤ 25 mm) with sPTB or TB, and normal cervix with CL > 25 mm were explored respectively using the sequence-based technique. The species richness and biodiversity within samples and between samples were compared, and the community state type (CST) system was further established.

The microbial biodiversity analysis showed that samples in the CI_sPTB group had highly sparse dissimilarity, with a prominently different distribution from the other two groups resulted in TB. Interestingly, the distributions of the CI_TB group and the Nm_TB group were quite similar with most overlapping region, which implied

the similarity between these two groups. In other words, pregnant women who delivered TB might share the similar microbial composition.

Based on the Jensen-Shannon distance (JSD), the community state types (CSTs) were clustered at the bacterial community level. The distribution of CSTs in each group revealed a potential link between the CST and the sPTB.

Microbial dysbiosis was frequent in pregnancies delivered sPTB, yet uncommon in pregnancies delivered TB. 50% of the CI_sPTB group had a microbial dysbiosis in cervical microbiome, in which, 30% were CST IV-A, and 20% were CST IV-B. CST IV-A was characterized with increased BV-related bacteria (*Sneathia, Prevotella, Megasphaera, Peptoniphilus, Parvimonas, Anaerococcus, Mycoplasma, etc.*), and decreased *Lactobacilli*. On the other hand, CST IV-B was characterized with the prominent higher relative abundance of *Gardnerella* and *Atopobium* and some other BV-related taxa, which had been found associated with metronidazole resistant BV (*132*), and increased the risk of PTB (*133*). Microbial dysbiosis rarely occurred in cervical microbiome from TB pregnancies. No CST IV-A was detected in both CI_TB and Nm_TB groups; and only 6.7% (1/15) of the CI_TB group and 7.7% (1/13) of the Nm_TB group were found colonized by CST IV-B, respectively.

CST I is the most common community type, consisting of 55% (6/11) in pregnancies with dilated cervix, 36% (5/14) in pregnancies with short cervix and 46% (6/13) in pregnancies with normal cervix. The potential protective role of CST I to prevent PTB has been noted. All the samples from pregnancies with short or normal CL and closed os delivered TB. For pregnancies with dilated cervix, in spite of 64% (7/11) of delivered sPTB, 36% (4/11) of pregnancies with cervical dilation retrieved to TB and

all of them were colonized by CST I which is *L. crispatus* dominant. However, still 1/3 of pregnancies with CST I resulted in sPTB. Both cases were detected members of *Ureaplasma*, a type of bacteria commonly associated with IAI, chorioamnionitis and extreme preterm (*134-136*). This finding also suggests the necessity to develop the test for some specific bacteria closely related to PTB.

The *L. iners* dominated CST III with the prevalence of 18% (2/11), 36% (5/14) and 31% (4/13) in pregnancies with dilated cervix, short cervix and normal cervix, respectively. The recruited pregnancies with CST III had varied clinical outcome in various cervical conditions. In women with dilated cervix, all the pregnancies with CST III delivered sPTB, while the pregnancies of women with closed cervix delivered TB no matter the cervical length was short or normal. This observation was consistent to previous reports. In 2014, Ljubomir Petricevic *et al* assessed the dominant vaginal Lactobacillus species in healthy women in early pregnancy in relation to pregnancy outcome utilizing denaturing gradient gel electrophoresis (DGGE). It was suggested that dominating *L. iners* in early pregnancy might be associated with PTB (*85*).

L. gasseri dominated CST II had low frequency, presenting 7% in both short cervix (1/14) and normal cervix (1/13). sPTB occurred in 50% of CST II. Pregnant woman with a short cervix and CST II cervical microbiome delivered sPTB, while the one with normal cervix delivered TB. Though the sample size was limited, the observation in our study seemed consistent to the report by Hans Verstraelen *et al*. A longitudinal analysis of the vaginal microflora (VMF) was performed in pregnancy, which suggested that *L. crispatus* could promote the stability of normal VMF and

that *L. gasseri* and/or *L. iners* were more conducive to the abnormal VMF. The latter lactobacilli was found with a ten-fold increased risk of conversion to abnormal VMF than others (RR 10.41, 95% Confidence Interval 1.39–78.12, p = 0.008) (*137*).

Interestingly, a de novo CST VI with the characterized dominated *Bifidobacterium* was created for one case in Nm_TB group. Bonnie Chaban *et al* in 2014 reported two cases with Bifidobacterium dominated in the vaginal microbiome of healthy Canadian women (*138*). *Bifidobacterium* was very strict anaerobes, and was generally considered to be beneficial members of intestinal microbiome (*139*), although their role in the vaginal microbiota had not yet been elucidated. It is conceivable that Bifidobacterium, lactic acid-producing bacteria, could have a protective or health-promoting effect on the vagina analogous to Lactobacillus. Bifidobacteria also appeared to play an important role in early infant health and development (*140-142*), and their presence in the vaginal microbiota of healthy, reproductive-aged women could provide a means of transferring from mother to newborn during birth.

The composition of vaginal microbiome associated with pregnancy may have functional (that is, metabolic, immune) implications for women (*143*). In this study, the functional profiling of the cervical microbiome was predicted based on the generated OTUs using Tax4Fun. 6,443 KEGG (Kyoto Encyclopedia of Genes and Genomes database) orthologues (KO) in total were identified. Among which, 104 increased KOs and 19 decreased KOs were found in CI_sPTB group with fold change ≥ 2 compared to the other two groups (Kruskal-Wallis multiple comparison, $p < 0.05$, $q < 0.05$). The distribution of functional categories for sPTB associated KO

markers was shown in Figure 4-10. The level of metabolism, such as carbohydrate, energy, nucleotide, amino acid, cofactors and vitamins, increased prominently in CI patients resulting in sPTB, as well as the level of cell function, involving replication and repair, translation, membrane transport and signal transduction; while some other metabolic activity like lipid metabolism, secondary metabolites biosynthesis seems decreased. Figure 4-11 presents the corresponding alterations of the metabolism pathways in CI_sPTB cases. The microbial functional change could reflect the changes of cervicovaginal micro-environment. Further validation concerning the metabolites in cervicovagina might be demanded, which could highlight more about the mechanism of preterm birth. The composition of cervical microbiome would be changed due to the cervical reshaping. In this study, due to the limited sample size, the general significant change of CSTs between different cervical conditions had not been noticed. However, the microbial dysbiosis (CST IV-A and B) was much more prevalent in CI condition, which indicated that the functional deficiency of cervix may affect cervicovaginal microbiome to the dysbiosis. Further research is required to determine the causal relationship between CI and decreased stability of the microbiome.

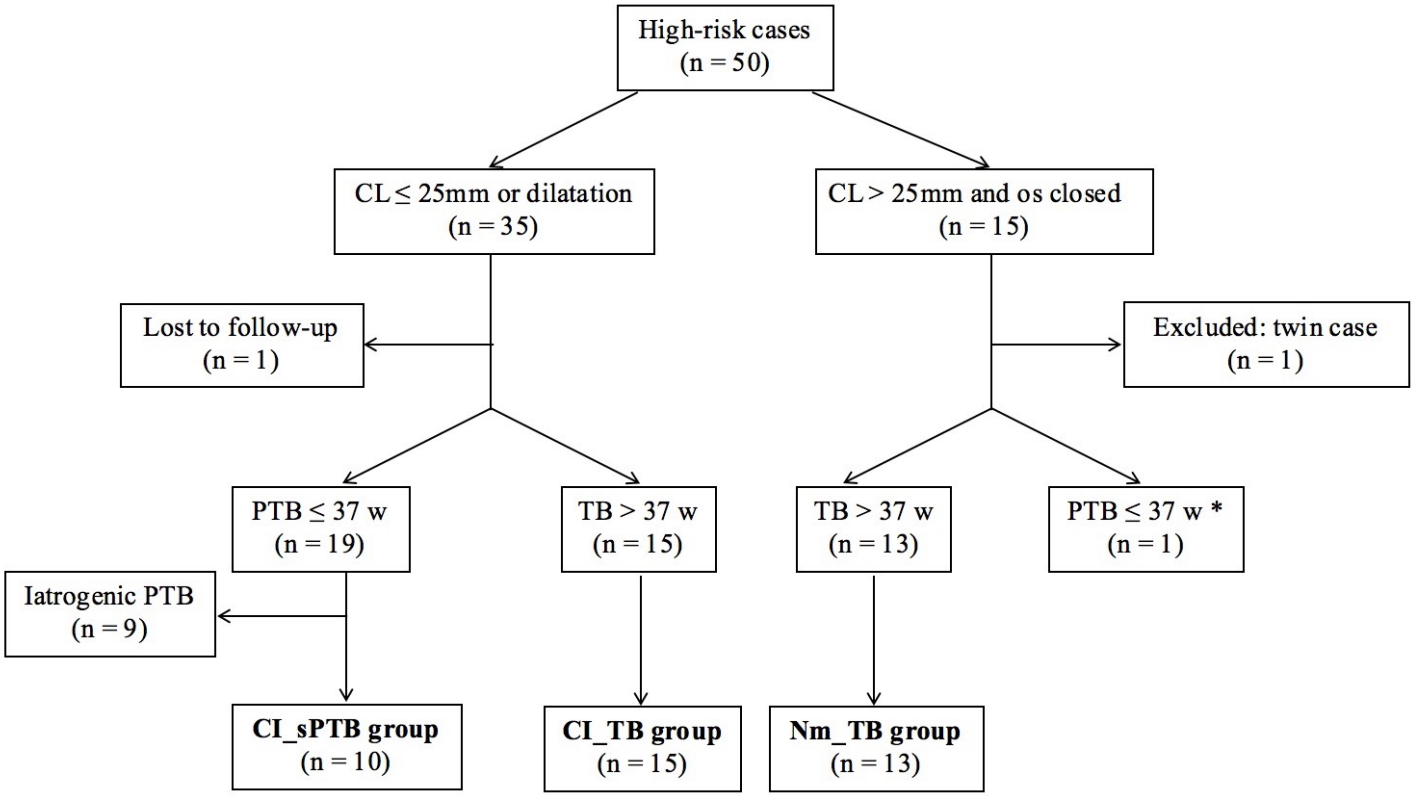
This is the first study about the cervical microbiome for pregnancy with cervical insufficiency. Technically, compared to previous reported 16S rRNA sequencing-based studies, this study has several improvements. It is more sensitive and reliable. As the more hyper-variant V4-V5 region was targeted, it could theoretically distinguish 99.8% of 9,244 typed bacterial taxa of known 16S rRNA pyrosequencing without apparent bias. The deeper sequencing also generates more features. After the sequencing data were processed, over 22,000 read counts on average were harvested

for each sample, which was ten-fold deeper than other similar reported researches, resulting in an increased coverage. It is more reliable for community state analysis. The strategy for CST generation in this study was OTU-based with higher sensitivity and reliability, while previous studies were commonly phylotype-based, which could be quickly processed but with increased classification mistakes (*144*).

In this study, the cervical bacterial community structure between CI and normal cervix was described. The microbial dysbiosis was more prevalent in pregnancy with CI with high possibility to deliver sPTB. The CI pregnancies with the *L. iners / gasseri* dominated CSTs in cervical microbiome had higher probability to deliver sPTB. These findings might help understand the pathogenesis of sPTB in CI pregnancies.

However, this study also has several limitations. Firstly, due to limited sample size, the regression analysis could not be carried out. Thus, some potential confounders, such as amniotic fluid sludge, severe funneling, the antibiotics use after cervical intervention and ethnicities/races, might affect the findings. Besides, the limited sample size of this study lacked the power to show the CST frequency change. A further validation with a larger sample size is required. Secondly, this study mainly focuses on the cervical microbiome in women with CI. To understand the mechanism better, the further exploration of the local micro-environment including the local metabolites, the immune-related cytokines, and other microorganisms like virus or fungi should be considered. More information involving the BV or AV related test should be tested. The longitudinal follow-up with cervical microbiome is also needed, which might give more information about the pathogenesis of PTB. Thirdly, though

prediction of the microbial functional profile has been made, the further survey of the cervical microbiome by the metagenome sequencing should be considered, which would provide more detailed and reliable information in microbial function.



* Not included into analysis due to the small sample size.

Figure 4-1 Patients recruited in this study.

Table 4-1 Key characteristics of participants

		CI_sPTB Group (<37wk) n=10	CI_TB Group (≥37 wk) n=15	Nm_TB Group (≥37 wk) n=13	p*	p[#]
Cervical length (mm), median (IQR)		6.95 (0.10, 19.7)	18.7 (15.0, 22.5)	29.2 (27.7, 33.8)	0.3	< 0.0001
Cervical os	**Dilated,** n (%)	7 (70.0)	4 (26.7)	0 (0.0)	< 0.0001	< 0.0001
	Closed, n (%)	3 (30.0)	11 (73.3)	13 (100.0)		
Intervention	**Cerclage,** n (%)	8 (80.0)	12 (80.0)	-	1.0	-
	Pessary, n (%)	2 (20.0)	3 (20.0)	-		
GA at sampling, median (IQR)		20.3 (18.5, 22.3)	21.6 (19.2, 22.5)	21.4 (21.1, 21.6)	0.6	0.5
GA at birth (weeks), median (IQR)		23.9 (20.6, 25.5)	38.6 (38.1, 39.7)	39.7 (39.4, 40.3)	< 0.0001	< 0.0001
Maternal age (years), median (IQR)		31.5 (30.3, 37.0)	33.0 (31.0, 35.0)	31.0 (28.0, 34.0)	0.6	0.2
Previous preterm birth, n (%)		4 (40.0)	2 (13.3)	-	0.2	-

CI, cervical insufficiency, treated with cerclage / pessary, indicated by cervical shortening (CL ≤ 25 mm) / cervical dilation;
sPTB, spontaneous preterm birth; **TB,** term birth. **GA,** gestational age; **IQR,** interquartile range.
* Comparison between group CI_sPTB and group CI_TB: Mann-Whitney rank sum test for continuous variables; Fisher exact test for categorical variables.
[#] Kruskal-Wallis test.

Table 4-2 Clinical information of participants: CI_sPTB group

Sample ID	Maternal age (years)	Cervix (dilated / closed)	Cervical length (mm)	Cervical intervention	GA at Mx (weeks)	Sampling-to-delivery (days)	GA at delivery (weeks)	GA at sampling (weeks)	Parity	History of PTB	Birthweight (g)
1MID-21	31	Dilated	41.6	cerclage	13 5/7	37	18 2/7	13	3	+	160
1MID-19	30	Dilated	18.6	cerclage	18 1/7	10	19 4/7	18 1/7	5	+	320
1MID-11	28	Dilated	0.1	cerclage	19 4/7	7	20 4/7	19 4/7	2	-	360
2MID-18	37	Dilated	0.1	cerclage	20 2/7	2	20 4/7	20 2/7	2	-	760
2MID-14	29	Dilated	0.1	cerclage	20 2/7	24	23 5/7	20 2/7	1	-	670
2MID-16	37	Dilated	23	cerclage	22 6/7	8	24	22 6/7	0	-	650
3MID-2	31	Closed	6.5	pessary	20 5/7	34	25 3/7	20 4/7	0	-	680
4MID-2	32	Closed	7.4	pessary	24 2/7	9	25 4/7	24 2/7	2	+	760
1MID-15	37	Dilated	0.1	cerclage	24	51	31 2/7	24	0	-	1780
3MID-3	35	Closed	20	cerclage	14 4/7	129	33	14 4/7	0	+	1755

GA, gestational age; **Mx,** management; **PTB,** preterm birth.

Table 4-2 Clinical information of participants: CI_TB group

Sample ID	Maternal age (years)	Cervix (dilated / closed)	Cervical length (mm)	Cervical intervention	GA at Mx (weeks)	Sampling-to-delivery (days)	GA at delivery (weeks)	GA at sampling (weeks)	Parity	History of PTB	Birthweight (g)
2MID-10	42	Dilated	0.1	cerclage	18 6/7	131	37 4/7	18 6/7	2	+	2800
4MID-3	33	Closed	22.3	pessary	19 4/7	126	37 4/7	19 4/7	1	+	2890
2MID-5	31	Dilated	20	cerclage	22 2/7	108	37 5/7	22 2/7	2	-	2800
2MID-7	36	Closed	23.8	cerclage	23 2/7	103	38	23 2/7	2	-	3800
2MID-21	34	Closed	23	cerclage	17 2/7	146	38 1/7	17 2/7	0	-	3000
4MID-14	35	Closed	18.7	cerclage	16	158	38 2/7	15 5/7	0	-	2690
1MID-6	30	Closed	22	cerclage	14	171	38 3/7	14	1	-	2970
2MID-13	30	Closed	14	cerclage	21 4/7	119	38 4/7	21 4/7	1	-	3480
1MID-5	33	Dilated	9	cerclage	22 5/7	112	38 5/7	22 5/7	3	-	3050
1MID-2	31	Dilated	16	cerclage	24	109	39 4/7	24	0	-	3100
4MID-1	38	Closed	5.5	pessary	21 5/7	126	39.71	21.71	1	-	3175
3MID-15	35	Closed	22.6	pessary	20 4/7	134	39 5/7	20 4/7	0	-	3850
2MID-15	30	Closed	24	cerclage	20 3/7	139	40 2/7	20 3/7	0	-	3300
2MID-19	34	Closed	16.3	cerclage	25 4/7	105	40 3/7	25 3/7	1	-	3220
1MID-14	33	Closed	16.2	cerclage	22 1/7	129	40 4/7	22 1/7	1	-	3840

GA, gestational age; **Mx,** management; **PTB,** preterm birth.

Table 4-2 Clinical information of participants: Nm_TB group

Sample ID	Maternal age (years)	Cervix (dilated / closed)	Cervical length (mm)	Cervical intervention	GA at Mx (weeks)	Sampling-to-delivery (days)	GA at delivery (weeks)	GA at sampling (weeks)	Parity	History of PTB	Birthweight (g)
4MID-7	39	Closed	29.2	no	-	118	38	21 1/7	1	-	3050
3MID-7	30	Closed	29.1	no	-	133	38 5/7	19 5/7	0	-	3580
4MID-8	27	Closed	31.2	no	-	129	39 2/7	20 6/7	0	-	2925
3MID-10	36	Closed	31.3	no	-	125	39 3/7	21 4/7	0	-	3136
4MID-13	34	Closed	41.5	no	-	127	39 4/7	21 3/7	0	-	2990
3MID-4	29	Closed	25.5	no	-	127	39 5/7	21 4/7	0	-	3285
4MID-4	32	Closed	25.6	no	-	127	39 5/7	21 4/7	0	-	3045
4MID-6	28	Closed	28.7	no	-	129	39 5/7	21 2/7	0	-	3220
3MID-5	31	Closed	26.3	no	-	131	40	21 2/7	0	-	3130
3MID-6	31	Closed	27.7	no	-	130	40 2/7	21 5/7	0	-	2785
3MID-14	27	Closed	43.1	no	-	133	40 3/7	21 3/7	0	-	3510
3MID-11	36	Closed	34.3	no	-	136	41 1/7	21 5/7	1	-	3320
4MID-10	28	Closed	33.8	no	-	142	41 2/7	21	0	-	3130

GA, gestational age; **Mx,** management; **PTB,** preterm birth.

Table 4-3 Sampling depth and microbial biodiversity

CI_sPTB group (n = 10)

Sample ID	1MID-11	1MID-15	1MID-19	1MID-21	2MID-14	2MID-16	2MID-18	3MID-2	3MID-3	4MID-2
Processed sampling depth	11750	11290	11489	15958	37115	32387	26891	23758	27459	23635
Coverage (%)	99.92	99.88	99.82	99.94	99.95	99.86	99.82	99.96	99.91	99.89
Number of observed OTUs (3%)	[1] 19	26	48	19	23	79	84	24	38	56
Phyla (18)	[2] 4	6	11	5	4	7	6	7	5	5
Genera (186)	[2] 12	16	23	10	8	20	25	17	13	21
Chao1 (3%)	[3] 26.08	36.95	45.70	68.41	17.11	24.45	32.25	13.44	17.63	17.46
LCI95	[4] 17.79	25.02	27.90	48.67	8.64	15.07	20.81	9.77	9.93	10.53
HCI95	[5] 64.29	83.77	115.53	131.17	57.87	66.21	78.89	35.74	55.20	52.53
Shannon index (3%)	[6] 0.39	0.13	0.54	0.36	0.01	0.03	0.08	0.07	0.12	0.18
Inverse Simpson (3%)	[7] 1.22	1.04	1.28	1.12	1.00	1.01	1.02	1.02	1.05	1.08
LCI95	[4] 1.21	1.03	1.26	1.11	1.00	1.00	1.02	1.02	1.04	1.07
HCI95	[5] 1.24	1.04	1.30	1.13	1.00	1.01	1.02	1.02	1.06	1.09

[1] OTUs: Operational Taxonomic Units at 3% nucleotide difference.
[2] Number of phyla and genera are based on taxonomic classification by Mothur, with the total number of phyla and genera detected in MetagenomeSeq.
[3] Chao1 is an estimator of the minimum richness and is based on the number of rare OTUs (singletons and doublets) within a sample.
[4] LCI95: lower limit of 95% Confidence Interval.
[5] HCI95: upper limit of 95% Confidence Interval.
[6] Shannon index: combines estimates of richness (total number of OTUs) and evenness (relative abundance). Here it returns a non-parametric estimate of the classical Shannon diversity index for an OTU definition.
[7] Inverse Simpson: represents the number of uniformly distributed OTUs that were required to have the same diversity as the actual community.

Table 4-3 Sampling depth and microbial biodiversity

CI_TB group (n = 15)

Sample ID	1MID-14	1MID-2	1MID-5	1MID-6	2MID-10	2MID-13	2MID-15	2MID-19	2MID-21	2MID-5	2MID-7	3MID-15	4MID-1	4MID-14	4MID-3
Processed sampling depth	13400	17570	13151	11863	28080	20896	32223	29623	34013	7456	34845	21355	25242	22133	14785
coverage (%)	99.90	99.94	99.92	99.96	99.95	99.96	99.94	99.97	99.95	99.91	99.95	99.85	99.96	99.97	99.96
number of observed OTUs (3%) [1]	28	13	23	11	20	16	33	15	30	17	28	49	20	15	18
Phyla (18) [2]	6	3	6	5	4	5	5	6	7	6	5	3	5	3	6
Genera (186) [2]	15	6	15	5	6	10	15	12	11	10	11	15	10	11	13
Chao1 (3%) [3]	15.89	29.30	80.45	107.06	12.01	29.44	21.20	25.40	23.44	24.82	13.68	63.48	25.22	44.63	11.33
LCI95 [4]	9.33	17.35	49.83	64.42	7.62	15.62	17.79	13.75	13.15	16.86	10.25	37.35	16.38	25.25	8.39
HCI95 [5]	49.24	78.61	180.50	239.77	36.89	85.20	39.34	74.90	69.64	60.94	34.70	152.84	63.96	117.22	30.16
Shannon index (3%) [6]	0.08	0.03	1.03	1.57	0.01	0.02	0.11	0.04	0.36	0.67	0.15	0.26	0.04	1.05	0.03
Inverse Simpson (3%) [7]	1.03	1.01	1.80	2.66	1.00	1.00	1.03	1.01	1.20	1.82	1.06	1.09	1.01	2.47	1.01
LCI95 [4]	1.02	1.00	1.76	2.58	1.00	1.00	1.02	1.01	1.18	1.79	1.05	1.08	1.00	2.44	1.00
HCI95 [5]	1.04	1.01	1.84	2.75	1.00	1.00	1.04	1.01	1.21	1.84	1.07	1.11	1.01	2.50	1.01

[1] OTUs: Operational Taxonomic Units at 3% nucleotide difference.
[2] Number of phyla and genera are based on taxonomic classification by Mothur, with the total number of phyla and genera detected in MetagenomeSeq.
[3] Chao1 is an estimator of the minimum richness and is based on the number of rare OTUs (singletons and doublets) within a sample.
[4] LCI95: lower limit of 95% Confidence Interval.
[5] HCI95: upper limit of 95% Confidence Interval.
[6] Shannon index: combines estimates of richness (total number of OTUs) and evenness (relative abundance). Here it returns a non-parametric estimate of the classical Shannon diversity index for an OTU definition.
[7] Inverse Simpson: represents the number of uniformly distributed OTUs that were required to have the same diversity as the actual community.

Table 4-3 Sampling depth and microbial biodiversity

CI_TB group (n = 15)

Sample ID	3MID-10	3MID-11	3MID-14	3MID-4	3MID-5	3MID-6	3MID-7	4MID-10	4MID-13	4MID-4	4MID-6	4MID-7	4MID-8
Processed sampling depth	25987	24048	21489	21232	22653	25170	17972	18019	29464	20480	16598	18595	26730
coverage (%)	99.94	99.94	99.97	99.98	99.98	99.98	99.97	99.93	99.96	99.97	99.86	99.96	99.97
number of observed OTUs (3%)[1]	23	26	15	12	8	13	16	20	19	14	40	23	20
Phyla (18)[2]	3	3	4	3	4	3	4	3	4	6	6	4	3
Genera (186)[2]	6	17	8	6	5	5	7	8	7	9	15	13	8
Chao1 (3%)[3]	6.34	11.55	16.54	19.65	25.50	15.38	13.93	60.59	20.46	14.68	61.85	22.75	19.51
LCI95[4]	4.26	8.62	12.44	12.15	14.98	9.60	10.55	42.62	15.65	9.53	35.99	18.64	12.34
HCI95[5]	21.50	30.44	40.06	55.99	71.61	45.97	34.35	126.86	47.42	42.65	151.44	46.17	54.51
Shannon index (3%)[6]	0.01	0.03	0.74	0.39	0.40	0.02	0.06	1.86	0.56	0.05	0.23	0.10	0.04
Inverse Simpson (3%)[7]	1.00	1.01	2.02	1.26	1.26	1.00	1.02	3.76	1.41	1.01	1.08	1.03	1.01
LCI95[4]	1.00	1.00	2.01	1.24	1.24	1.00	1.01	3.64	1.39	1.01	1.08	1.02	1.01
HCI95[5]	1.00	1.01	2.03	1.28	1.28	1.01	1.02	3.89	1.44	1.02	1.09	1.03	1.01

[1] OTUs: Operational Taxonomic Units at 3% nucleotide difference.
[2] Number of phyla and genera are based on taxonomic classification by Mothur, with the total number of phyla and genera detected in MetagenomeSeq.
[3] Chao1 is an estimator of the minimum richness and is based on the number of rare OTUs (singletons and doublets) within a sample.
[4] LCI95: lower limit of 95% Confidence Interval.
[5] HCI95: upper limit of 95% Confidence Interval.
[6] Shannon index: combines estimates of richness (total number of OTUs) and evenness (relative abundance). Here it returns a non-parametric estimate of the classical Shannon diversity index for an OTU definition.
[7] Inverse Simpson: represents the number of uniformly distributed OTUs that were required to have the same diversity as the actual community.

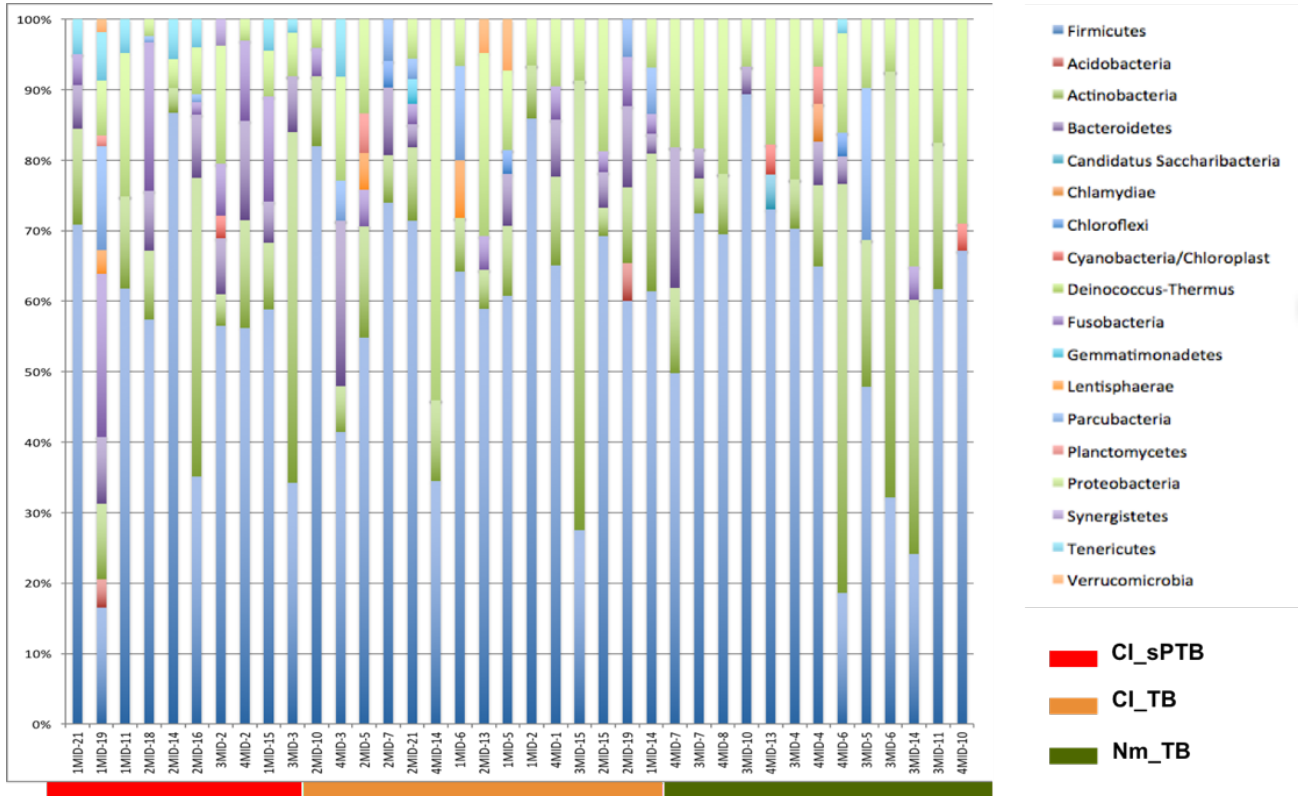


Figure 4-2 Statistics on the phylum level.
(A) Relative abundance of bacterial phyla in all individuals under study.

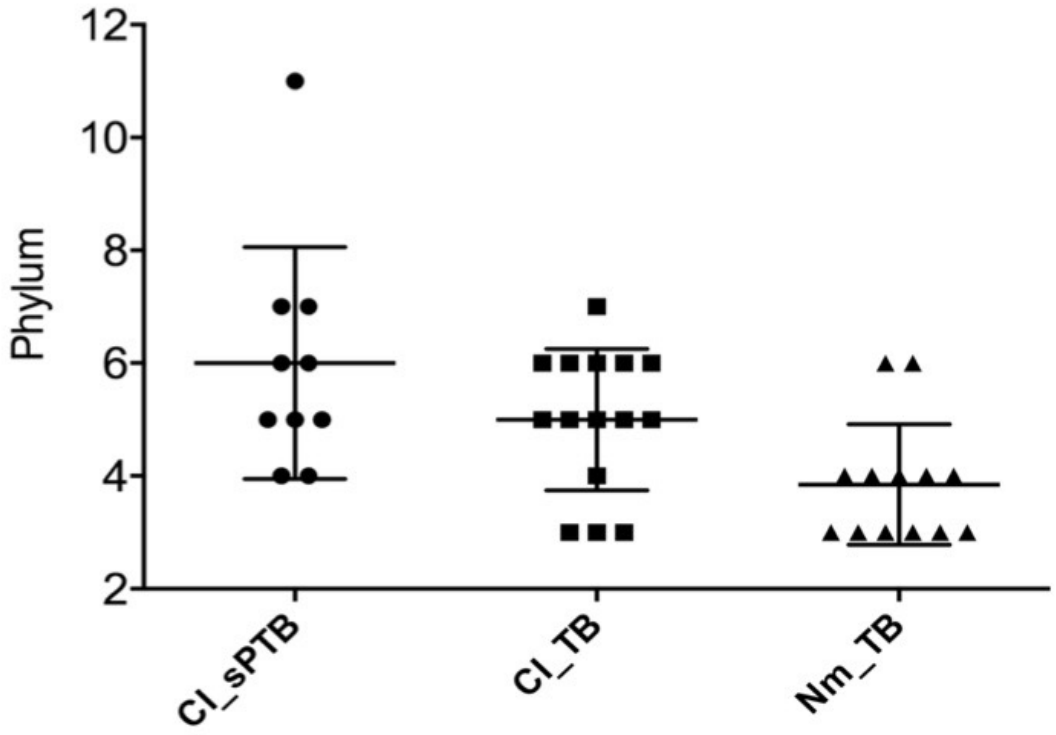


Figure 4-2 Statistics on the phylum level.
(B) Phylum numbers among the three groups.
Kruskal-Wallis test, $p = 0.006$;
Post-hoc test (SNK): CI_sPTB vs CI_TB: $p > 0.05$; CI_sPTB vs Nm_TB: $p = 0.001$; CI_TB vs. Nm_TB: $p = 0.043$.

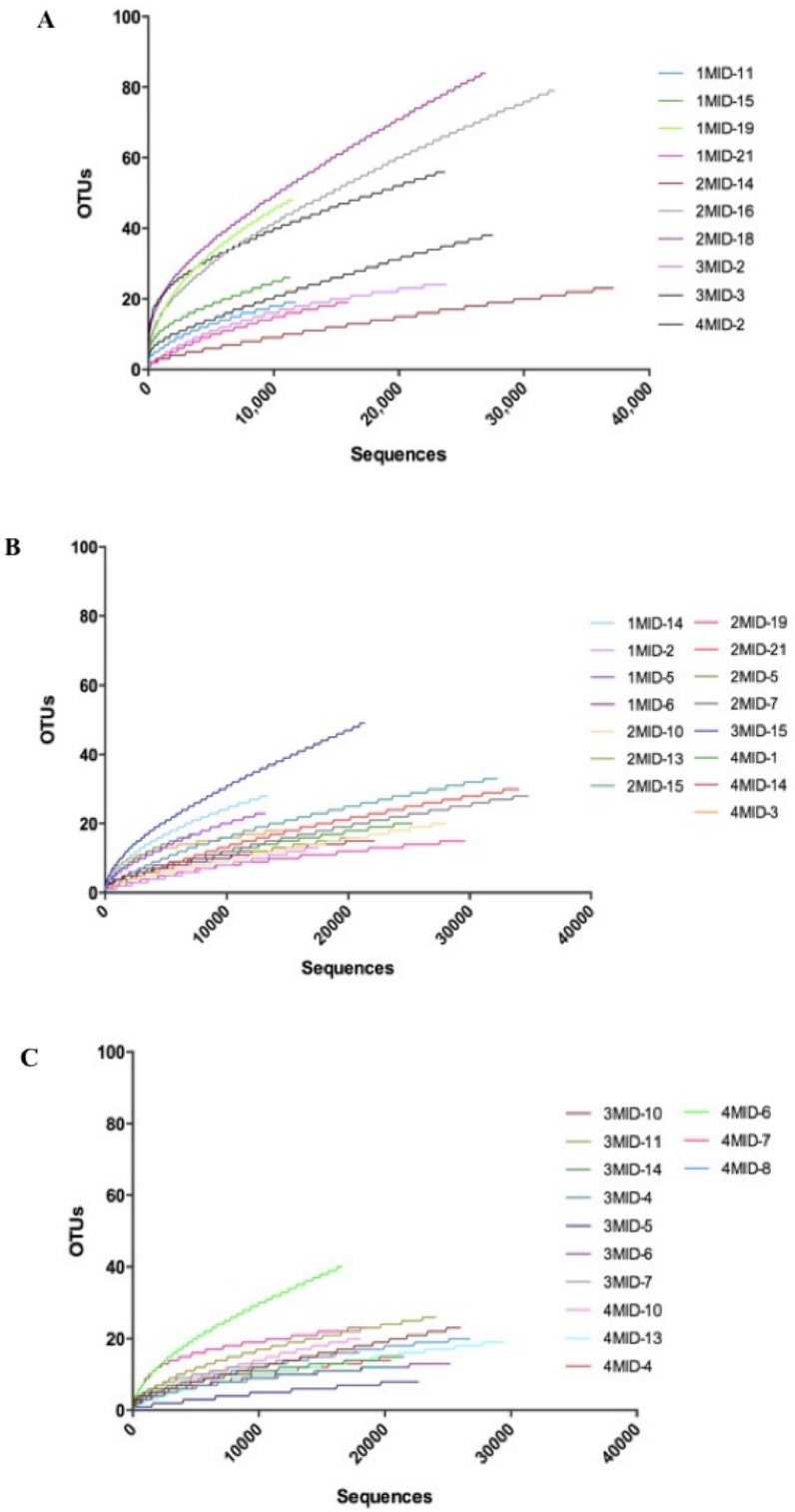


Figure 4-3 Number of OTUs as function of the total number of sequences.

(A) Rarefaction curves of individual samples for the **CI_sPTB** groups; (**B)** Rarefaction curves of individual samples for the **CI_TB** groups; (**C)** Rarefaction curves of individual samples for the **Nm_TB** groups. Based on the raw sequencing data, CI_sPTB has relatively higher richness than the other two groups.

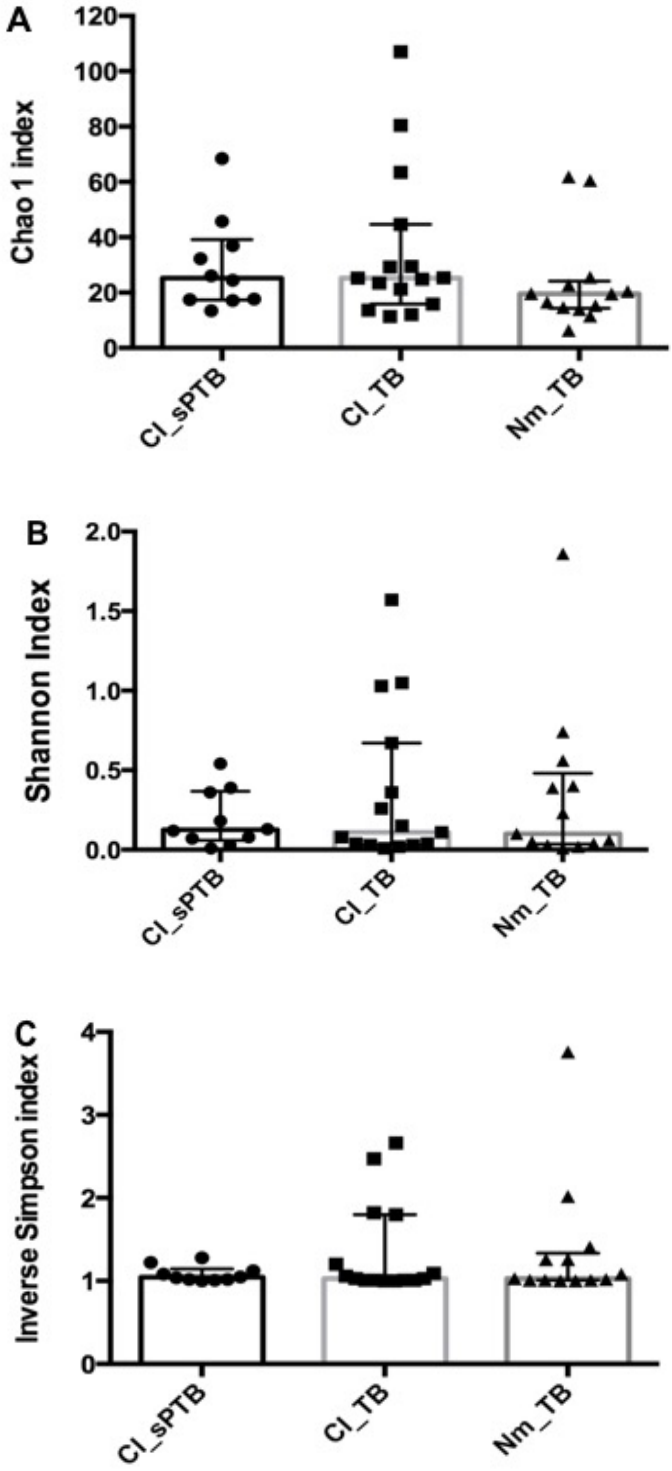


Figure 4-4 Alpha-diversity of the cervical microbiome among three groups (CI_sPTB, CI_TB, Nm_TB groups).
(A) Chao1 index (p = 0.23, Kruskal-Wallis test);
(**B) Shannon index** (p = 0.99, Kruskal-Wallis test);
(**C) Inverse Simpson index** (p = 1.00, Kruskal-Wallis test).

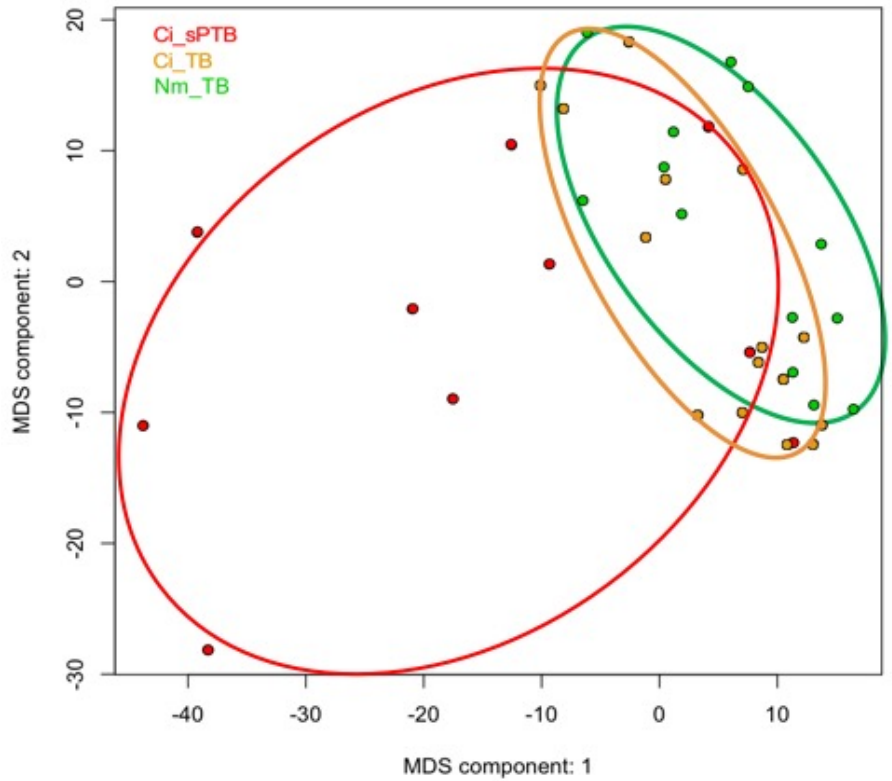


Figure 4-5 MDS analysis for recruited samples.
MDS analysis stands for the dissimilarity between communities of samples**.**
Red dots: CI_sPTB group; Yellow dots: CI_TB group; Green dots: Nm_TB group

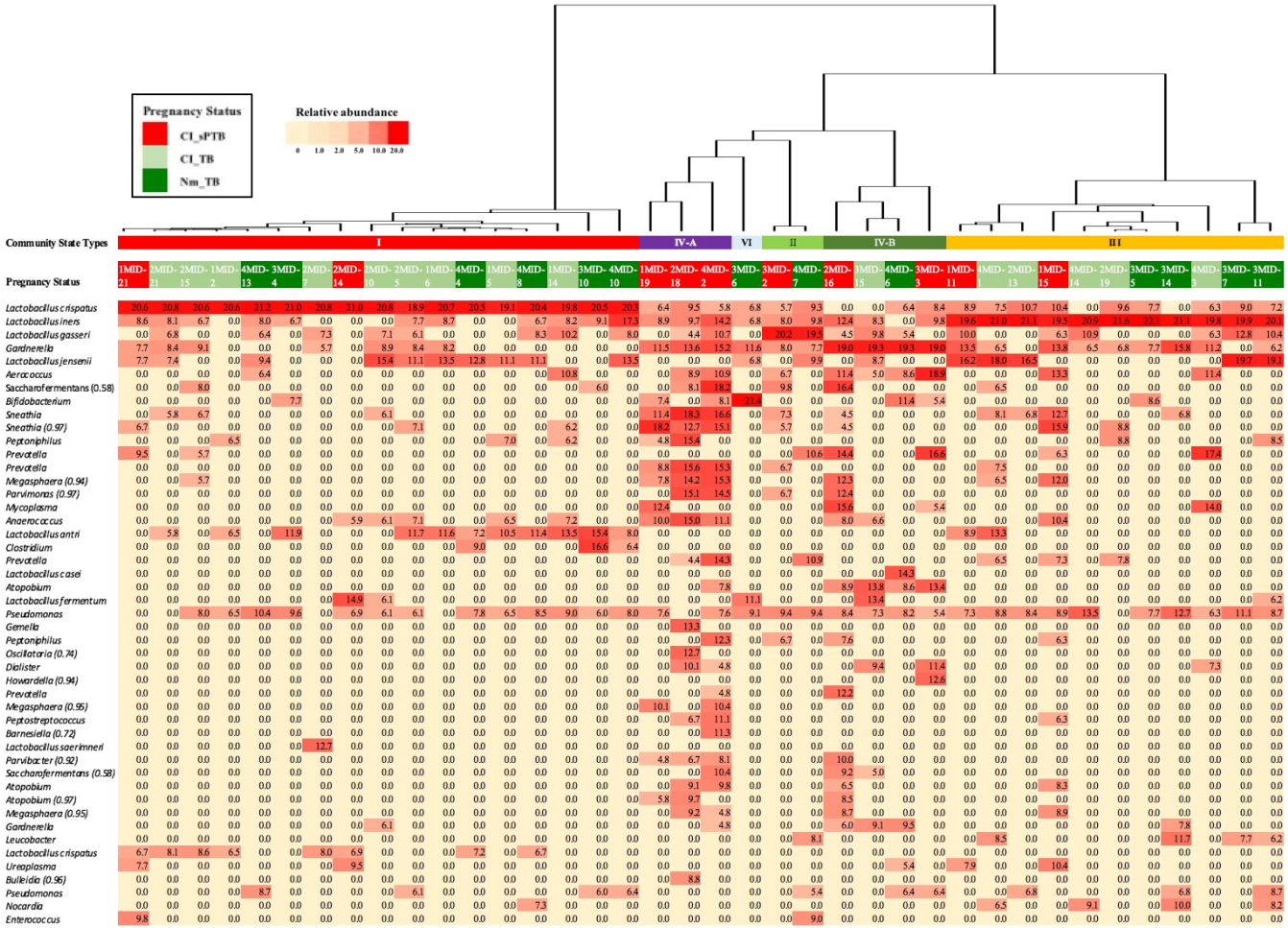


Figure 4-6 Heatmap of relative abundance of microbial taxa found in the mid-trimester cervical samples.

Ward linkage hierarchical clustering of Jensen-Shannon metric identified six CSTs (CST I, II, III, IV-A, IV-B and IV). The upper color bar shows the six CSTs, while the lower color bar shows the pregnancy status.

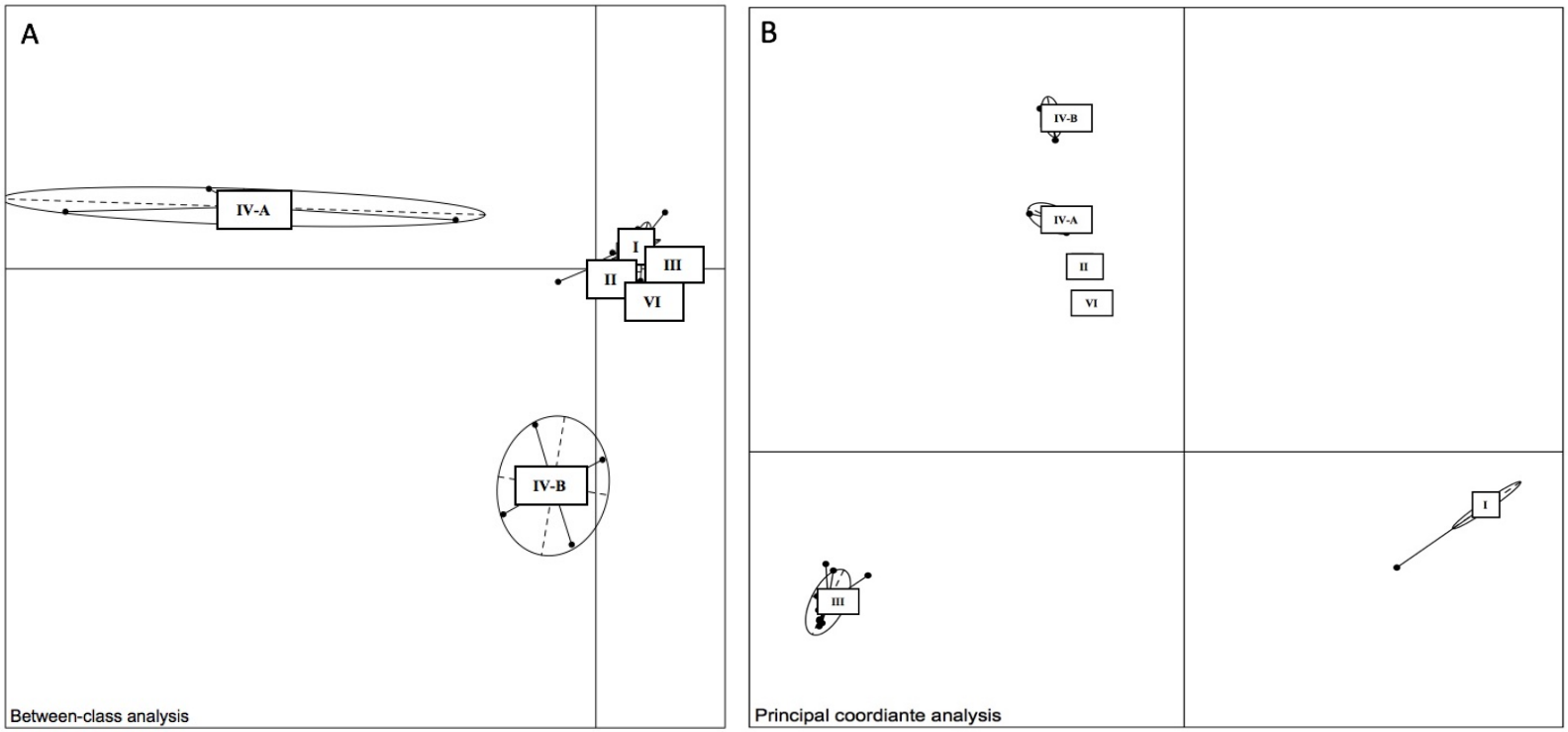
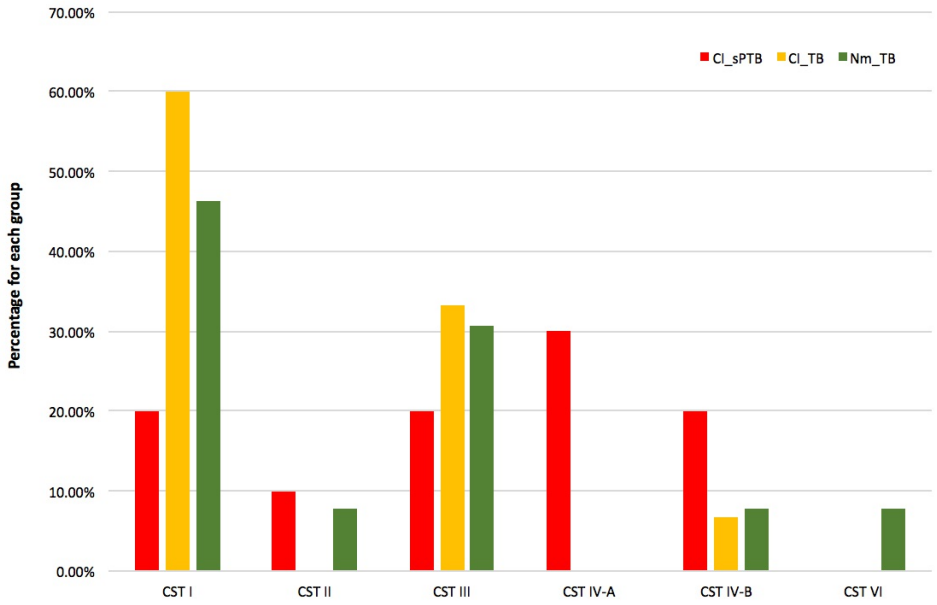


Figure 4-7 BCA and PCoA analysis for recruited samples according to JSD.

(A) The BCA for recruited samples according to JSD;
(B) PCoA of recruited samples according to JSD.



CST/Pregnancy status	I	II	III	IV-A	IV-B	VI	Total
CI_sPTB	2 (20.0%)	1 (10.0%)	2 (20.0%)	3 (30.0%)	2 (20.0%)	0 (0.0%)	10
CI_TB	9 (60.0%)	0 (0.0%)	5 (33.3%)	0 (0.0%)	1 (6.7%)	0 (0.0%)	15
Nm_TB	6 (46.2%)	1 (7.7%)	4 (30.7%)	0 (0.0%)	1 (7.7%)	1 (7.7%)	13

Figure 4-8 CSTs distribution among three groups (CI_sPTB, CI_TB, Nm_TB).

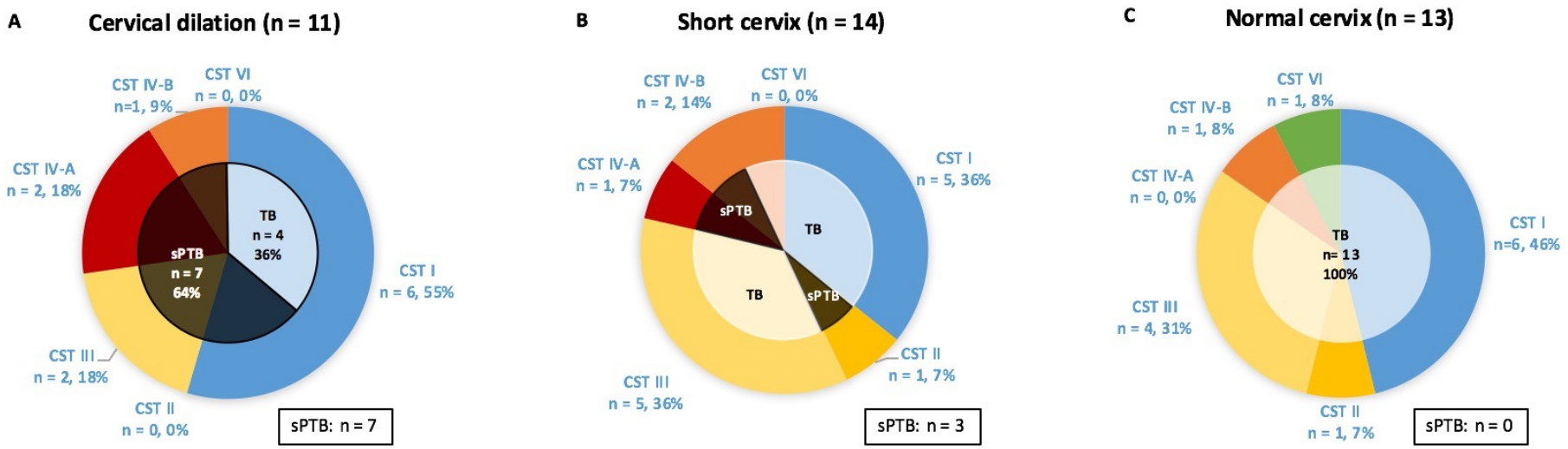


Figure 4-9 CSTs distribution and sPTB rate in various cervical condition.
(A) Cervical dilation (n = 11); **(B)** Short cervix (n = 14); (**C**) Normal cervix (n = 13).

Characteristics for each community state type (CST):
CST I: *L. crispatus* dominated; CST II: *L. gasseri* dominated; CST III: *L. iners* dominated; CST IV-A: decreased *Lactobacillus spp.* & increased BV related bacteria; CST IV-B: decreased *Lactobacillus spp.* & prominent increased *Atopobium* and *Gardnerella* with a few other BV related taxa; CST VI: *Bifidobacterium*.

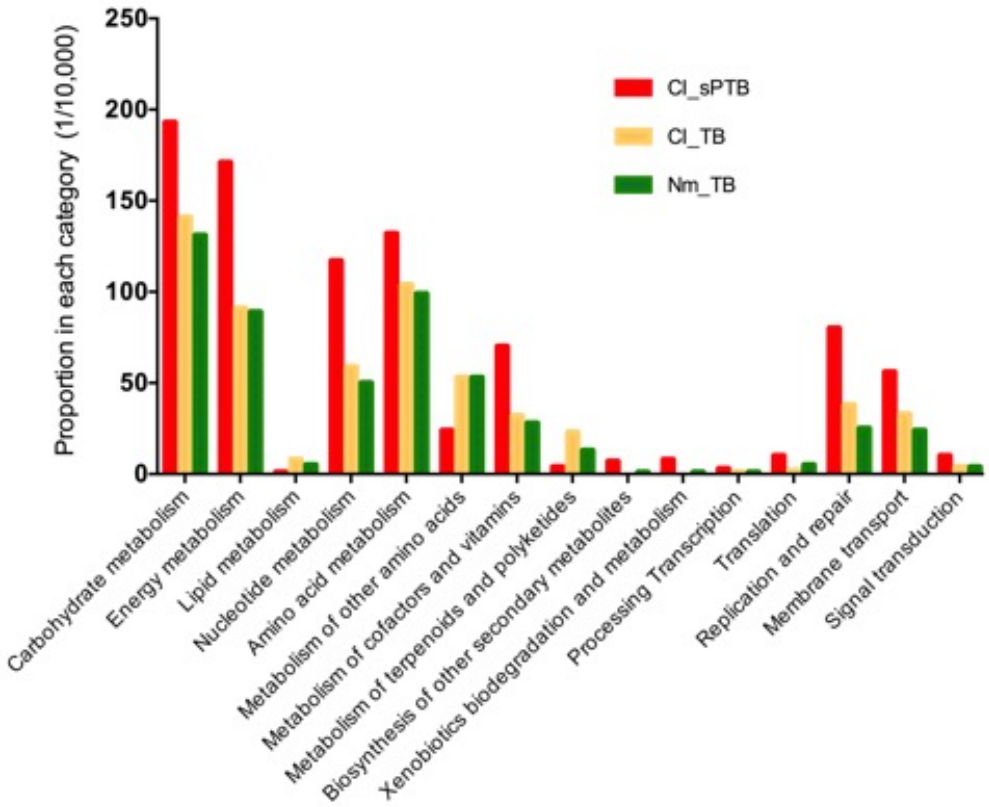


Figure 4-10 The distribution of functional categories for sPTB-associated KO markers.

CI_sPTB group has enhanced functions mainly in: i) metabolism level: carbohydrate, energy, nucleotide, amino acid, cofactors and vitamins; ii) genetic level: replication and repair, translation; iii) cell level: membrane transport, signal transduction.

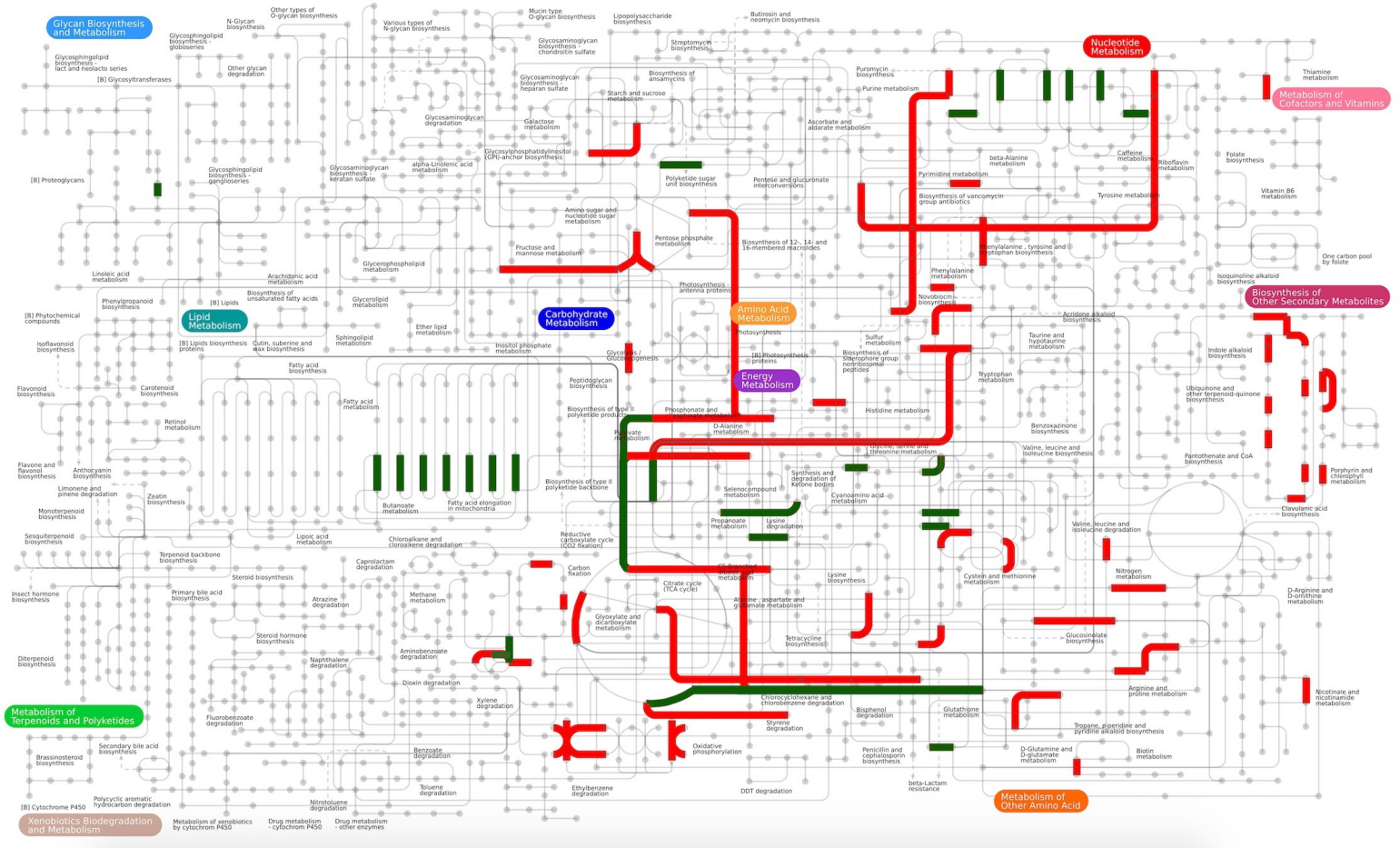


Figure 4-11 The altered functional pathways in CI_sPTB groups.
Red: Pathway with increased activity; Green: Pathway with decreased activity.

Chapter 5 Cervical microbiome signature identification for successful cervical intervention

5.1 Introduction

PTB is the major cause of neonatal mortality and morbidity (*145*). Neonates born preterm are currently facing an increasing risk of short-term complications attributed to the immaturity of multiple organ systems and neurodevelopmental disorder, such as cerebral palsy, intellectual disabilities, as well as vision and hearing impairments (*146, 147*). In Asia, the incidence of preterm birth generally varies from 7.2% to 13.6% (*148*).

Though smoking cessation, the reduction of iatrogenic preterm birth and progesterone supplementation are efficient for PTB prevention. Also, the placement of a cerclage/pessary is considered as a more direct and effective treatment, which is mainly applied to pregnant women with cervical insufficiency (CI) in the mid-trimester (*149*). However, the existing evidences show that though cervical intervention can reduce preterm birth in CI population, a certain proportion of them cannot benefit from it (*34, 111*). It implies that the current screening system of CI based on the ultrasound and history is not good enough. In addition, recent evidence has demonstrated that the cerclage might increase intra-amniotic infection, and arouse severe maternal sepsis (*111, 150*), which further emphasizes on the importance to establish a more reliable system of CI screening. Therefore, to identify

the candidates who could truly benefit from cervical intervention is crucial for clinical decision.

For years, studies in cultured bacteria, immune factors from amniotic fluid and urogenital tract have suggested the microbial-induced inflammation as one of the leading causes to preterm birth (*55*). Nonetheless, the culture-based screening and treatment strategy for BV have failed to reduce the PTB rates (*151*). Benefiting from the development of Human Microbiome Project (HMP), the microbiome in the reproductive tract of human females has been disclosed. In 2012, Gajer *et al*. described the temporal dynamics of human vaginal microbiome in 32 non-pregnant and reproductive-age women (*76*). In 2014, Hyman *et al*. showed the diversity of vaginal microbiome and its correlation with preterm birth (*152*). Recently, Aagaard *et al*. demonstrated the consistent presence of microbiome in placentas from healthy pregnancies (*153*). All the above reports imply the important role of "uncultivable" bacteria in the pathogenic mechanism of PTB, which might also be the potential marker to identify the patients who could benefit from cervical intervention.

Herein, 16S rRNA pyrosequencing, as a culture-independent tool, was performed to explore the endocervical microbiome in 25 Asian pregnant women with CI. The perinatal outcome was also observed to identify its correlation with the bacteria communities.

5.2 Methods

5.2.1 Microbial signature in CI_sPTB group

After the generation of OTUs in Mothur, the read counts of each taxon were further normalized into the $\log_2$-scaled relative abundance using the MetagenomeSeq's CSS normalization strategy (*154*), which is based on the ZIG model, and could make the differentially abundant OTUs comparable between groups of multiple samples. The nonparametric statistics (Mann-Whitney test) was performed to identify the difference of bacteria abundance between the group resulting in spontaneous preterm birth and the group resulting in term birth. Q-value was utilized for multiple testing correction in R (*155*). A microbial predictor was further developed based on these differently abundant taxa, Figure 5-2 shows the development of LA7, the The log10 scaled relative abundance of each taxon was first transformed into the linear scale. The linear scaled abundance of the seven increased taxa were summed and further transformed into the log10 scaled LA7.

5.2.2 Prediction for successful intervention

The performance of the microbial signature was then evaluated for prediction of the successful cervical intervention. The receiver operating characteristic (ROC) curve was depicted for estimation of the clinical diagnostic value. The Kaplan-Meier analysis was performed to compute the proportion of continued pregnancies in CI patients (Figure 5-3).

5.3 Results

The key clinical characteristics of the CI_sPTB group and the CI_TB group are shown in Table 4-1. To identify the difference of bacteria abundance between the CI_sPTB group and CI_TB group, nonparametric statistic (Mann-Whitney test) was performed, followed by adjustment for multiple testing using the False Discovery Rate (FDR) method. Seven taxa were found to increase significantly in the sPTB group, belonging to the genera of *Sneathia, Parvimonas, Ureaplasma, Atopobium, Peptoniphilus, Megasphaera and Paraeggerthella*, respectively ($p = 0.01$, $q = 0.044$). Figure 5-1 lists the genus information, and relative abundance of the seven bacteria amongst the 25 patients. The microbial signature LA7, which stands for the abundance of the seven differentially abundant taxa, was established as the potential predictor for the successful cervical intervention.

This internal validation showed that the combined marker LA7 presented quite different values in the CI_sPTB group and CI_TB group, with the median (IQR, interquartile range) 3.35 (2.45 - 4.71) and 0.845 (0.845 - 0.845), relatively (Mann-Whitney rank sum test, $p < 0.0001$) (Figure 5-3A). The ROC analysis showed that the area under curve reached 0.92 (95% Confidence Interval 0.79 to 1.05) ($p = 0.0005$) (Figure 5-3B). Using the LA7 > 2.26 as a threshold in defining a positive test result, the sPTB cases with 90% of sensitivity and 93% of specificity (likelihood ratio, 13.5) could be identified. To exclude the confounder from the varied intervention between cerclage and pessary, a linear logistic regression was conducted, which suggested that the cervical intervention would not affect the clinical outcome ($p = 1$).

The performance on prediction of pregnant maturity and latency was estimated via the Kaplan-Meier survival analysis. Compared to the positive cases of seven-taxa marker LA7, the LA7 negative (cutoff < 2.26) cases were more likely to retrieve to maturity with a median gestational age at 38.43 weeks vs. 23.86 weeks (Log-rank (Mantel-Cox) test, p = 0.0049, Hazard Ratio (logrank) 2.79, 95% Confidence Interval 1.708 to 12.45) (Figure 5-3C), as well as to remain undelivered for the longer period of 129 days vs. 17 days (Log-rank (Mantel-Cox) test, p < 0.0001, Hazard Ratio (logrank) 5.74, 95% Confidence Interval 15.46 to 201.6) (Figure 5-3D).

5.4 Discussion

In this part of study, seven differentially abundant bacteria were identified via the comparison between the CI_sPTB group and the CI_TB group. A cervical microbiome signature (LA7) was then developed. The pilot study reveals that LA7-positive patients delivered earlier after intervention than LA7-negative patients, which might help to distinguish the CI patients failing to benefit from the cerclage / pessary intervention.

For years, preterm birth is the major causes of poor neonatal outcomes. However, the existing prediction and prevention system mainly depends on the obstetric history of miscarriage or preterm birth and ultrasound manifestation, which only decreases around 20% of preterm birth and approximately 30% of high risk women would deliver preterm even though the cerclage provided (*34*). One objective of our study was to explore whether and which types of microbiome in CI pregnancies may be associated with subsequent spontaneous preterm birth (sPTB) (CI_sPTB group) even after intervention. Here, we used the pyrosequencing based method to has the huge

potential to deliberate the microbiological lab from the traditional culture-based procedure, and has the possibility to identify a large number of pathogens which were unavailable in old days.

Based on the homo-ethnic database, the observation on the phyla-level hinted a slight change between CI_sPTB and CI_TB groups. The decrease of *Firmicutes* and increase of *Actinobacteria* implied a clue for microbial differences in the two groups. The seven taxa identified in our present study were individually reported in previous research utilizing the fetal membrane and amniotic fluid. Hence further scientific evidence was added to our study to support the potential pathogenicity of *Atopobium (133, 156)*, *Ureaplasma* (*134, 157*), *Sneathia (158, 159)*, *Aerococcus (160)*, *Parvimonas* (*161*), *Peptoniphilus* (*162, 163*) and *Megasphaera* (*164, 165*) in preterm birth. However, except the well-known *Ureaplasma*, other species as above were usually difficult for traditional culture-based isolation, leaving a significant portion of preterm births with elusive etiology.

Unlike previous studies, our study identified the seven bacterial taxa prospectively in the endocervix of CI pregnancies. Our finding also strengthened the support for the ascending infection theory of preterm birth. Compared to the traditional method which commonly identified the pathogen in tissue, such as fetal membrane and amniotic fluid, our means could be a potential tool to predict the pathogen before intervention or childbirth, which would help the clinical decision, especially in the CI condition demanded by operational intervention.

This is a case-control study with limited sample size. Based on the results, further explorations are needed. First of all, to convince the screening effect of the seven

taxa, an external validation with larger sample size is needed. Secondly, our study has only observed the cervical microbiome in Asian, which means the conditions in other ethnicities are still unknown. Considering the diversity of microbiome in different races, the performance of the LA7 might be inconsistent with various populations. Besides, in this study, we recruited cases in both Hong Kong and Korea. The ethnic difference between these two populations could not be totally excluded and should be taken into account. Thus, we suggest the further validation through ethnicities, or taking ethnic group as a covariate of the microbiome dataset if the sample size is sufficient for regression analysis. Thirdly, though the literature implies the potential pathogenicity of the seven disclosed bacterial taxa, the exact underlying mechanism, especially their effect towards the host immune system and the metabolism of the cervicovaginal micro-environment, remains unknown. More technical tools are demanded for the exploration of human immunological alteration and the microbial metabolites. Else, based upon the novel understanding of microbiome, it is suspected that the probiotics might help improve the perinatal outcome through changing the microbial community structure. Further exploration should be planned.

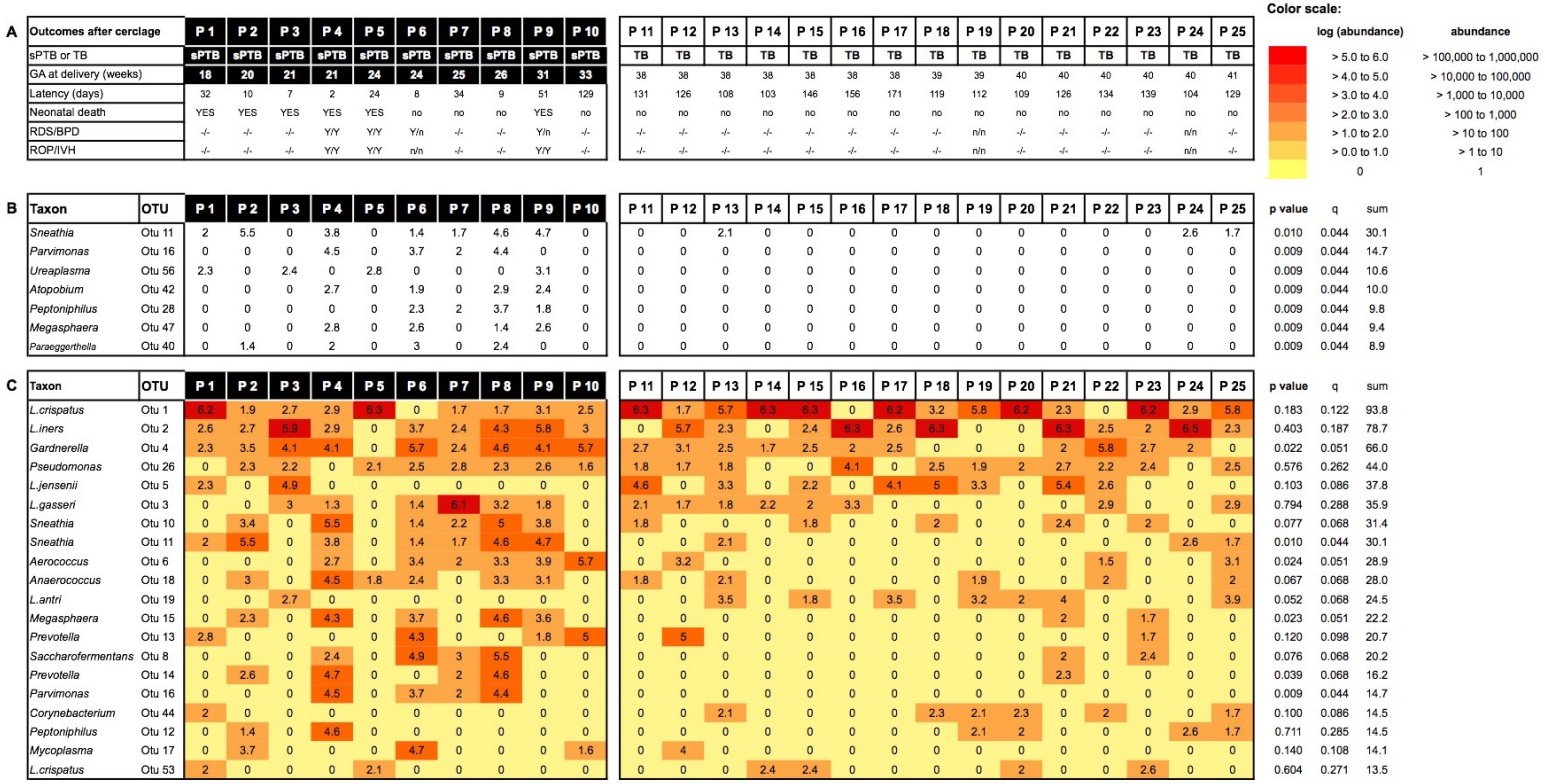


Figure 5-1 Differentially abundant bacterial taxa between CI_sPTB and CI_TB groups.

(A) The clinical outcomes of 25 patients after cervical intervention. Left: CI_sPTB group; Right: CI_TB group; **(B)** Differentially relative abundant bacterial taxa between CI_sPTB group and CI_TB group; **(C)** The twenty most abundant bacterial taxa/species identified in both groups after CSS normalization.

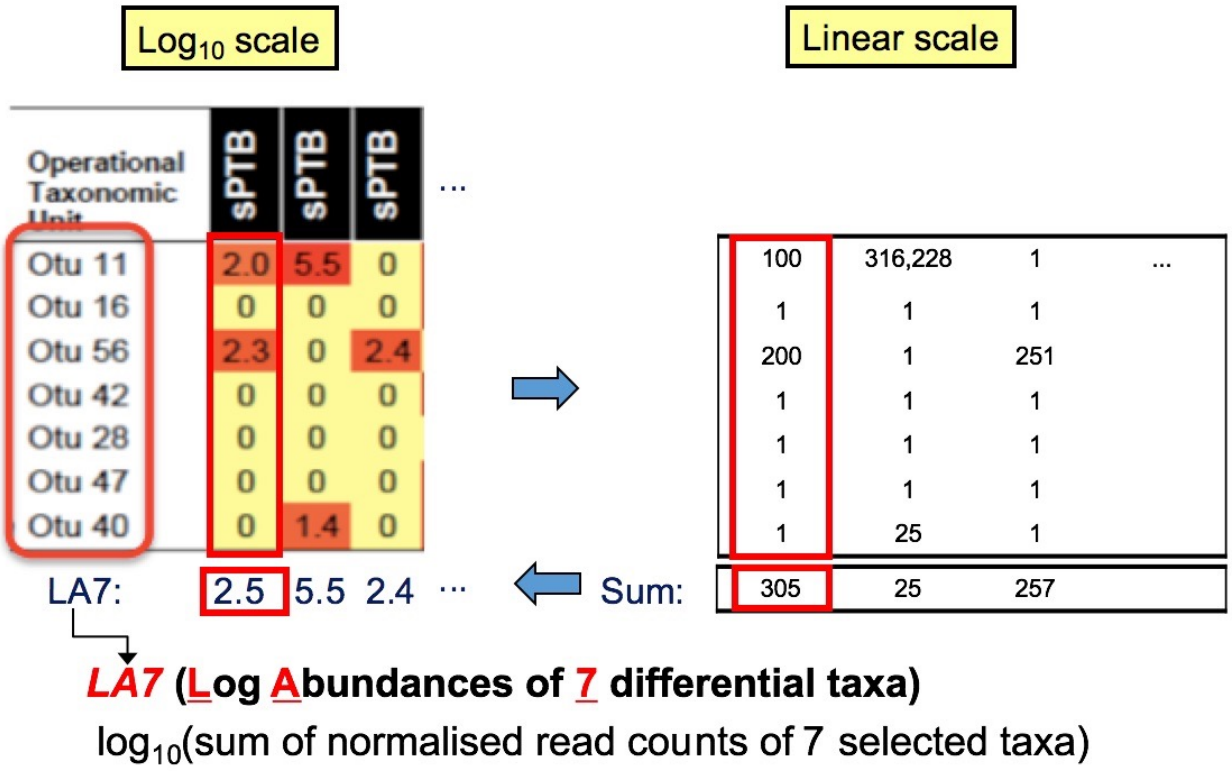


Figure 5-2 Summarization of the abundances of the seven differentially abundant taxa per sample.

The $\log_{10}$ scaled relative abundance of each taxon was first transformed into the linear scale. The linear scaled abundance of the seven increased taxa were summed and further transformed into the $\log_{10}$ scaled LA7.

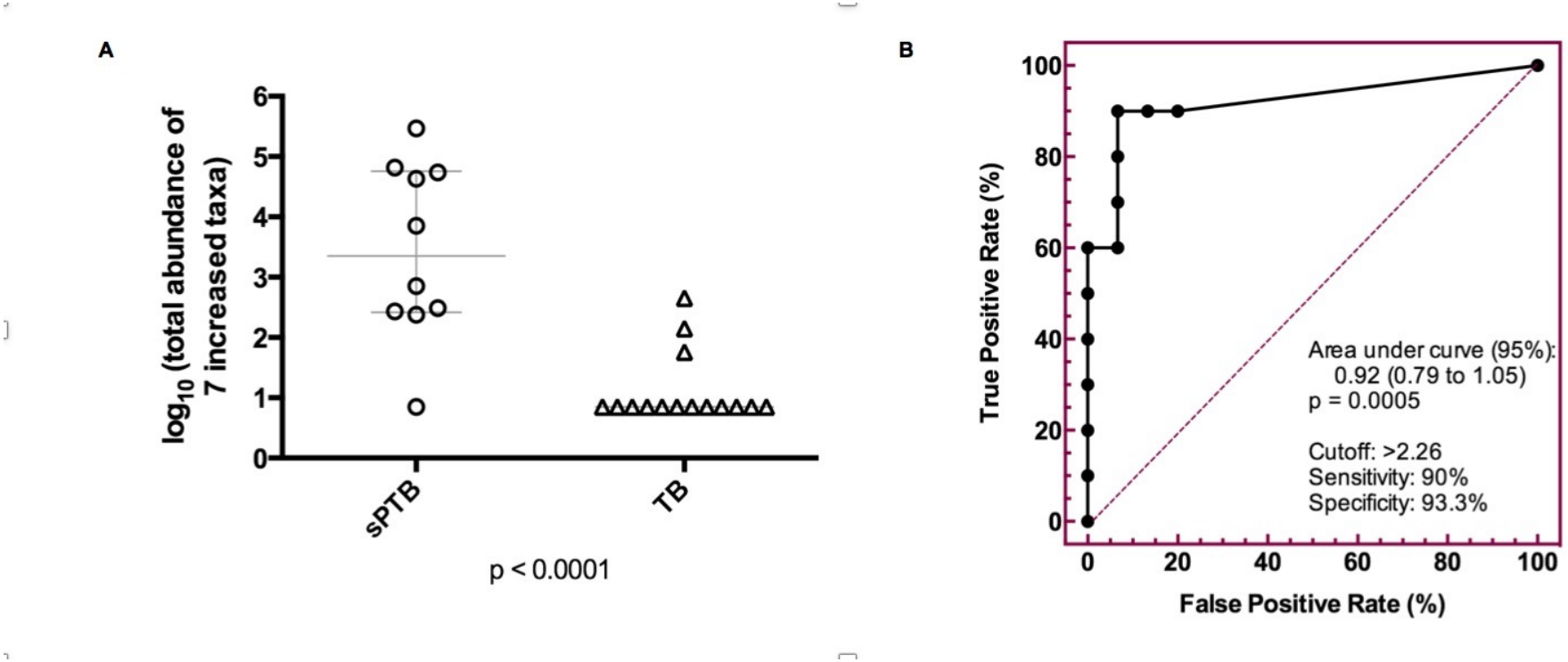


Figure 5-3 The microbial signature LA7 as the predictor for successful cervical intervention.

(A) Dot plot of LA7 in CI_sPTB group and CI_TB group, median (IQR, interquartile range) 3.35 (2.45 - 4.71) and 0.845 (0.845 - 0.845), relatively (Mann-Whitney rank sum test, $p < 0.0001$); **(B)** ROC curve of LA7 in distinguishing CI patients in the sPTB group from those in the TB group.

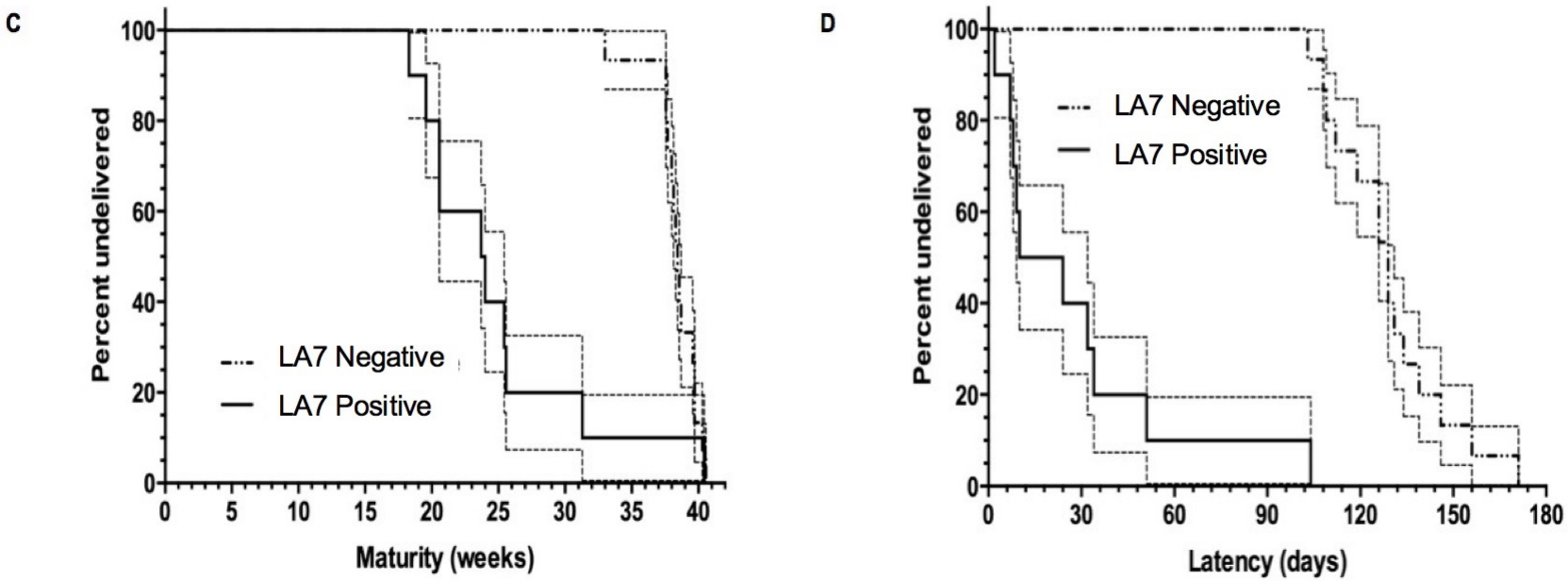


Figure 5-3 The microbial signature LA7 as the predictor for successful cervical intervention. (C) The Kaplan Meier curves of proportion of continued pregnancies in CI patients with LA7 negative (cutoff < 2.26) and CI patients with LA7 positive (cutoff > 2.26). Log-rank (Mantel-Cox) test showed the LA7 negative group have more chance to retrieve to maturity, compared to the LA7 positive group, with the median survival 38.43 weeks vs. 23.86 weeks ($p = 0.0049$, Hazard Ratio (logrank) 2.79, 95% Confidence Interval 1.708 to 12.45); **(D)** The Kaplan Meier curves of proportion of continued pregnancies in CI patients with LA7 negative (cutoff < 2.26) and CI patients with LA7 positive (cutoff > 2.26). Log-rank (Mantel-Cox) test showed the LA7 negative group have more chance to have the longer pregnant latency, compared to the LA7 positive group, with the median survival 129 days vs. 17 days ($p < 0.0001$, Hazard Ratio (logrank) 5.74, 95% Confidence Interval 15.46 to 201.6).

Section IV: Concluding remarks

Chapter 6 Conclusion and Future work

Using 16S rRNA sequencing, the characteristics of cervical microbiomes have been explored in three groups of mid-trimester pregnant women: i) the CI patients delivered sPTB, ii) the CI patients delivered TB, and iii) the normal pregnant women with normal cervical length and delivered TB. From which, we noticed that:

i) Compared to the other two groups, the CI patients delivered sPTB owned the cervical microbiome with higher biodiversity, which indicates the microbial dysbiosis existing;

ii) The microbial composition in varied cervical condition might be related to the clinical outcome, in which, the *L. crispatus* dominated composition (CST I) is most stable with less prevalent sPTB, while the BV-associated microbial status is with the highest risk of PTB;

iii) Seven bacterial taxa (LA7) were identified to significantly increase in the CI_sPTB group than the CI_TB group, which reveals the potential clinical application in predicting sPTB after cerclage / pessary intervention.

Several works should be done in future:

i) To further explore the underlying mechanism of ascending infection and intra-amniotic infection, the simultaneously monitoring of the immunologic condition should be estimated in pregnant women with CI;

ii) To further investigate the pathogenesis of sPTB and the potential protective mechanism based on varied cervical microbial compositions, the animal-based study should be considered;

iii) To validate the predictive value of the seven bacterial taxa in successful cervical intervention for CI patients, more mid-trimester pregnant women would be recruited.

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