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Characteristics of the vaginal microbiota associated with recurrent spontaneous preterm birth: a prospective cohort study

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Abstract

Background Failure to understand the causes of recurrent spontaneous preterm birth (sPTB) has limited effective interventions. This study aimed to examine the relationship between the vaginal microbiota and the risk of sPTB recurrence.

Methods A prospective cohort study was conducted involving 152 pregnant women at a high risk of sPTB recurrence due to a history of sPTB between 16 and 27^{+6} weeks of gestation. Vaginal swabs were collected sequentially during early pregnancy (before 16 weeks) and late pregnancy (16–24 weeks) for 16 S ribosomal RNA (16 S rRNA) sequencing. The vaginal microbiota was subsequently compared between the recurrence and non-recurrence groups and the results analysed longitudinally.

Results Fifty-three (34.9%) participants experienced recurrent sPTB. Using random forest classification models and the linear discriminant analysis effect size method, *Lactobacillus iners* (*L. iners*) and *Lactobacillus crispatus* (*L. crispatus*) were identified as featured species that distinguished the recurrent group from the non-recurrent group. Following the hierarchical clustering of the vaginal microbiota into six community state types (CSTs), in the recurrent group, CST III (dominated by *L. iners*) was more prevalent in early pregnancy, whereas in late pregnancy, CST IVA and CST IVB (dominated by *nonlactobacilli*) were more prevalent. In contrast, CST IA (dominated by *L. crispatus*) was more prevalent in the non-recurrent group. The six CSTs was simplified into three vaginal community types, *L. iners* dominant type exhibited decreased instability and a greater likelihood of transitioning to the non-*lactobacillus* dominant type compared with other (non-*iners*) *Lactobacilli* dominant types.

Conclusions *L. iners* dominance in the vaginal microbiota before 16 weeks of gestation is associated with an increased risk of recurrent sPTB, partly because of its propensity to transition to an unfavorable non-*Lactobacillus*-dominant state. This finding highlights the potential role of vaginal microbiota as an intervention target for reducing the risk of recurrent sPTB in early pregnancy.

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Keywords Spontaneous preterm birth, Recurrence, Vaginal microbiota, Vaginal community state type, *Lactobacillus iners*, Dynamics

Introduction

Preterm birth (PB) is the primary cause of death in children aged < 5 years. Approximately 1.1 million preterm babies die annually [1], and 65–75% of all PBs are spontaneous [2]. Providing both short- and long-term care to preterm newborns has economic significance. Moreover, preterm infants are more likely to experience neurobehavioral abnormalities [3].

Spontaneous preterm birth (sPTB) tends to reoccur with a recurrence rate of 15% to > 50%, which is inversely correlated with the number of gestational weeks in the most recent PB [4]. Unfortunately, the recommended prophylactic treatments, including cerclage and the use of progesterone [2, 5] show limited effectiveness, mostly because of an incomplete understanding of the factors causing sPTB recurrence. sPTBs have various probable causes, and despite thorough analysis, no clear reason has been identified in approximately half of the affected patients [6]. Investigating risk factors for recurrence is essential for effective prevention.

Microbial invasion of the amniotic cavity is a significant contributor to approximately 25–40% of sPTB [7]. Some studies have speculated that intrauterine infections are primarily caused by the ascent of vaginal bacteria into the uterine cavity [7]. Studies on the association between bacterial vaginosis (BV) and sPTB have revealed the influence of vaginal microbiota (VMB) on the risk of sPTB [8]. The relationship between sequencing-based VMB and sPTB was demonstrated using 16 S rRNA sequencing [9–20]. However, the effects of VMB disruption on the risk of sPTB recurrence have not been thoroughly studied. Several studies have investigated the features of recurrence-associated VMB in pregnant women with a history of sPTB; however, the conclusions were inconsistent [9, 12, 21]. The samples used in the investigations were obtained after approximately ≥ 16 weeks. Obtaining samples early in pregnancy will be beneficial for studying the characteristics of recurrence-associated VMB during the early stages of pregnancy. The participants in these studies were women who had preterm deliveries before 34 or 37 gestational weeks. sPTB recurrence rates in women with a history of sPTB before 28 gestational weeks are high with an early onset, thereby substantial challenge necessitating a careful consideration.

Traditionally, a healthy VMB is characterized by low bacterial diversity and dominance by *Lactobacillus* species, including *Lactobacillus* (*L.*) crispatus, *L. gasseri*, *L. iners* , and *L. jensenii* [22, 23]. Diverse VMB, particularly those dominated by non-*Lactobacillus* (non-*L.*) species, has been associated with an increased risk of sPTB, with effects varying by ethnicity [16, 20]. Although the protective effects of *Lactobacillus* species are well-documented, some studies have identified an association between the dominance of vaginal *L. iners* and an elevated risk of sPTB [9, 13, 15, 24]. In contrast, others have not found such relationship [16]. Furthermore, although the VMB is generally stable, it is dynamic. Studies have demonstrated that vaginal *L. iners* exhibited a less stable dominance than *L. crispatus* [25, 26].

In this study, we hypothesised that certain VMB characteristics would persist among high-risk women and contribute to an increased risk of recurrence. Therefore, this study aimed to investigate the VMB characteristics associated with sPTB recurrence in early pregnancy, in women with a history of spontaneous births before 28 weeks of gestation and examine the longitudinal dynamics of these characteristics.

Methods

Study cohort and sample collection

This study aimed to investigate the VMB characteristics associated with sPTB recurrence in early pregnancy, in women with a history of spontaneous births before 28 weeks of gestation and examine the longitudinal dynamics of these characteristics. A cohort of pregnant women was prospectively recruited between October 2018 and December 2022 at the Shanghai First Maternity and Infant Hospital. This study involved high-risk women with a history of miscarriage, birth following spontaneous labour, or preterm pre-labour rupture of membranes at 16^{+0}–27^{+6} weeks. The inclusion criteria include a singleton pregnancy and presenting to the hospital before 16 weeks' gestation. Exclusion criteria include (1) history of cervical surgery; (2) major foetal anomalies; (3) positive for human immunodeficiency virus or hepatitis C virus status; (4) use of antibiotics, steroids, or illegal drugs; and (5) severe obstetric or medical problems. For previous spontaneous second-trimester miscarriages or PB and pregnancy outcomes, the lower limit of 16 weeks was used because the mechanism of spontaneous pregnancy loss at gestational weeks 16–19 may be similar to that at gestational weeks 20-26 [27]. Parity was defined as

the number of pregnancies that progressed to at least 20 weeks of gestation.

Data on gestational age at sampling, interventions for PB, pregnancy complications, and gestation at birth were obtained from the hospital information system. The intervention choice was at the attending clinician's discretion according to the related guidelines and participant consent. Other exclusion criteria included loss to follow-up, caregiver-initiated PB, or failure in the quality control of the VMB data. Finally, 152 participants were included in the study (Fig. 1).

Vaginal swabs were obtained from pregnant women before 16 weeks of gestation (designated as early pregnancy) and 16–24 weeks of gestation (designated as late pregnancy) for 16 S rRNA sequencing. No sexual intercourse or vaginal hygiene practices within the 4 weeks before sample collection. Vaginal swabs were obtained from the posterior fornix using Dacron swabs by a research coordinator. These swabs were immediately frozen at $-80°C$ until shipping. The samples were transported on dry ice.

Sequencing, processing, and taxonomic assignment

Total bacterial DNA was extracted from the ectocervical swabs using the HiPure Stool/Soil DNA Mini Kit (Magen Biotechnology Co., Ltd., China). Polymerase chain

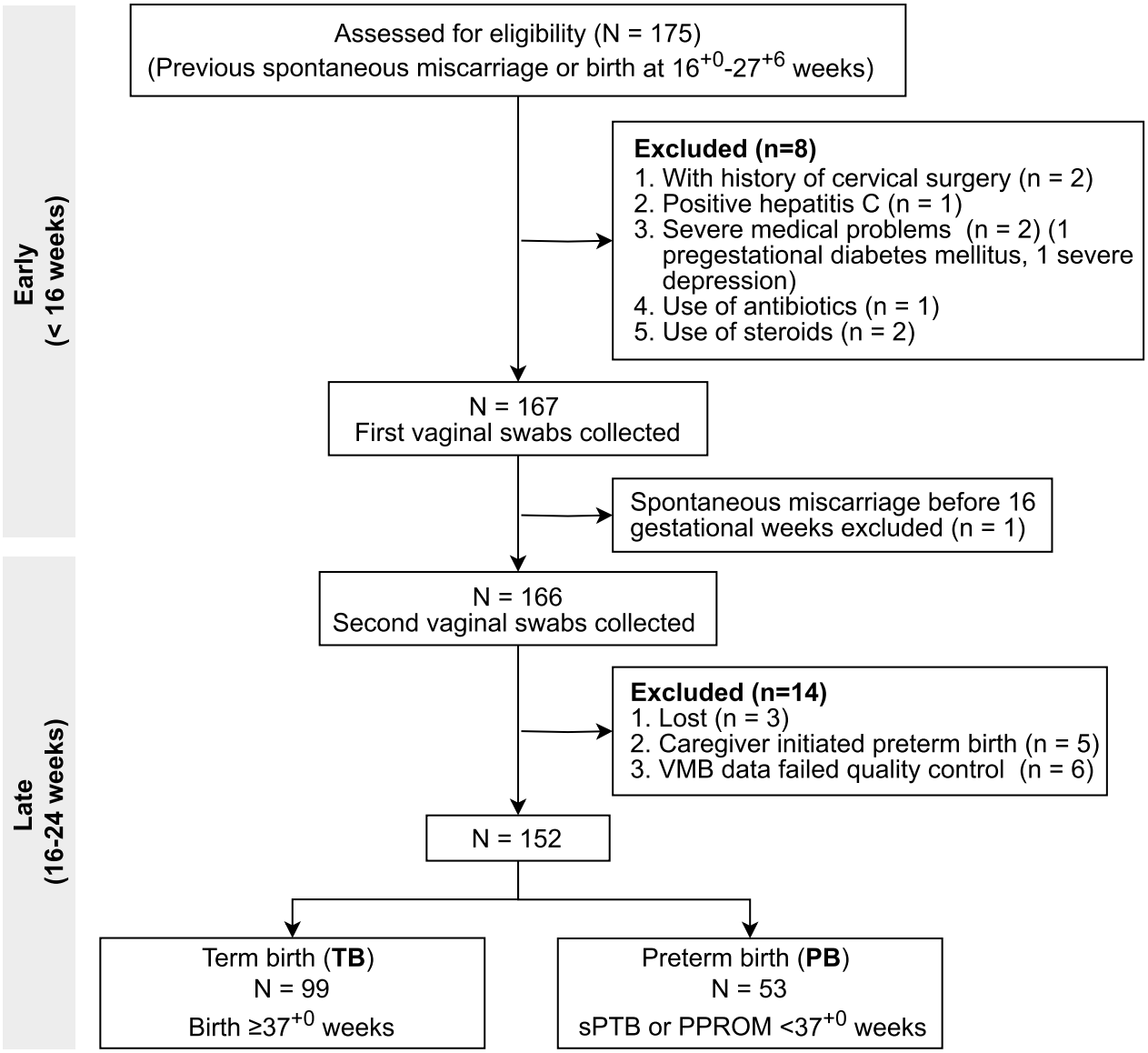

Fig. 1 Overview of participant recruitment and sample collection process

reaction amplification of the V3-V4 variable regions of the 16 S rRNA genes was performed using the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). Amplicons were visualized on a 2% agarose gel, quantified, and purified before they were loaded on the Illumina MiSeq PE300 platform to generate 300 bp paired-end reads (SY-410-1003, Illumina, San Diego, CA, USA). All samples were sequenced in a single batch. Negative controls were established during the sampling and library construction. Quality control of the reads was performed using R package DADA2. The reads were filtered and trimmed before denoising, merging, and removing chimeric sequences. Amplicon sequence variants (ASVs) were annotated using RDP Classifier v2.2 SILVA138 with a confidence threshold of 0.8. All the samples were resampled to equal sequencing depths. ASVs with less than 0.001% of the total sequence across all samples were discarded.

Bioinformatic and statistical analyses

Variations in microbial communities (beta diversity) between the sPTB and term birth (TB) groups during early and late pregnancy were visualized using Robust Aitchison principal coordinate analysis via the cmdscale R function [28]. Ellipses were 95% confidence intervals. Differences in beta diversity were tested using permutational multivariate analysis of variance (PERMANOVA) via the adonis2 function in the vegan package. Alpha diversity indices (Observed, Chao1, Shannon, Simpson) were calculated using the estimate_richness function in phyloseq, and the Mann–Whitney U test was used to compare these measures between groups. To identify featured species, we utilized random forest models to prioritise variables with importance according to the mean decrease in the Gini index. The linear discriminant analysis effect size (LEfSe) method was used to calculate the effect sizes. LEfSe analysis was conducted using the Microeco package, with an effect size threshold set at a logarithmic linear discriminant analysis (LDA) score of 2.0 for early pregnancy and 4.0 for late pregnancy. Feature selection was also conducted using support vector machine based on recursive feature elimination (SVM-RFE) with a linear kernel [29]. The relative abundances of *Lactobacillus species* were compared using the Mann–Whitney U test following a centred log-ratio (CLR) transformation. To control for false discovery rate, the Benjamini-Hochberg procedure was applied to adjust the P values. A species-level correlation network analysis was conducted using the SparCC algorithm via the ggClusterNet package, based on the top 25 species and a correlation threshold of 0.1 at P-value 0.05 [30].

Jensen-Shannon divergence was employed as a metric to quantify between-sample differences in microbial composition, and hierarchical clustering with Ward's linkage method was used to categorise each sample into one of six vaginal community state types (CSTs) using the vegan package. The composition of the CSTs is illustrated using heatmaps based on the top 20 species. The prevalence of CSTs between the sPTB and TB groups was compared for early and late pregnancies using the chi-square test or Fisher's exact test. The time to delivery of CSTs was visualized using Kaplan-Meier curves generated using the ggsurvplot function in the Survminer package. Hazard ratios with 95% confidence intervals and P values were estimated using the Cox proportional hazards model with the Cox function.

To facilitate longitudinal dynamic analysis, the CSTs were classified into three vaginal community types (vagitypes) based on their composition: *L. iners* vagitype (dominated by *L. iners*), other-*L.* vagitype (dominated by non-*iners L.*), and non-*L.* vagitype (dominated by the non-*L.* species). The transitions among these three vagitypes from early to late pregnancy were illustrated using a Sankey diagram generated using the ggSankeyGrad package, and transition heatmaps were produced using Seaborn in Python.

Results

Participants

Among the 152 participants identified to be at a high risk of sPTB, 28.9% (44/152) had a history of second trimester spontaneous miscarriage between 16 and 19^{+6} weeks, while the remaining participants had a history of sPTB between 20 and 27^{+6} weeks of gestation. In the current pregnancy, sPTBs before 37 weeks occurred in 34.9% (53/152) of the participants (mean 27^{+1} weeks, standard deviation (SD) ± 6^{+3} weeks, range 16^{+3}–36^{+4} weeks). Participants with TB and PB had comparable demographic characteristics (Table 1). Vaginal swabs were longitudinally collected from 152 participants before 16 weeks (152 samples, mean 12^{+4} weeks, SD ± 1^{+5} weeks, range 6^{+2}–15^{+4} weeks) and between 16 and 24 weeks (152 samples, mean 20^{+1} weeks, SD ± 2^{+0} weeks, range 16^{+1}–23^{+4} weeks).

Investigating the characteristics of recurrence-associated VMB

A total of 304 samples were analysed, and the bacterial communities differed between the TB and PB groups during early (PERMANOVA F= 2.326; R2= 0.015, P=0.014) and late pregnancy (PERMANOVA F= 3.463; R2= 0.023; P=0.001) (Figure 2A) at the species level. Alpha diversity (Observed, Chao1, Shannon, Simpson) differed between the PB and TB groups in late pregnancy, whereas no significant difference was observed in early pregnancy (Additional file 1 and Figure 2B).

Table 1 Demographic characteristics of the term and preterm birth groups

Characteristic	Term birth (n = 99)	Preterm birth (n = 53)	P value
Age, years, mean ± SD	33.1±4.2	32.2±3.5	0.164
BMI, kg/m^2, mean ± SD	22.9±3.8	23.6±3.7	0.286
Gravidity, median (IQR)	2 (1-2)	2 (1-2)	0.431
Parity, median (IQR)	1 (0-1)	1 (0-1)	0.861
Gestation of previous spontaneous pregnancy loss[a]			0.953
16^{+0}–19^{+6} weeks	28 (28.3)	16 (30.2)	
20^{+0}–27^{+6} weeks	71 (71.7)	37 (69.8)	
Infertility treatments	15 (15.2)	9 (17.0)	0.951
Gestational diabetes	16 (16.2)	10 (18.9)	0.844
Gestation at sample (weeks), *mean* ± *SD* (range)			
< 16^{+0} weeks	$12^{+4} \pm 1^{+5}$ (6^{+2}–15^{+4})	$12^{+5} \pm 1^{+6}$ (8^{+2}–15^{+3})	0.439
16^{+0}–24^{+0} weeks	$20^{+0} \pm 2^{+1}$(16^{+1}–23^{+4})	$20^{+1} \pm 1^{+6}$ (16^{+1}–23^{+4})	0.894
Intervention			
Progesterone	34 (34.3)	25 (47.2)	0.170
Cerclage	44 (44.4)	21 (39.6)	0.689
Post-intervention samples			
Post-progesterone samples[b]	25/198 (12.6)	19/106 (17.9)	0.280
Post-cerclage samples[b]	36/198 (18.2)	14/106 (13.2)	0.341
GA at delivery, weeks, median (range)	39^{+0} (37^{+0}–41^{+0})	26^{+6} (16^{+3}–36^{+4})	< 0.001

BMI body mass index; GA, gestational age, *IQR* interquartile range, *PB* preterm birth, *SD* standard deviation, *TB* term birth

The data are expressed as n (%) for categorical variables

[a] Refers to the latest miscarriage or preterm birth

[b] The proportion refers to the ratio of post-intervention samples to the total number of samples collected during both early and late pregnancy

P-values were determined using the t-test (normally distributed) or Mann–Whitney U test (non-normally distributed) for continuous variables and the chi-squared test or Fisher's exact test for categorical variables

Ranking of species based on the importance in the random forest models as determined by the mean decrease in Gini scores (Figure 2C) and LDA scores from the LEfSe analysis (Figure 2D) demonstrated that the most abundant *Lactobacillus* species, notably *L. iners* and *L. crispatus* , demonstrated significant discriminative importance between the TB and PB groups. *L. iners* continued to be the most discriminative feature in early pregnancy when using SVM-RFE.

Following CLR transformation, the Mann–Whitney U test indicated that the PB group had a greater abundance of *L. iners* (P=0.010, q=0.040) than the TB group during early pregnancy; however, during late pregnancy, the elevated abundance of *L. iners* in the PB group did

(See figure on next page.)

Fig. 2 Identifying differential species between groups with recurrence and those without recurrence. **A** Principal coordinate analysis (PCoA) plot of beta diversity based on robust Aitchison distance for the preterm and term birth groups in the early and late stages of pregnancy with p-values determined by analysis of similarities (Adonis2). **B** Beeswarm boxplot of alpha diversity values (Observed, Chao1, Shannon, Simpson) by outcome in early and late pregnancy, with line and whiskers representing the median and inter-quartile range, respectively. **C** Random forest plots highlighting the top 15 microbial biomarkers distinguishing the preterm group from the term group. Species were ranked in descending order of their importance according to the mean decrease in their Gini score. (**D**) Linear discriminant analysis (LDA) score determined by the LDA effect size (LEfSe) analysis showing biomarkers between the preterm and term group. The threshold for LDA score was > 2 in early pregnancy and > 4 in late pregnancy. The letter before the taxa indicates taxonomic level: "p__" for phylum; "c__" for class; "o__" for order; "f__" for family; "g__" for genus; and "s__" for species. **E** Box plots comparing the relative abundance of *Lactobacillus crispatus* and *Lactobacillus iners* by pregnancy outcome in early and late pregnancy. **F** Bacterial co-occurrence networks (SparCC correlation, the most abundant 25 species, p value < 0.05, and correlation > 0.1). The node represents bacterial species. The size of a node is proportional to its abundance. The edge color represents positive (green) and negative (red) correlations, and the edge thickness is equivalent to the correlation value. Statistical significance is shown as *P value < 0.05, **P value < 0.01, ***P value < 0.001

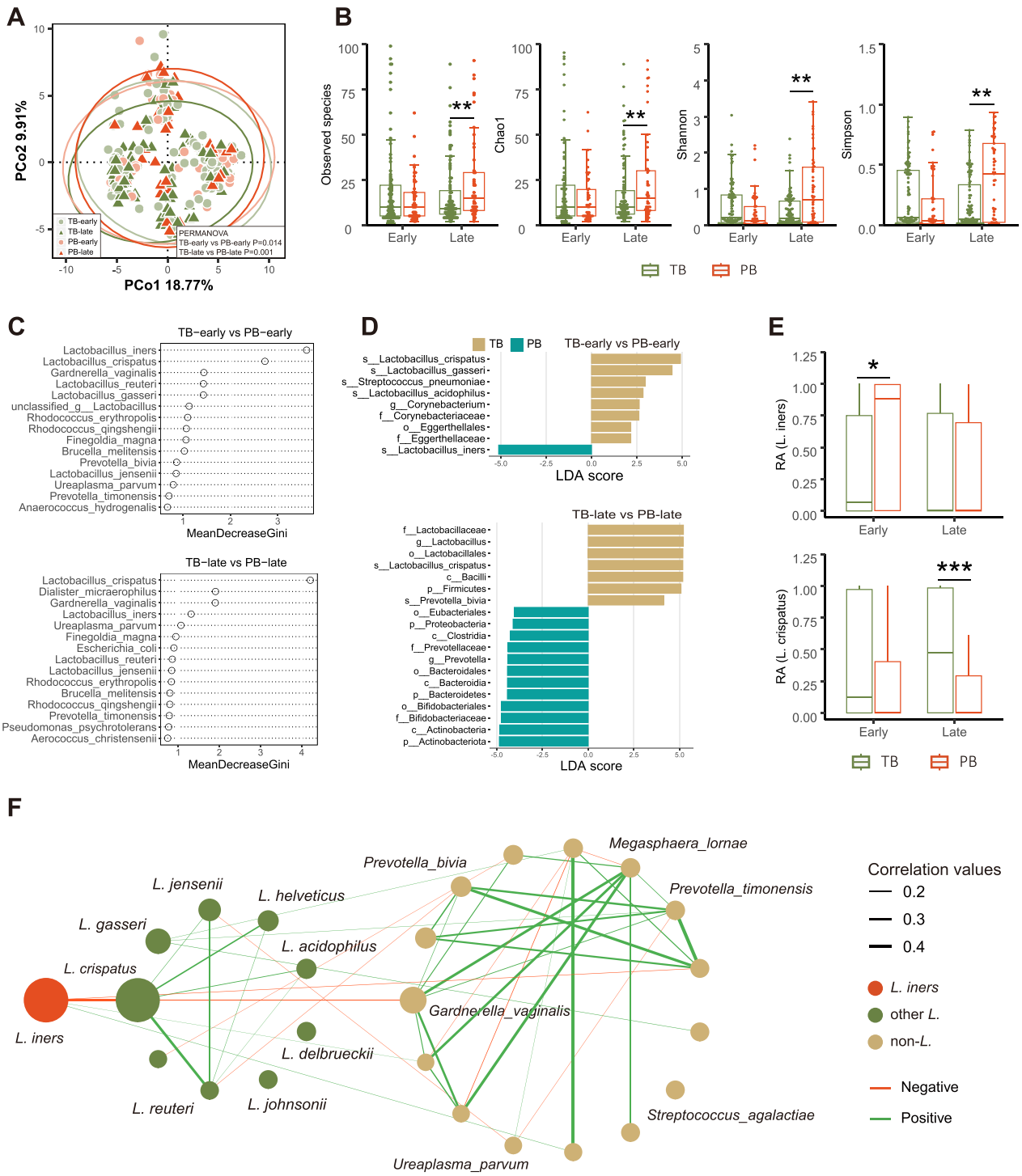

Fig. 2 (See legend on previous page.)

not persist. In contrast, *L. crispatus* was more abundant in the TB group than in the PB group, with a statistical significance observed only in late pregnancy (P<0.001, q=0.001) (Additional file 2 and Figure 2E). Network analysis revealed a negative correlation between *L. iners* and *L. crispatus* (r= −0.629, P<0.001) and between *L. crispatus* and some non-*L.* species (Figure 2F).

Using hierarchical clustering based on Jensen-Shannon divergence, 304 samples were clustered into six CSTs: IA (*L. crispatus* , 34.5%), IB (*L. crispatus* and *L. iners*

, 13.2%), II (*L. gasseri*, 3.6%), III (*L. iners* , 31.9%), IVA (*Gardenerella*, 6.3%), and IVB (diverse species, 10.5%). Heatmaps of the vaginal species in early and late pregnancy associated with CST and delivery outcomes are presented in Figure 3A. During early pregnancy, *L. iners* dominant CST III was significantly more prevalent in the PB group than in the TB control group (29/53, 57.4% vs. 29/99, 29.3%, P=0.004, q=0.024) (Table 2 and Figure 3B). In contrast, *L. crispatus* dominant CST IA was more prevalent in the TB group than in the PB group (37/99, 37.4% vs. 11/53, 20.8%), although this difference was not statistically significant (P=0.055, q=0.165). CST III was correlated with shorter pregnancy durations than CST IA (Figure 3C). In late pregnancy, the prevalence of CST III was comparable between the two groups. However, CST IVA (6/53, 15.1% vs. 2/99, 2.0%, P=0.004, q=0.008) and CST IVB (10/53, 30.2% vs. 6/99, 6.1%, P<0.001, q=0.001) were more prevalent in the PB group than in the TB group (Table 2 and Figure 3B). CST IA was significantly more common in the TB group than in the PB group (47/99, 47.5% vs. 10/53, 18.9%, P<0.001, q=0.003). CST IVA and IVB had shorter pregnancy durations than CST IA (Figure 3C).

Table 2 Prevalence of six vaginal community state types in the term and preterm groups

Vaginal community state types	Total	TB (n=99)	PB (n=53)	P value	q value
Early pregnancy (< 16^{+0} weeks)					
CST IA	48 (31.6)	37 (37.4)	11 (20.8)	0.055	0.165
CST IB	22 (14.5)	15 (15.2)	7 (13.2)	0.934	0.934
CST II	5 (3.3)	5 (5.1)	0 (0.0)	0.163	0.326
CST III	58 (38.2)	29 (29.3)	29 (54.7)	0.004	0.024
CST IVA	9 (5.9)	7 (7.1)	2 (3.8)	0.497	0.746
CST IVB	10 (6.6)	6 (6.1)	4 (7.6)	0.740	0.888
Late pregnancy (16–24 weeks)					
CST IA	57 (37.5)	47 (47.5)	10 (18.9)	< 0.001	0.003
CST IB	18 (11.8)	14 (14.1)	4 (7.6)	0.349	0.524
CST II	6 (4.0)	5 (5.1)	1 (1.9)	0.665	0.798
CST III	39 (25.7)	25 (25.3)	14 (26.4)	1.000	1.000
CST IVA	10 (6.6)	2 (2.0)	8 (15.1)	0.004	0.008
CST IVB	22 (14.5)	6 (6.1)	16 (30.2)	< 0.001	0.001

CST community state type, *PB* preterm birth, *TB* term birth

The data are expressed as n (%) for categorical variables

P-values were determined using the chi-squared test or Fisher's exact test. The q values were determined using the Benjamini-Hochberg procedure and the P values for early and late pregnancy were adjusted independently

Longitudinal dynamic characteristics of vaginal community state types associated with recurrence risk

The heatmaps generated by the relative abundance of the vaginal microbiota in early and late pregnancy indicate longitudinal changes (Figure 3A). For further longitudinal dynamic analysis, six CSTs were classified into three vagitypes: *L. iners* vagitype (including CST III, dominated by *L. iners* , 31.9%), other-*L.* vagitype (including CST IA, CST IB, and CST II, dominated by non-*iners L.*, 51.3%), and non-*L.* vagitype (including the CST IVA and CST IVB, which were dominated by non-*L.* species, 16.8%) (Figure 4A).

A comparison of the prevalence of the nine transition types in the PB and TB groups (Additional file 3 and Figure 4B) revealed that the persistence of the other-*L.* vagitype was more common in the TB group than in the PB group (52/99, 52.5% vs. 11/53, 20.8%, P<0.001, q=0.001). Rate of transition to non-*L.* vagitype from the *L. iners* vagitype was greater in the PB group than in the TB group (14/53, 26.4% vs. 1/99, 1.0%, P<0.001, q=0.001).

A Sankey diagram illustrates the longitudinal variation in the three vagitypes from early to late pregnancy (Figure 4C). Transition heatmaps reveal that the other-*L.* vagitype exhibited greater stability than the *L. iners* vagitype (63/75, 84.0% vs. 28/58, 48.3%, P<0.001), whereas the *L. iners* vagitype demonstrated a greater frequency of transition to the non-*L.* vagitype (15/58, 25.9% vs. 6/75, 8.0%, P=0.010) than the other-*L.* vagitype (Table 3, Figure 4D). In addition, the other-*L.* vagitype exhibited greater stability in the TB group than in the PB group (52/57, 91.2% vs. 11/18, 61.1%, P=0.006), whereas the *L. iners* vagitype exhibited a higher rate of transition to the non-*L.* type (14/29, 48.2% vs. 1/29, 3.5%, P<0.001) in the PB group than in the TB group (Table 3, Figure 4D).

Discussion

Our findings revealed that the dominance of *L. iners* in the vagina before 16 gestational weeks was associated with an increased risk of recurrent sPTB compared with the dominance of non-*iners L.* species. The inherent instability of the *L. iners* dominant vagitype, coupled with its tendency to transform to non-*L.* vagitype, contributed to an elevated risk of recurrence.

Despite numerous studies examining the association between vaginal *L. iners* dominance and sPTB, the impact of vaginal *L. iners* dominance on sPTB recurrence has only been explored in a limited number of studies. These studies involved collecting vaginal swabs at various stages of pregnancy, leading to inconsistent findings. Kindinger et al. identified a pathogenic effect of *L iners* dominance at 16 weeks of gestation, which was associated with an increased risk of sPTB recurrence [12]. In contrast, Goodfellow et al. did not detect a similar pathogenic

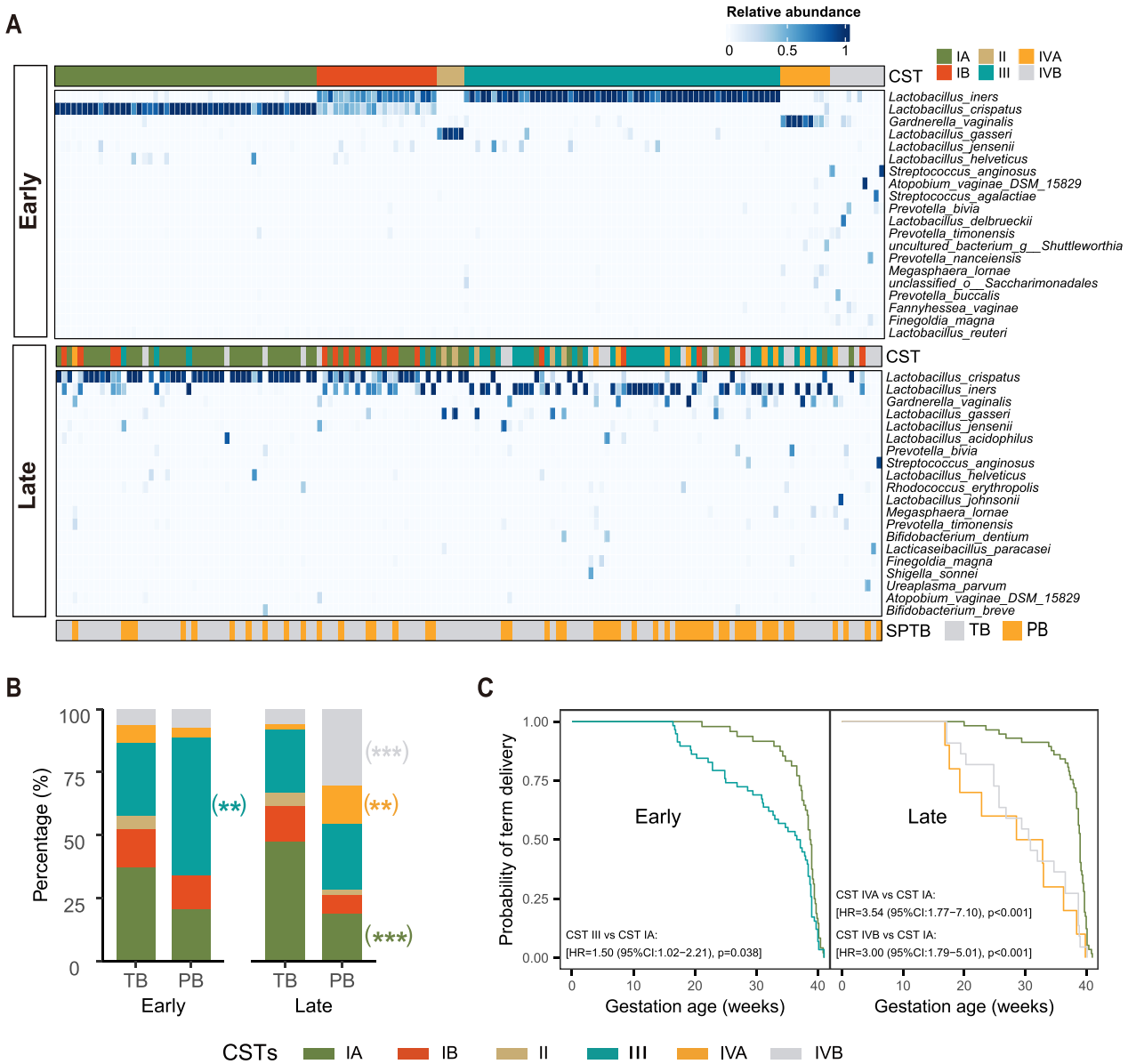

Fig. 3 Community state types (CSTs) and risk of recurrent sPTB. **A** Heatmaps displaying the relative abundance of the most abundant 20 vaginal species in early and late pregnancy. Hierarchical clustering of Jensen-Shannon distances with Ward linkage was used to determine six CSTs: IA (*L. crispatus*), IB (*L. crispatus* and *L. iners*), II (*L. gasseri*), III (*L. iners*), IVA (*Gardenerella*), and IVB (diverse non-*L.* species). The outcome of birth is indicated below the heatmap. **B** Barplot comparing the prevalence of CSTs between the preterm and term groups in early and late pregnancy. **C** Kaplan-Meier survival plot Kaplan-Meier survival plot comparing gestation at delivery between CST III and CST IA in the early stage; between CST IVA or CST IVB and CST IA in late stages. P-values estimated using Cox proportional hazard regression models. Statistical significance is shown as **P value < 0.01, ***P value < 0.001

role for *L. iners* in samples collected between 15 and 22 weeks of gestation [9]. Schuster et al. collected vaginal swabs during the first and second trimesters and found that microbe-related etiological contributions might be limited. However, this finding should be considered carefully since these women underwent additional preventive treatments, such as administration of progesterone and treatment for bacterial vaginosis [21]. In this study, we examined the relationship between vaginal *L. iners* dominance and risk of sPTB recurrence before 16 weeks and between 16 and 24 weeks. Our analysis revealed a significant correlation between *L. iners* dominance and risk of recurrence before 16 gestational weeks; however, this correlation was not observed between 16 and 24 weeks.

Table 3 Comparison of transitions between vaginal microbiota community types dominated by *Lactobacillus iners* and other *Lactobacillus* species

	Other-*L.* vagitype				*L. iners* vagitype				
	Total (n = 75)	TB (n = 57)	PB (n = 18)	P value TB vs. PB	Total (n = 58)	TB (n = 29)	PB (n = 29)	P value TB vs. PB	P value other-*L.* vs. *L. iners*
Stability	63 (84.0)	52 (91.2)	11 (61.1)	0.006	28 (48.3)	17 (58.6)	11 (37.9)	0.189	< 0.001
Transition to non-*L.* vagitype	6 (8.0)	2 (3.5)	4 (22.2)	0.027	15 (25.9)	1 (3.5)	14 (48.2)	< 0.001	0.010

PB preterm birth, *TB* term birth, vagitype, vaginal community type; *L. iners* vagitype, *L. iners* dominant vagitype; other-*L.* vagitype, non-*iners L.* dominant vagitype; non-*L.* vagitype, non-*L.* dominant vagitype

The data are expressed as n (%) for categorical variables

P-values were determined using the chi-squared test or Fisher's exact test

The varying associations between *L. iners* dominance and recurrence risk at different stages of pregnancy may be attributed to the dynamics of VMB. Specifically, *L. iners* dominance during early pregnancy appeared to be unstable and more prone to transitioning to a state dominated by non-*L.* species later in pregnancy. This transition results in a diminished association between *L. iners* and recurrence risk while concurrently strengthening the relationship between non-*L.* species and the risk of recurrence.

Previous studies have indicated that the dominance of vaginal *L. iners* is characterised by lower stability and reduced capacity to inhibit the growth of non-*L.* pathogens; however, existing evidence remains limited. In non-pregnant women, epidemiological evidence from meta-analyses indicates that *L. iners*-dominated VMB may be more susceptible to BV and sexually transmitted infections compared with *L. crispatus*-dominated VMB [31, 32]. In pregnant women, Kindinger et al. reported a 74% stability rate for vaginal *L. iners* dominance, whereas the instability rate for *L. crispatus* dominance was 92% [12]. Our study reached similar conclusions; however, the stability rate of *L. iners* dominance was 48%, which was notably lower than the rates reported in previous studies. The inconsistent instability in *L. iners* dominance is likely attributable to variations in strains and heterogeneous host factors across the studies [33–36]. Participants in our study were those with a history of spontaneous pregnancy loss at earlier gestational weeks. They displayed a higher recurrence rate (34.9% vs. 21.0%) and an earlier delivery (mean gestational age of 27^{+1} weeks vs. 32^{+6} weeks), in comparison with those in the study by Kindinger et al. This suggests a greater likelihood of unstable *L. iners* strains and less favourable host characteristics among these participants.

The predominance of *L. iners* in the vagina was associated with an elevated risk of sPTB through multiple mechanisms. Unlike other *L.* species, *L. iners* cannot produce D-lactic acid, hydrogen peroxide, bacteriocins, and other antimicrobial compounds, which reduces its effectiveness in inhibiting the proliferation of potentially pathogenic bacteria [37, 38]; Furthermore, *L. iners* may interact with the host immune system and affect cytokine production and cervical epithelial barrier function [39]. Additionally, *L. iners* dominance in the vagina may alter the expression of extracellular matrix metalloproteinase inducer and matrix metalloproteinase, which are involved in cervical remodelling and translocation of bacteria into the uterus [40]. The proliferation of certain pathogenic non-*L.* species in the vagina, cervical remodelling, and the invasion of pathogenic species into the uterus are recognised mechanisms that contribute to sPTB. The risk of sPTB recurrence associated with vaginal dominance of *L. iners* is primarily attributed to its remarkable ability to persist in the vagina. *L. iners* demonstrates a superior ability to adhere to vaginal epithelial cells, facilitated by its binding to fibronectin, compared with other *L.* species [41]. Furthermore, comparative genomic studies have revealed that *L. iners* retains a high number of core genes that may undergo gain or loss in response to environmental pressures, in contrast to other *L.* species. This genetic adaptability might contribute to survival in diverse vaginal environments [42]. Notably, *L. iners* has been observed to persist even after antibiotic treatment [43, 44]

The findings of this study have significant implications for the clinical management of women with a history of sPTB who are at an elevated risk of recurrence and for future research endeavours. Integrating these microbiota profiles into prenatal care protocols could enable early identification of pregnancies at risk. Further investigations are necessary to ascertain whether interventions aimed at transitioning the VMB from a state dominated by *L. iners* to one dominated by other *L.* species during early pregnancy could effectively

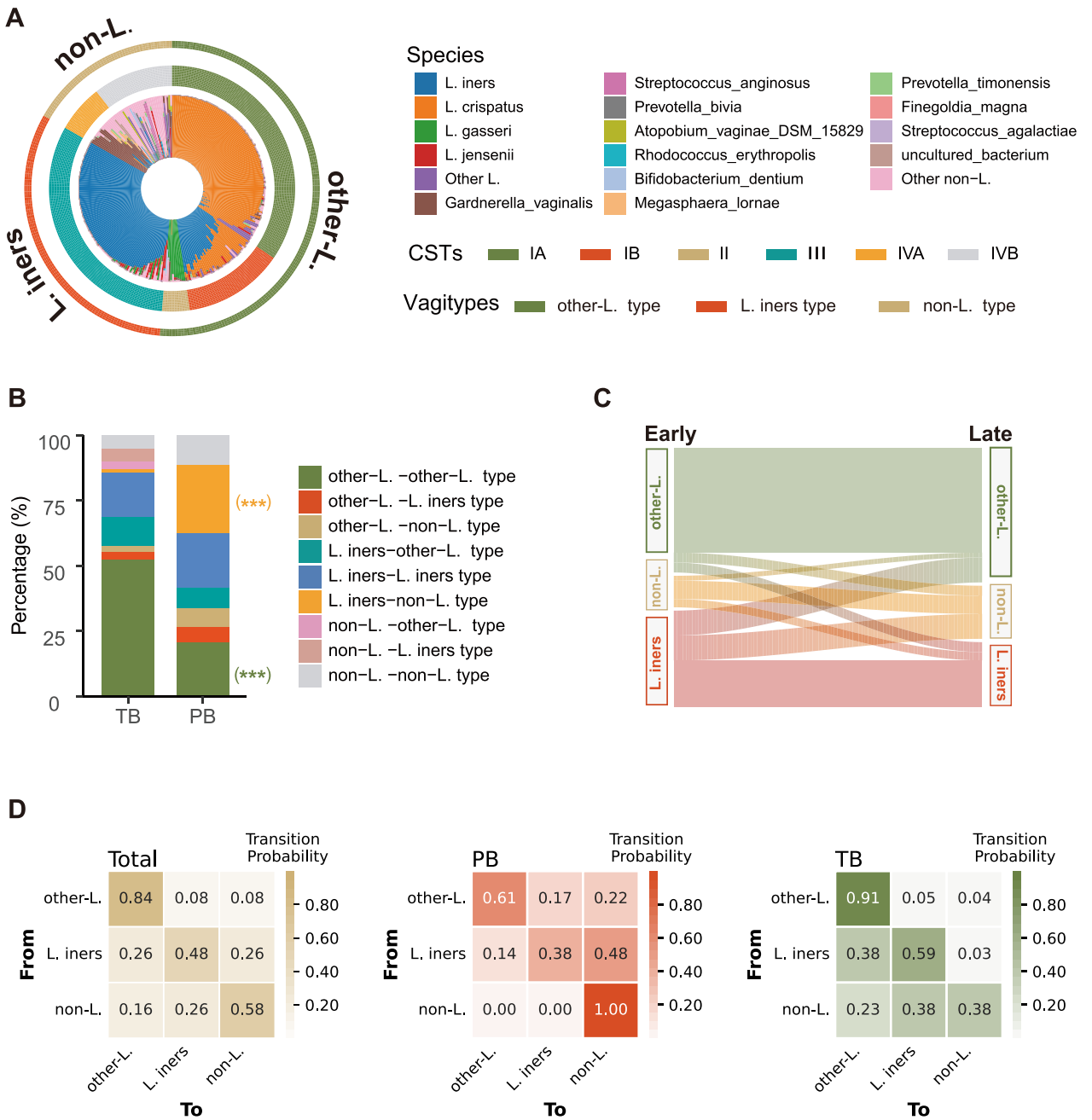

Fig. 4 Stratification of CSTs into three vagitypes and longitudinal analysis. **A** Grouping the six CSTs into three vagitypes based on the dominant species. **B** Bar plots comparing the prevalence of each transition type in the preterm and term groups. **C** Sanky diagram showing the longitudinal changes of three vaginal community types between early and late pregnancy. **D** Transition probabilities of shifting from one vagitype to another for all participants, those with preterm birth, and those with term birth. Statistical significance is shown as ***P value < 0.001

reduce the risk of recurrent sPTB [45]. Additionally, given that the pathogenic properties of *L. iners* are strain-specific, further studies are essential to identify these pathogenic strains, thereby enhancing the precision of prediction and prevention strategies [46].

The present study has some limitations. Initially, we aimed to investigate the relationship between VMB and the recurrence of early and late sPTB separately, since early sPTB is more closely associated with infection compared to late sPTB and may present recurrence-associated VMB characteristics that differ from those observed

in late sPTB [47]. However, the sample size was insufficient for analysis. Furthermore, prophylactic cerclage [48, 49] and progesterone administration [12, 50] may influence the VMB, potentially confounding the association between VMB and sPTB. However, it is important to note that only a small proportion of samples were collected after these interventions, and these samples exhibited comparable percentages between the PB and TB groups. Throughout the study, participants consistently adhered to standard treatment protocols, rendering this limitation unavoidable.Finally, the study was conducted on women with a high risk of sPTB recurrence, and further investigation is required before its application to the broader population.

Conclusion

The predominance of *L. iners* in the vagina during early pregnancy was associated with an increased risk of recurrent sPTB compared with that of other *L.* species. The propensity of *L. iners* to transition to non-*L.* partially explains its association with a heightened risk of sPTB recurrence. These findings have significant implications for future research on VMB-targeted preventive interventions during early pregnancy, with the goal of reducing the risk of sPTB recurrence. However, the underlying mechanisms linking microbiota composition to sPTB require further investigation. In addition, conducting interventional trials to determine the efficacy of microbiota-targeted strategies in clinical settings is essential.

Abbreviations

sPTB	Spontaneous preterm birth
16 S	16 S ribosomal RNA
CST	Community state types
PB	Preterm birth
BV	Bacterial vaginosis
VMB	Vaginal microbiota
L.	Lactobacillus
ASV	Amplicon sequence variant
LDA	Least discriminant analysis
PERMANOVA	Permutational multivariate analysis of variance
LEfSe	Linear discriminant analysis effect size
SVM-RFE	Support vector machine based on recursive feature elimination
CLR	Centered log-ratio
Vagitype	Vaginal community type
TB	Term birth

SD Standard deviationSupplementary Information

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Additional file 1.

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Author contributions

HY: Conceptualisation, methodology, supervision, and funding acquisition. FZ: Formal analysis, writing-reviewing and editing. XHL: Project administration, writing-reviewing and editing. XJ: Conceptualisation, formal analysis, writing-review and editing. YRB: Writing - original draft. XL: Writing - review and editing. XXQ: Data curation. XYM: Data curation. JQD: Formal analysis. LYW: Visualisation and validation. All authors read and approved the final manuscript.

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Data availibility

The sequencing data supporting the conclusions of this study are available at the NCBI Sequence Read Archive (SRA) BioProject accession number PRJNA1185444 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1185444). Additional information required to reanalyse the data reported in this study is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shanghai First Maternity and Infant Hospital (KS18137) and was performed according to the reviewed protocol. Written informed consent was obtained from all the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no Competing interests.

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