

LACTIN-V (Lactobacillus crispatus CTV-05) leads to microbial and immune modulation in pregnant women at risk of spontaneous preterm birth:FLIP-1 study

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Additional Declarations: Yes there is potential Competing Interest. PRB, DAM, and TPP hold a patent regarding *Lactobacillus crispatus* composition for use in pregnant subjects. International application number: PCT/GB2022/050453. International publication number: WO 2022/175680. ZT, PRB and DAM hold a patent for Rapid evaporative ionisation mass spectroscopy (REIMS) and Desorption Electrospray Ionisation Mass Spectroscopy (DESI-MS) analysis of swabs and biopsy samples. International application number: PCT/GB2016/050621 International publication number: WO2016142691A1. GDSC, ZT, PRB, and DAM hold a patent on metabolic signatures for prediction of mucosal inflammation and microbial composition. International application number: PCT/GB2022/051833 International publication number: WO2023285832. TPP and LL are employees of Osel, Inc., the company developing LACTIN-V. PPL is the Executive Chairman of Osel, Inc. JRM has received consultancy fees from EnteroBiotix Ltd., Cultech Ltd., and Crescent Enterprise.

Abstract

Preterm birth (PTB) remains a leading cause of neonatal morbidity and mortality, with no new preventive therapeutics introduced since the 1970s. Evidence increasingly implicates adverse vaginal microbiome-immune interactions in its aetiology. We report the effects of the live biotherapeutic, *Lactobacillus crispatus* CTV-05 (LACTIN-V), in an observational study of pregnant individuals at high-risk of PTB (n=61). LACTIN-V treatment was associated with a shift towards *L. crispatus*-dominated microbiota, which was sustained at eight weeks post-cessation and confirmed to be viable CTV-05. Engraftment of LACTIN-V was accompanied by significant reductions in cervicovaginal fluid concentrations of IL-1 β , IL-6, C3a, C3b/iC3b, C4, and Factor H, changes not seen in untreated time-matched controls. We also highlight the utility of metabolic profiling by Desorption Electrospray Ionisation in identifying individuals most likely to benefit from treatment and for assessing therapeutic response. The reduced PTB rate observed with LACTIN-V administration supports its use in preventing microbiome-immune-mediated PTB.

Main

Preterm birth (PTB) is a major burden to global health, occurring in 9.9% of all births, affecting 13.4 million babies each year (1). It is the leading cause of death in children under the age of five and a significant cause of short- and long-term morbidity (1). The majority of PTBs occur following the spontaneous onset of contractions, with or without premature rupture of fetal membranes (2). There are many aetiological drivers that contribute to the syndrome of PTB, with microbial and inflammation driven pathways considered to be common causal factors in spontaneous PTBs (sPTBs) (2, 3).

Predictive biomarkers and preventive therapies for PTB remain limited, and a lack of precision-medicine approaches contribute to PTB rates remaining stagnant for decades (1). Available cervicovaginal fluid biomarkers including fetal fibronectin, phosphorylated insulin-like growth factor binding protein-1, and placental alpha microglobulin-1, do not reflect biochemical features of the cervicovaginal microbial-immune *milieu*. Likewise, the mechanisms of action of the main preventive clinical interventions; cervical cerclage and progesterone, do not directly target microbial-immune mechanisms. Consequently, there is an urgent need to develop novel predictive and preventive strategies that specifically target microbial-immune-driven pathways to PTB.

Composition of the vaginal microbiota is a potentially modifiable risk factor for PTB. *Lactobacillus crispatus* (Community State Type I, (CST I)) dominance of the vaginal microbiome is associated with decreased risk of sPTB, whilst *L. iners* dominance (CST III) or diverse, *Lactobacillus* depleted, polymicrobial communities (CST IV) are associated with increased risk of sPTB, especially early sPTB <34 weeks of pregnancy (4, 5). Recent cohort studies have highlighted a complex inter-relationship between suboptimal vaginal microbiome composition and activation of host immune and inflammatory responses implicated in the early onset of human parturition and birth (6-8).

LACTIN-V, a live biotherapeutic containing *Lactobacillus crispatus* strain CTV-05, has been shown to modulate vaginal microbial composition in non-pregnant populations (9-12), with some also reporting effects on cervicovaginal cytokine and chemokine concentrations (13-15). We have previously reported that LACTIN-V is safe, well-accepted, and associated with only low rates of mild and tolerable side effects in a first in-pregnancy, single-arm, open-label feasibility study of women at high risk of sPTB (the FLIP-1 Study) (16). Here we report on the colonisation dynamics and immune-modulatory effects of LACTIN-V and compare these findings with a time-matched, non-LACTIN-V treated cohort of pregnant women also at risk of sPTB. We also examine the capacity of direct swab analysis by Desorption Electrospray Ionisation Mass Spectrometry (DESI-MS) to capture cervicovaginal mucosal metabolic characteristics useful for predicting host treatment response to LACTIN-V.

Results

Study cohort

A total of 61 women attending the PTB prevention clinic at Queen Charlotte's and Chelsea Hospital due to being at high risk of sPTB were included in the FLIP-1 study. Most participants had a history of prior PTB and/or mid trimester loss (MTL) (n=29, 47.5%) or a history of previous cervical excisional treatment (n=28, 45.9%). Demographics of the study cohort, and the reported solicited and unsolicited maternal adverse events were reported previously (16). The schedule of treatment and timepoints of sample collection is summarised in Extended Data Fig 1. Vaginal swabs were collected from participants throughout the study, including prior to treatment with LACTIN-V (T0). A total of 39 women (63.9%) provided samples at all seven timepoints: T0 pre-treatment, T1, T2, T3, T4, T5 and T6 (delivery), with median gestational ages of 13⁺⁵, 15⁺¹, 18⁺¹, 20⁺², 28⁺², 36⁺¹, 38⁺³, respectively. Samples were provided by 60 women pre-loading dose and again one week later (T0 and 1, n=60). 54 women provided samples at T2, and 54, 53, 48, 41 at T3, T4, T5, T6 respectively. Of the 13 women who did not provide samples at T5, four (6.5%) had delivered or miscarried prior. Extended Data Fig. 2 summarises the number of participant samples analysed for microbial composition (n=382), immune mediator analyses (n=234) and metabolic profiles (n=361).

LACTIN-V leads to persistent vaginal colonisation in pregnancy

Metataxonomic profiling of vaginal bacteria was performed on 382 swabs, generating a total of 6,919,048 reads, and an average of 18,160 reads per sample (Extended Data Fig. 3). Prior to the loading dose (T0), the CST proportions within the study population were 47.54% (n=29) for CST I (*L. crispatus* dominated), 6.56% (n=4) for CST II (*L. gasseri* dominated), 22.95% (n=14) for CST III (*L. iners* dominated), 16.39% (n=10) for CST IV (diverse polymicrobial), and 6.56% (n=4) for CST V (*L. jensenii* dominated) (Fig. 1a,b). The proportion of participants with CST I increased to 90.9% at T1, and remained high (85.9%) at T4, 8 weeks following cessation of LACTIN-V administration.

Colonisation status was defined by the congruency of targeted PCR assay and Amplicon Sequence Variant (ASV) based detection of *L. crispatus* CTV-05, presented in Fig. 1a. For samples displaying discrepancies between PCR and ASV results, *L. crispatus* CTV-05 was deemed to be detected if the *L. crispatus* CTV-05 copy number was ≥ 4277 per swab. This threshold was selected due to it being the highest load detected in any patients prior to LACTIN-V treatment where ASV-1 and ASV-2 were both detected, likely representing naturally occurring *L. crispatus* CTV-05. Viability of engrafted LACTIN-V was demonstrated by confirmation of CTV-05 from cultured isolates derived from vaginal swabs collected eight weeks post-treatment. Examination of CST transitions revealed a shift in the proportion of women classed as CST I from 29/61 (47.5%) at baseline to 55/61 (90.1%) following loading dose (Fig. 1b). While no change in total bacterial 16S rRNA copies/ml was seen following the loading dose at T1 or thereafter (Fig. 1c), a significant increase in LACTIN-V copies/ml was seen after the loading phase (T1) ($p < 0.0001$) (Fig. 1d). A bimodal distribution was seen during the maintenance phase (T2 – T5) indicative of women showing either persistently high or gradually declining LACTIN-V copy numbers. Median LACTIN-V copy numbers declined at delivery, approximately 18 weeks after final administration.

Determination of LACTIN-V colonisers and non-colonisers

To identify groups of participants with distinct LACTIN-V colonisation patterns, clustering analysis was performed on log-transformed LACTIN-V copies/ml for all samples. K-means clustering was applied to the processed dataset with $k = 2$ clusters, as this configuration provided the optimal average silhouette score across cluster numbers ($k = 2-10$). The two resulting clusters were interpreted as colonisers and non-colonisers, based on the direction and magnitude of change in $\log_{10}(\text{LACTIN-V})$ levels over time. Cluster membership was then merged back into the full longitudinal dataset for visualisation and downstream analyses. CST classification prior to LACTIN-V treatment initiation was a significant determinant of subsequent colonisation response ($p = 0.002$) (Fig. 2a). Participants with CST II, III, IV-A, IV-C, and V were more likely to colonise compared to those with a starting CST I and IV-B, the latter characterised by high relative abundances of *Fannyhessea vaginae* (basionym: *Atopobium vaginae*), *Gardnerella vaginalis*, and other bacterial vaginosis (BV)-associated taxa. Although total bacterial load did not differ significantly between colonisers and non-colonisers, a significant increase in LACTIN-V bacterial load was seen across timepoints following pre-loading dose (T0) through until delivery (T6) in colonisers, whereas no significant changes were seen from T2 in non-colonisers (Fig. 2b). When determining coloniser and non-coloniser status by CST and sub-CST classifications, there were differential responses. Notably 18 of 29 (62%) of women who were already deemed CST I at T0 were classified as non-colonisers (Fig. 2c). One woman with CST III and three women with CST IV-B were also deemed non-colonisers, respectively.

LACTIN-V modulates the cervicovaginal immune *milieu* during pregnancy

LACTIN-V colonisers demonstrated immune modulation consistent with attenuated inflammation characterised by a reduction in cervicovaginal (CVF) concentrations of IL-1 β , IL-6, C4, C3a, and C3b/iC3b (Fig. 3a). These differences were observed across post-treatment timepoints, but only in those classified as colonised by LACTIN-V. Furthermore, reduced CVF concentrations of inflammatory mediators followed the classification of CST at T0, whereby those who commenced the study with pre-existing CST I did not demonstrate changes, whereas those who were non-CST I had a reduced median concentration (Fig. 3b).

Since this was a single-arm study, with recruitment partly occurring during the COVID-19 pandemic when PTB rates were reported to have been reduced (17), we also analysed pregnancy outcomes, vaginal microbial composition and immune mediator status for a time-period matched cohort of non-LACTIN-V treated patients who were part of the VMET-2 study. These patients also attended the PTB prevention clinic due to being at high-risk of PTB and therefore served as an appropriate non-LACTIN-V treated control population. A total of 262 patients were recruited to VMET-2 during the recruitment period for FLIP-1, 53 of whom had matched longitudinal samples for comparative microbiome and immune analysis. Two timepoints aligned between the FLIP-1 and VMET-2 studies corresponding to the FLIP-1 T0 (12⁺⁰-16⁺⁶ weeks gestation), and FLIP-1 T3 (20⁺⁰-24⁺⁶ weeks gestation), which were used for comparative analyses. In the FLIP-1 study, the combined MTL or sPTB <34⁺⁰ was 4.9% (n=3/61) whereas in the VMET-2 study it was 9.9% (n=26/262) (Extended Data Table 1). In both cohorts, similar CST distribution was observed at T0 (Fig. 4a). In the VMET-2 study, CST proportions remained stable between T0 and T3, unlike the FLIP-1 study cohort where a major shift to CST I was seen during the same time-period (p=0.002). While a statistically significant reduction in CVF concentrations of C4, C3a, C3b/iC3b, and Factor H was observed in FLIP-1 participants between T0 and T3, no changes were seen in the VMET-2 study cohort (Fig. 4b-g and Extended Data Table 2)

LACTIN-V-induced microbiome transitions modify the mucosal metabolic environment

We next evaluated whether LACTIN-V-induced *L. crispatus* dominance in the vagina led to changes in the mucosal metabolome. Metabolic profiles were acquired using direct swab analysis by DESI-MS (18) (n=361) yielding a total of 3656 mass-to-charge ratio (*m/z*) features. In agreement with previous findings in the VMET-2 cohort (19), random forest classifiers could be trained to discriminate between bacterial communities dominated by *Lactobacillus* spp. and CST IV using the metabolic profiles (Fig. 5a, ROC AUC=0.977). Previously described metabolic signatures associated with mucosal inflammation could also be replicated, using random forest regression models trained to predict the mucosal concentration of various cytokines and complement proteins (Fig. 5b-d).

A paired comparison of mass spectral feature intensities between T0 and T1 in the women, whose CST assignment changed following LACTIN-V treatment, highlighted a total of 220 statistically significant differences (Benjamini-Hochberg q-value < 0.05). Matching the theoretical *m/z* with a tolerance of 15 ppm, we identified 23 of these that could be matched to the set of previously annotated markers (19) with four representative examples are shown in Fig. 5e-h. A total of 15 out of 20 previously reported

metabolic markers of CVF IL-1 β concentration were again found to significantly correlate with Luminex-measured IL-1 β levels in the FLIP-1 patient samples (Spearman correlation test q-value < 0.05). Metabolic markers correlated with concentrations of other immune analytes, with examples of IL-1 β C4, Factor H and C3b/iC3b also presented in Fig. 5i-l.

Discussion

Here, we present evidence of effective, persistent vaginal colonisation with *L. crispatus* CTV-05 (LACTIN-V), and associated attenuation of local inflammation in a pregnant population. Recent cohort studies have highlighted the complex causal relationship between an adverse vaginal microbiome and maternal immune responses that can lead adverse pregnancy outcomes (8, 20-22). This is thought to be partly driven by maternal inflammatory response to pathobionts, evidenced by heightened cervicovaginal inflammation in sub-optimal CSTs (especially CST III and IV) and in cases of early membrane rupture, silent cervical dilation and sPTB. In contrast, *L. crispatus* dominance promotes lactic-acid-mediated acidity, suppression of pathogen colonisation and immune homeostasis (23-25).

The live biotherapeutic *L. crispatus* CTV-05 (LACTIN-V) was initially developed as an adjunct to antibiotics in the treatment of recurrent urinary tract infections (rUTI) and BV (26). Isolated from the vagina of a healthy, non-pregnant woman, the strain is a homofermenter of glucose to D- and L-lactic acid and adheres to vaginal epithelial cells (27). Phase I and 2a double blinded randomised placebo-controlled trials using LACTIN-V in pre-menopausal women with a history of rUTI showed no serious adverse events, high compliance, and reduced rates of rUTI, especially in association with high level colonisation with *L. crispatus* (11, 28). In parallel, several studies have reported the safety, tolerability and good colonisation rates in women with BV, with reduced rates of recurrence (9, 12, 15, 29).

In this study, we used the LACTIN-V formulation of 2×10^9 CFU delivered via applicator with a loading phase of five days, consistent with previous studies (9, 12, 15), and a maintenance phase consisting of weekly dosing for six weeks. Colonisation by LACTIN-V was predominantly achieved in women starting treatment with CST III and CST IV, both of which are associated with an increased risk of sPTB. Unique to other studies, we also demonstrate LACTIN-V remained viable and was culturable from vaginal swabs eight weeks following cessation of treatment.

Of the women who started treatment with CST IV, 7/10 (70%) transitioned to CST I following loading dose, consistent with the colonisation efficiency post loading dose of 78% reported in women with BV (9). All except one woman transitioned from CST III to CST I following treatment, consistent with the recognised mutually antagonistic colonisation patterns of *L. iners* and *L. crispatus*. Their genomic differences imply distinct metabolic capabilities influencing competitive fitness and niche partitioning (30), an example being that oleic acid selectively inhibits *L. iners* yet promotes *L. crispatus* growth (31).

Three women (5%) transitioned to CST IV from CST I after the maintenance phase of dosing, suggesting that a minority of women may benefit from prolonged maintenance dosing. In our study, following a six-week maintenance dosing phase, CST I persisted in 86% and 88% of the cohort at eight and sixteen

weeks after treatment cessation respectively. Of women who were originally CST IV, CST I persistence was seen in 57% and 62.5% at eight and sixteen weeks post-treatment respectively. This is similar to the rates of *L. crispatus* CTV-05 detectable at week 24, (approximately 12 weeks post cessation of dosing) of 48% of women treated for 10 weeks for BV (12).

Colonisation rates likely vary between cohorts due to differences in baseline microbiota and host factors affecting *L. crispatus* engraftment. We defined colonisers as women with sustained high concentrations of *L. crispatus* CTV-05 over time. Non-CST I profiles were most susceptible, except CST IV-B, characterised as having a high relative abundance of *G. vaginalis*, moderate *F. vaginae* and low *Candidatus Lachnocurva vaginae*(32, 33). In contrast, CST IV-A (high prevalence of *Ca. L. vaginae*) pre LACTIN-V treatment has been associated with poor response and lack of benefit for BV in non-pregnant women (34). The decline in LACTIN-V copies seen at delivery is likely to reflect the duration of time post LACTIN-V cessation, yet only six women reversed to non-CST I following maintenance. It is unclear what factors influence duration of colonisation, however this lends support for minimally invasive monitoring of microbe-host interactions through pregnancy to monitor response to treatment. Limited sample size prevented analysis of many factors influencing colonisation, including oestrogen action, glycogen levels and availability, ethnicity, sociodemographics, parity, sexual intercourse, interpregnancy interval, secretor status and ABO blood group (35-41).

Our study differed from prior LACTIN-V trials in that participants were pregnant, not screened for BV, and did not receive pre-treatment antibiotics. Data from previous non-pregnant trials indicates that antibiotic treatment appears necessary for successful LACTIN-V colonisation and reduction recurrence of BV in non-pregnant women (42). In our study, high transition from CST IV to CST I in the absence of antibiotics suggests LACTIN-V may be effective as a primary and sole therapy during pregnancy. It is possible that engraftment in pregnancy is supported by the high estrogenic state of pregnancy and/or abstinence from intercourse, which was a trial requirement.

Previous studies have reported sustained reductions in IL-1 α in response to LACTIN-V treatment (13, 15). A nested sub-study from the Cohen 2020 trial (12) demonstrated distinct immune profiles between colonisation-resistant and permissive women, which was closely linked to abundance of BV associated species (14). Secondary analysis of successfully treated women in this LACTIN-V phase 2b trial showed IL-1 α , IL-1 β and TNF- α increased as *Lactobacillus* abundance declined (34). Consistent with these cytokine changes, we observed significant reductions in IL-1 β , IL-6, C4, C3a, C3b/iC3b, and Factor H, most marked in those who colonised by LACTIN-V, supporting the concept that LACTIN-V promotes a mucosal immune-quiescent state. This aligns with observational data showing reduced innate immune and complement activation in CST I (*L. crispatus*-dominant) compared with CST III and IV profiles (6-8). Furthermore, complement activation at the cervicovaginal interface is implicated in cervical shortening and sPTB (8). C4, produced by the liver, epithelial cells, and immune cells, leads to the formation of C3 convertase which results in amplification of the complement cascade and cleavage of C3 to C3a and C3b (43). C3a is an anaphylatoxin promoting inflammation and chemoattracts neutrophils, whereas C3b leads to enhanced phagocytosis by opsonising pathogens, and further drives amplification of the

complement cascade. It is thus feasible that LACTIN-V suppression of the complement pathways contributed to the lower rates of sPTB seen in this study. In our study, lower concentration of the regulator Factor H, which is primarily produced in the liver, may reflect consumption of this factor and its activity being located on cell surfaces (44). It is also possible that surface layer proteins present on *L. crispatus* strains, which selectively bind to the anti-inflammatory receptor DC-SIGN, further contributing to the dampened immune state observed with LACTIN-V colonisation (45).

Although not powered to assess PTB outcomes, this feasibility study observed a 50% reduction in PTB <37 and <34 weeks compared with historical controls. To address potential confounding from COVID-19 pandemic-related reductions in sPTB, we also included a time-matched cohort in the analysis. Consistent with this observation, sPTB/MTL <34 was also 50% lower, 3/61 (4.9%), compared to time-matched controls 26/262 (9.9%). Furthermore, the non-treatment cohort showed no significant change in microbial composition or local immune markers during pregnancy, partially mitigating the absence of placebo control.

Introducing novel therapies in pregnancy remains challenging due to concerns among patients, clinicians, sponsors and regulators about potential fetal harm and adverse pregnancy outcomes. Our study, like other LACTIN-V studies found adverse effects to be mild, with reports of a transient change in discharge being the most commonly reported side effect, and acceptability rates to be high (12) (15, 16, 46)

A unique aspect of our current study is that we explored the effects of intervention on the mucosal metabolome. This was achieved using a rapid DESI-MS based platform for swab analysis. DESI-MS offers a preparation-free platform capable of profiling cervicovaginal metabolic signatures that reflect both vaginal microbiome composition and mucosal immune activation. In large pregnancy cohorts, DESI-MS distinguished *Lactobacillus*-dominant from dysbiotic bacterial communities. It also identified metabolite patterns associated with elevated inflammatory mediators such as IL-1 β , IL-8, complement proteins and IgG subclasses, all of which are enriched in women with *Lactobacillus*-depleted microbiota and increased PTB susceptibility (19). This study provided a unique interventional validation of these findings by confirming associations between metabolites and CST or host immune response, and their response to compositional shifts. This shows that DESI-MS can function as a predictive tool for detecting metabolic surrogates of microbiome dysbiosis and local immune activation, to precisely identify women who may benefit from antenatal treatment with LACTIN-V.

Study limitations include the lack of a placebo arm for the most robust comparison, and the small number of participants, which limits the ability to investigate factors influencing the success of LACTIN-V colonisation and its relationship to PTB. These aspects will be evaluated in a larger placebo-controlled Phase 2 study, <https://www.isrctn.com/ISRCTN13610566>. Strengths of the study include the completeness of longitudinal sampling, allowing us to report robustly the microbe-immune trajectory from baseline through loading and maintenance phases and three post exposure timepoints.

Conclusion

In this feasibility study, we show that *Lactobacillus crispatus* CTV-05 (LACTIN-V) establishes sustained vaginal colonisation in pregnancy, promotes a shift toward a protective CST I profile, is associated with reduced cervicovaginal inflammation, and accordingly may reduce early PTB. These findings support a biologically plausible strategy to prevent sPTB by restoring a *Lactobacillus*-dominant, immune-quiescent mucosal environment. We also demonstrate that DESI-MS metabolomic profiling can rapidly identify microbial and inflammatory signatures, offering potential as a point-of-care approach for patient selection and monitoring. Together, these data provide compelling preliminary evidence that targeted microbiome modulation with LACTIN-V, coupled with metabolomic stratification and represents a promising therapeutic strategy for sPTB prevention, now advancing to Phase 2 evaluation.

Methods

Study design

Women at high risk for sPTB were recruited from PTB prevention clinics at Queen Charlotte's and Chelsea Hospital between September 2019 and April 2021. Women provided written informed consent as part of the FLIP-1 study (**F**easibility, safety, tolerance and assessment of **L**ACTIN-V use **I**n a cohort of **P**regnant women at risk of PTB), which was ethically approved by the NHS Health Research Authority London-Surrey Borders Research Ethics Committee, REC 19/LO/1018. ClinicalTrials.gov ID, NCT03992534. Inclusion criteria were women at risk of preterm labour who were referred to the prematurity clinic, and exclusion criteria were HIV positive women, women unable to provide informed consent, women aged <18, and women receiving antibiotic treatment within 1 week of recruitment.

Women were eligible for recruitment between 11⁺⁰ and 15⁺⁶ weeks of pregnancy and were recruited at their first visit to the PTB prevention clinic, with a median gestational age of 13⁺⁵ (IQR 13⁺⁰-14⁺⁴).

Since this was a single arm study, we compared microbial composition and immune mediator concentrations at two gestational age aligned timepoints from the VMET-2 (Vaginal Microbiome and Metabonome in pregnancy) study which was recruiting in parallel for the matched duration of the FLIP-1 study as non-LACTIN-V treated controls. Ethical approval was obtained from the NHS Health Research Authority London-Stanmore Research Ethics Committee, REC 14/LO/0328. Timepoint A had samples collected between 12⁺⁰-16⁺⁰ weeks, and timepoint B had samples collected between 20⁺⁰ and 23⁺⁶. Inclusion criteria were the same as the FLIP-1 study, and exclusion criteria were HIV-positive women, women under 18, women who had vaginal bleeding in the preceding week, and women who had had sexual intercourse in the preceding three days.

Study procedure and sample collection

During the consent process, women were informed of the administration schedule for LACTIN-V use. The dosing schedule commenced with a loading dose phase consisting of five consecutive daily doses

of LACTIN-V, followed by a maintenance phase consisting of six weekly doses of LACTIN-V (Extended Data Fig.1). A supply of pre-filled applicators containing *Lactobacillus crispatus* CTV-05 powder at a dose of 2×10^9 CFU preserved with inactive ingredients was provided to the participant. Consenting women were provided with 11 vaginal applicators to be stored at 2-8°C in a standard refrigerator. Each participant was asked to report adverse events and antibiotic use during pregnancy. Participants were followed up in the PTB prevention clinic, and vaginal swabs were collected prior to the loading dose (Timepoint 0, T0), and at 6 subsequent longitudinal timepoints; T1 (Median GA 15^{+1} [IQR 14^{+3} - 16^{+1}]), T2 (18^{+1} [17^{+1} - 18^{+6}]), T3 (20^{+2} [19^{+2} - 20^{+6}]), T4 (28^{+2} [27^{+5} - 29^{+4}]), T5 (36^{+1} [34^{+6} - 36^{+3}]), and T6 (at delivery (38^{+3} [37^{+4} - 39^{+2}]). Cervicovaginal fluid (CVF) was sampled using BBL™ CultureSwab™ MaxV Liquid Amies (Becton, Dickinson and Company, Oxford UK) and Transwab (MW170 with rayon blue lid, Medical Wire & Equipment, Corsham, UK). Swabs were stored at -80°C until use. Standard clinical care was continued in the PTB prevention clinic, with the use of progesterone and/or cervical cerclage offered where clinically indicated. LACTIN-V was provided by OSEL Inc. California.

Sample analysis

Vaginal Microbial Composition

DNA extraction was performed from CVF collected using Transport Swabs with Liquid Amies medium (VWR international Ltd). The swab was thawed on ice and the content of the Amies Liquid transport medium was collected and centrifuged at 6200g x 10 minutes at 4°C, with the pellet resuspended in 300µL of lysis master mix (Lysozyme (10mg/mL, Sigma), Mutanolysin (25000u/mL, Sigma), Lysostaphin (4000U/mL) TE₅₀ (10mM TRIS/50mM EDTA), 12% Triton, and PBS) followed by incubation for one hour in a 37°C water bath. An additional mechanical lysis step was performed using 0.1mm glass beads (VWR) for 1 minute at 25Hz in a Tissue Lyser LT (Qiagen, Manchester, UK). DNA was extracted using QIAamp DNA Mini Kit (Qiagen), following manufacturer's instructions.

Whole genomic DNA was sequenced for the V1-V2 hypervariable regions of 16S rRNA on an Illumina MiSeq platform (Illumina, Inc. San Diego, California) and performed at Research and Testing Laboratories (RTL Genomics, Texas, USA). The forward primer set (28f-YM) consisted of a mixture of the following primers mixed at a 4:1:1:1 ratio; 28F-Borrellia GAGTTTGATCCTGGCTTAG; 28F-Chlorlex GAATTTGATCTTGGTTCAG; 28F-Bifido GGGTTCGATTCTGGCTCAG; 28F-YM GAGTTTGATCNTGGCTCAG. The reverse primer consisted of 388R GCTGCCTCCCGTAGGAGT. Microbiome analysis included the use of Cutadapt for primer sequences trimming, and ASV (amplicon sequence variant) counts per sample were calculated using the QIIME2 bioinformatics pipeline (version 2022.2.1) (47). DADA2 (version 2022.2.0) was utilised for denoising and the SILVA database was used for taxonomic classification of ASVs (48, 49). The VALENCIA centroid classification algorithm was used to classify samples into the following community state types: CST I (*L. crispatus*), CST II (*L. gasseri*), CST III (*L. iners*), CST IV (reduced lactobacilli and increased diversity), and CST V (*L. jensenii*). Subclassifications of CST I (CST I - A and -B), and CST IV (IV -A, -B and -C) were also described (33).

Bacterial Load

Bacterial load was quantified at each study timepoint from vaginal swabs using the BactQuant qPCR assay targeting the 16S rRNA gene to estimate total bacterial abundance. A standard curve was established by 10-fold serial dilutions of *Escherichia coli* DNA (Sigma) ranging from 3.0×10^8 copies. PCR was performed using 5 μ L DNA template from each *E. coli* standards and extracted DNA from the vaginal swabs in duplicate using the ABI StepOne[®] qPCR system. The final PCR reaction mixture (20 μ L) contained 10 μ L Platinum Supermix UDG (Life Tech), 0.36 μ L of 100uM BactQUANT forward and reverse primers (Sigma) 5' CCT ACG GGA GGC AGC A 3' and 5' GGA CTA CCG GGT ATC TAA TC 3' respectively, 0.045 μ L of 10 mmol/ μ L BactQUANT probe 5' 6FAM-CAG CAG CCG CGG TA-MGBNFQ (Life Tech), 0.4 μ L of 100mmol/ μ L Rox (Life Tech) and 3.835 μ L bacterial DNA free water PCR grade (Qiagen). The PCR cycle conditions were set to hold at 50°C for 2 minutes, 95°C for 10 seconds, 40 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds. Copy numbers were calculated from Ct values against the *E. coli* standard curve and adjusted for dilution and elution volume to yield genome copies per swab. Relative abundance of dominant taxa (>95% of reads) was corrected for species-specific 16S rRNA gene operon copy numbers using the Michigan rrn database.

Colonisation status

Colonisation with *Lactobacillus crispatus* CTV-05 was assessed using complementary molecular approaches. Detection of *L. crispatus* CTV-05 was performed by (a) metataxonomic sequencing to identify a strain-specific amplicon sequence variant (ASV-2) unique to *L. crispatus* CTV-05, and (b) PCR-based methods. Gene targets for strain and species-specific PCR were selected using genes identified in *Lactobacillus crispatus* CTV-05 and *L. crispatus*-specific regions which were absent in other sequenced bacterial strains and with low numbers of homologs in vaginal metagenome datasets. Primer sequences consisted of forward and reverse primers targeting *L. crispatus* CTV-05 strain (LBKG_01471, 5'-GCTGTTGCAGCCAGACAGTT-3', 5'-TCTCTGGGACATCCATAAGTTG-3', 206 bp). DNA was extracted from vaginal swabs (T0–T6) and confirmed by PCR amplification of 16S rRNA gene using universal primers, 5' AGAGTTTGATCCTGGCTCAG 3' and 5' GCTGCCTCCCGTAGGAGT 3' (Sigma) with appropriate positive (*L. crispatus* CTV-05, Group B *Streptococcus* (GBS)) and negative (DEPC water, AE buffer, blank swab) controls. Targeted PCR employed strain specific primers, amplifying a 206 bp region unique to *L. crispatus* CTV-05 and confirmed strain specificity against 24 *L. crispatus* clinical isolates. Quantitative PCR targeting the same sequence quantified *L. crispatus* CTV-05 load per swab using a standard curve generated from reference strain DNA. PCR reactions were setup in duplicate with 25mL QuantiTect SYBR Green RT-PCR Master Mix (Qiagen), 2.5mL forward primer (10mM), 2.5 mMDNA extract and 10mL H₂O for a total volume of 50mL. Following initial denaturation at 95°C for 15 minutes, PCR cycling consisted of 40 cycles at 95°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds. Detection frequencies were compared across timepoints using both ASV-2 metataxonomic data and PCR results to assess colonisation dynamics following LACTIN-V administration. We also used mathematical modelling to determine colonisation using log transformed LACTIN-V ASV-2 data from all timepoints accounting for trajectories following treatment. Data fell into two clusters: Cluster 1 with low copy

numbers which we deemed as 'non-colonisers', and Cluster 2 with persistently high copy numbers, which we deemed as 'colonisers'.

Detection of viable *Lactobacillus crispatus* CTV-05

At T4 (28 weeks' gestation) Liquid Amies vaginal swab was collected for culture. Vaginal swabs were streaked onto MRS plates and incubated for 48 hours at 30°C under anaerobic condition generated by using Oxoid AnaeroGen Compact Sachet in an airtight jar (Thermo Scientific.) Following this, 2-3 representative colonies were removed and resuspended with 2mL of PBS. Samples were centrifuged at 1600g for 10 mins at 4°C and pellets used for DNA extraction as described previously (n=55). Using the species-specific PCR, 47/55 (85%) women had detectable bacterial DNA amplicons of the expected molecular weight when performing PCR with *L. crispatus* CTV-05 primers (as above) indicating the presence of viable *L. crispatus* CTV-05 eight weeks after last administration of LACTIN-V. ASV-2 was detected for 32/55 women (58%).

Immune Response

The immune *milieu* was analysed from CVF using supernatants collected from the vaginal swabs as stated above. Measured analytes included: cytokines (IL-8, IL-6, IL-1 β), Human Premixed Cytokine Panel (R&D Systems/Bio-Techne, Gillingham, UK) and complement proteins C3a (Human ProcartaPlex Simplex Kit, Invitrogen), C2, C4b, C5, C5a, Factor D, MBL, Factor I (Human Complement Magnetic Bead Panel 1, Milliplex Millipore), C1q, C3, C3b/iC3b, C4, Factor H (Human Complement Magnetic Bead Panel 2, Milliplex Millipore), and matrix metalloproteinases: (MMP-2, MMP-8, MMP-9, R&D Systems/ Bio-Techne). Kits were used following manufacturer's instructions. All samples were analysed neat except IL-8 which was measured as a single-plex assay following a 10-fold dilution (Calibrator Diluent RD6-52). All samples were analysed in duplicate. Acquisition was performed with Magpix® or Luminex 200 analysers, equipped with xPONENT® software.

Raw data were examined for points with poor replicate agreement and potential outliers by assessing the percentage coefficient of variation (%CV) of replicates [$\%CV = \text{STDev}/\text{Mean} \times 100$] and percentage recovery [$\%Recovery = (\text{Observed Standard Value}/\text{Expected Standard Value}) \times 100$]. All R^2 values were above 0.970. Concentrations below the lower limit of detection were assigned half the assay's sensitivity threshold. Coefficient of variation was calculated across all plates (Microsoft Excel v.2211) for standards, and an internal pool calibrator was used to assess the potential for plate-to plate variation.

Desorption Electrospray Ionisation Mass spectrometry (DESI-MS)

DESI-MS analyses for the FLIP-1 cohort were performed using an ACQUITY RDa/BioAccord Time-of-Flight mass spectrometer (Waters Corporation, Wilmslow, UK). For swab analysis and as part of the DESI stage, a roboticised sampling device was used. Swabs were placed into a rotating holder positioned orthogonally in front of the MS inlet capillary with a swab–capillary distance of approximately 2 mm. The DESI sprayer tip was pointed to the swab centre with a tip-sample distance of 1.5–2 mm and

a distance between the tip and the inlet capillary of 2 mm. The entire surface of the swabs was analysed by DESI-MS through clockwise rotation of the swab toward the MS capillary using a methanol/water (95:5, v/v) solvent at 30 $\mu\text{L min}^{-1}$. Data were acquired in both positive and negative ion modes in the m/z range of 50-2000. Data acquisition parameters were: capillary voltage 0.8-1.2 kV, gas pressure at 4 bar, source temperature at 120°C, and gas desolvation at 450°C. Samples from individual patients were analysed consecutively, but the order in which different patients' samples were analysed was randomised by gestational age, parity, and microbial composition using a Python-based script. RAW files were converted to .mzML with proteoWizard, prior to processing using custom Python scripts (50). Individual scans were merged using a Python implementation of the MALDIquant (51) *binPeaks* algorithm and averaged to generate a single spectrum. The m/z values were re-calibrated using a linear function fitted robustly with the RANSAC algorithm (52) and a reference list of previously annotated m/z values. The spectrum profiles were then assembled into a data matrix of intensities using again the MALDIquant *binPeaks* algorithm.

Statistical analysis

Statistical analyses were performed using R (version 4.5.2) and Graphpad Prism v10.3.1. Statistical significance was assumed at $p < 0.05$. Univariable comparisons of categorical data were completed using Chi-square test or Fisher's exact test. Continuous data was assessed for normality using Kolmogorov-Smirnov tests and presented as mean (+/- standard deviation) or median (+/- inter-quartile range). Immune mediator analyses were performed using two-tailed Wilcoxon-Mann-Whitney with Bonferroni correction for multiple testing or the paired two tailed Wilcoxon signed-rank test. Metabolite differences associated with CST transitions were tested using paired Wilcoxon signed-rank tests. The Benjamini-Hochberg false discovery correction method was used to account for multiple testing when testing mass spectral features. The random forest classifiers and regression model were trained and cross-validated using repeated ($n=15$) stratified 7-fold cross-validation via the *caret* (v7.0.1) model training interface, and the *randomForest* (v4.7.1.2) and *ranger* (v0.17.0) packages. All random forest models used 1000 trees, and *mtry* was fixed to the square-root or one-third of the feature number, for the classifier and regression models, respectively. The regression models also used *splitrule* "variance" and a fixed *min.node.size* of 5. Figures were created using the packages *ggplot2* (v3.5.2), *patchwork* (v1.3.2), and *plotROC* (v2.3.3).

Declarations

Reporting Summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The datasets generated and analysed in this current study have been deposited in the European Nucleotide Archive under the accession code PRJEB109300, PRJEB83084, PRJEB89979, PRJEB90668, and in MetaboLights. This study used pre-existing software and did not generate new custom code or algorithms. Any additional information reported is available from the lead contact upon reasonable request. Source data are provided In Excel tables for each figure.

Code availability

The data analysis scripts are available via GitHub <https://github.com/katiacapuccini/FLIP-1-safety-trial-Imperial>.

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Author contributions

LS, PRB, and DAM conceptualized the study and developed the experimental design. Clinical sampling and coordination of metadata were performed by EB, RLL, KM, LS. Experiments were performed by EB, RLL, LAR, KC, VB, BGM, EM and YSL. Data processing, analysis and/or interpretation was performed EB, RLL, KC, EM, LAR, JRM, KM, SN, GDSC, YSL, BGM, LS, PL, TPP, DAM, and PRB. RL, EB, GDSC, KC, LS, SN and DAM prepared all figures and tables. RLL and LS wrote the first draft of the manuscript. All authors critically reviewed, read and approved the final manuscript.

Competing Interests

PRB, DAM, and TPP hold a patent regarding *Lactobacillus crispatus* composition for use in pregnant subjects. International application number: PCT/GB2022/050453. International publication number: WO 2022/175680. ZT, PRB and DAM hold a patent for Rapid evaporative ionisation mass spectroscopy (REIMS) and Desorption Electrospray Ionisation Mass Spectroscopy (DESI-MS) analysis of swabs and biopsy samples. International application number: PCT/GB2016/050621 International publication number: WO2016142691A1. GDSC, ZT, PRB, and DAM hold a patent on metabolic signatures for prediction of mucosal inflammation and microbial composition. International application number: PCT/GB2022/051833 International publication number: WO2023285832. TPP and LL are employees of Osel, Inc., the company developing LACTIN-V. PPL is the Executive Chairman of Osel, Inc. JRM has received consultancy fees from EnteroBiotix Ltd., Cultech Ltd., and Crescent Enterprise.

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Figures

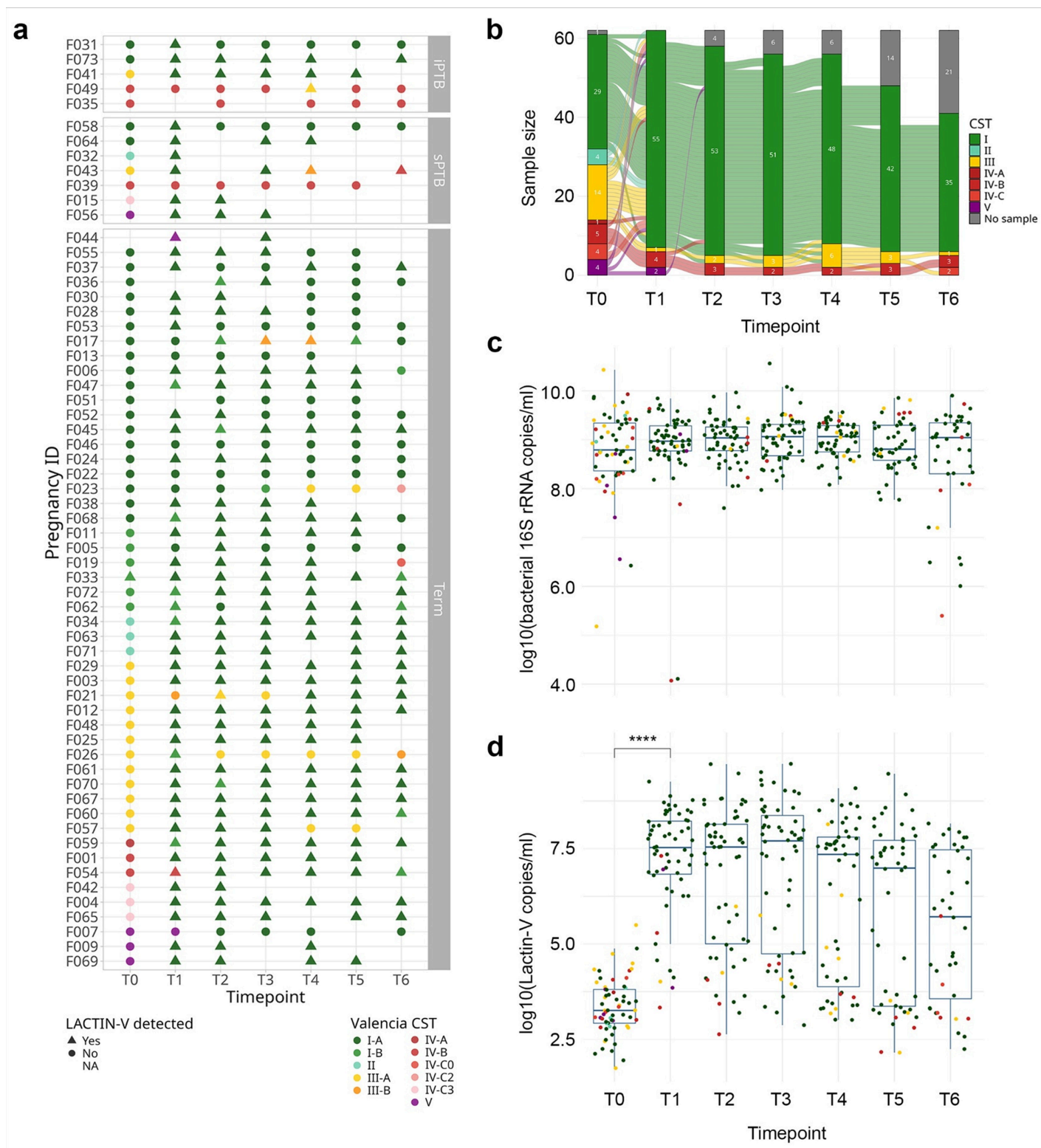


Figure 1

Microbial composition prior to and following LACTIN-V administration

a, Longitudinal dot plot showing community state type (CST; colour-coded) and detection of the LACTIN-V amplicon sequence variant (ASV; triangles) in each participant. Participants are grouped by pregnancy outcome: IPTB (iatrogenic preterm birth), SPTB (spontaneous preterm birth or mid-trimester

loss), and term (≥ 37 weeks). Participant F033 exhibited baseline detection of the predominant *Lactobacillus crispatus* strain included in LACTIN-V (CTV-05). **b**, Sankey plot depicting CST transitions across the FLIP-1 study timepoints. **c**, Box-and-whisker plots showing bacterial load ($\log_{10}$) across FLIP-1 timepoints, with individual participants colour-coded by CST. **d**, Box-and-whisker plots showing LACTIN-V copies/mL for each participant at each timepoint, with CST assignment indicated by colour. Statistical analyses: Two tailed Wilcoxon-Mann-Whitney.

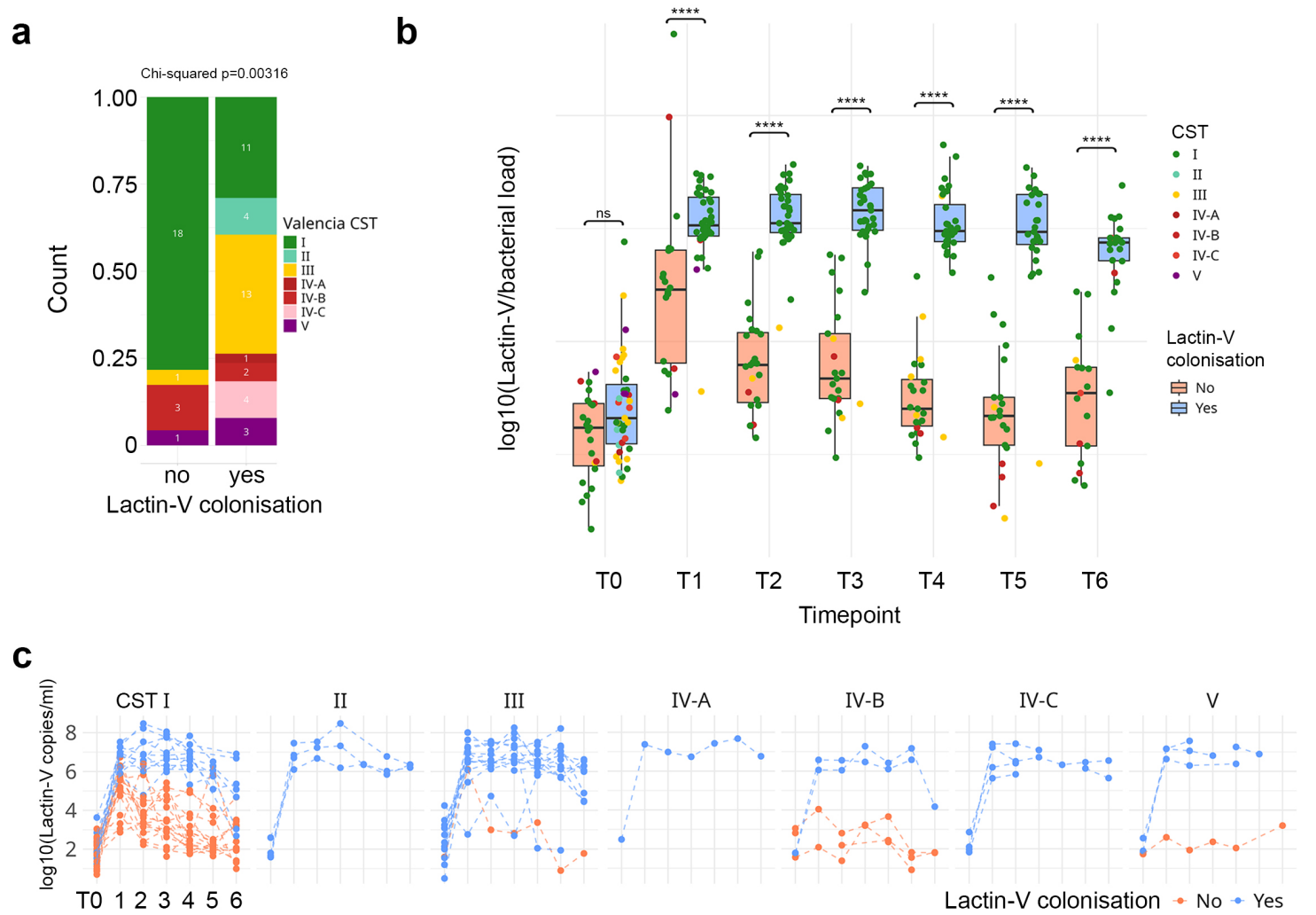


Figure 2

LACTIN-V responder and non-responder trajectory

a, Stacked bar chart showing the distribution of vaginal community state types (CSTs) at timepoint 0 in participants who became LACTIN-V colonisers and non-colonisers. Differences in CST proportions between the two groups were assessed using a non-parametric chi-squared test ($P = 0.00236$). **b**, Box-and-whisker plots showing LACTIN-V copies per mL ($\log_{10}$ scale) in colonisers (blue) and non-colonisers (orange) at each FLIP-1 study timepoint. ($****P < 0.0001$; Two tailed Wilcoxon-Mann-Whitney. **c**, Line graphs showing LACTIN-V copies per mL ($\log_{10}$ scale) stratified by CST, with colonisers shown in blue and non-colonisers shown in orange.

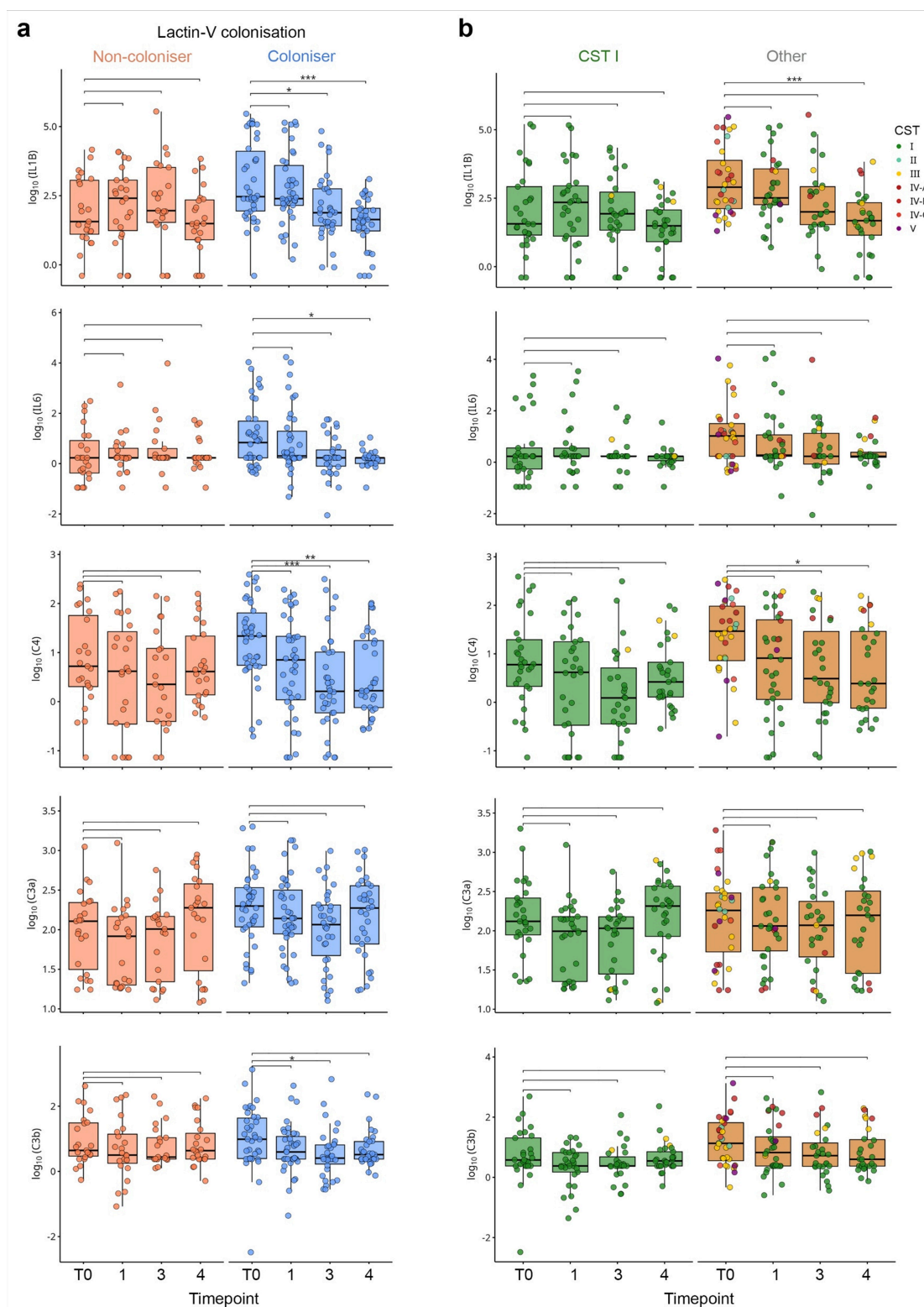


Figure 3

Immune milieu by responder status and CST

a, Box-and-whisker plots showing cervicovaginal concentrations (log₁₀ scale) of the immune mediators IL-1β, IL-6, C4, C3a, and C3b/iC3b in non-colonisers (orange) and colonisers (blue). **b**, Box-and-whisker plots showing concentrations of IL-1β, IL-6, C4, C3a and C3b/iC3b at timepoint 0, stratified by CST

Concentrations are shown at timepoint 0 (prior to treatment), timepoint 1 (post-loading phase), timepoint 3 (post-maintenance phase) and timepoint 4 (8 weeks after cessation of LACTIN-V). Statistical significance was assessed using Two tailed Wilcoxon-Mann-Whitney. P-values were adjusted for multiple testing with the Bonferroni correction. (ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

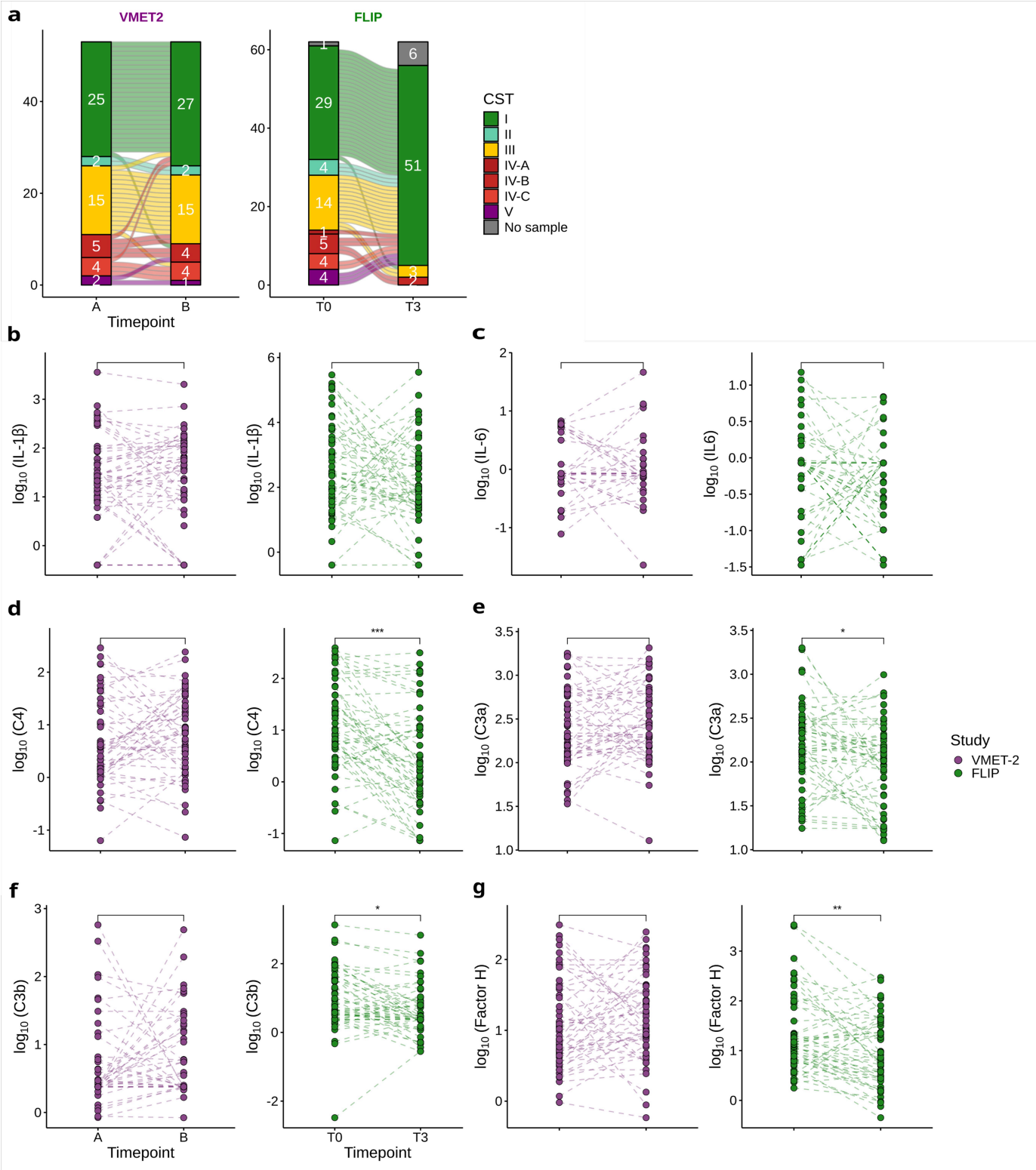


Figure 4

Comparisons in microbial composition and immune milieu with non-treated time-matched controls

a, Sankey plot showing community state type (CST) distributions at two matched longitudinal timepoints in the VMET-2 study: timepoint A (12+0 to 16+6 weeks' gestation) and timepoint B (20+0 to 24+6 weeks' gestation). Timepoints correspond to FLIP-1 study timepoint 0 (12+0 to 16+6 weeks' gestation) and timepoint 3 (19+0 to 23+6 weeks' gestation). **b-g**, Paired comparisons of six cervicovaginal immune mediators (IL-1 β , IL-6, C4, C3a, C3b/iC3b and Factor H; log10 scale) across longitudinal timepoints in the VMET-2 and FLIP-1 studies. Data points from VMET-2 participants are shown in purple, and those from FLIP-1 participants in green. Statistical significance was assessed using the paired Wilcoxon signed-rank test two tailed (ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

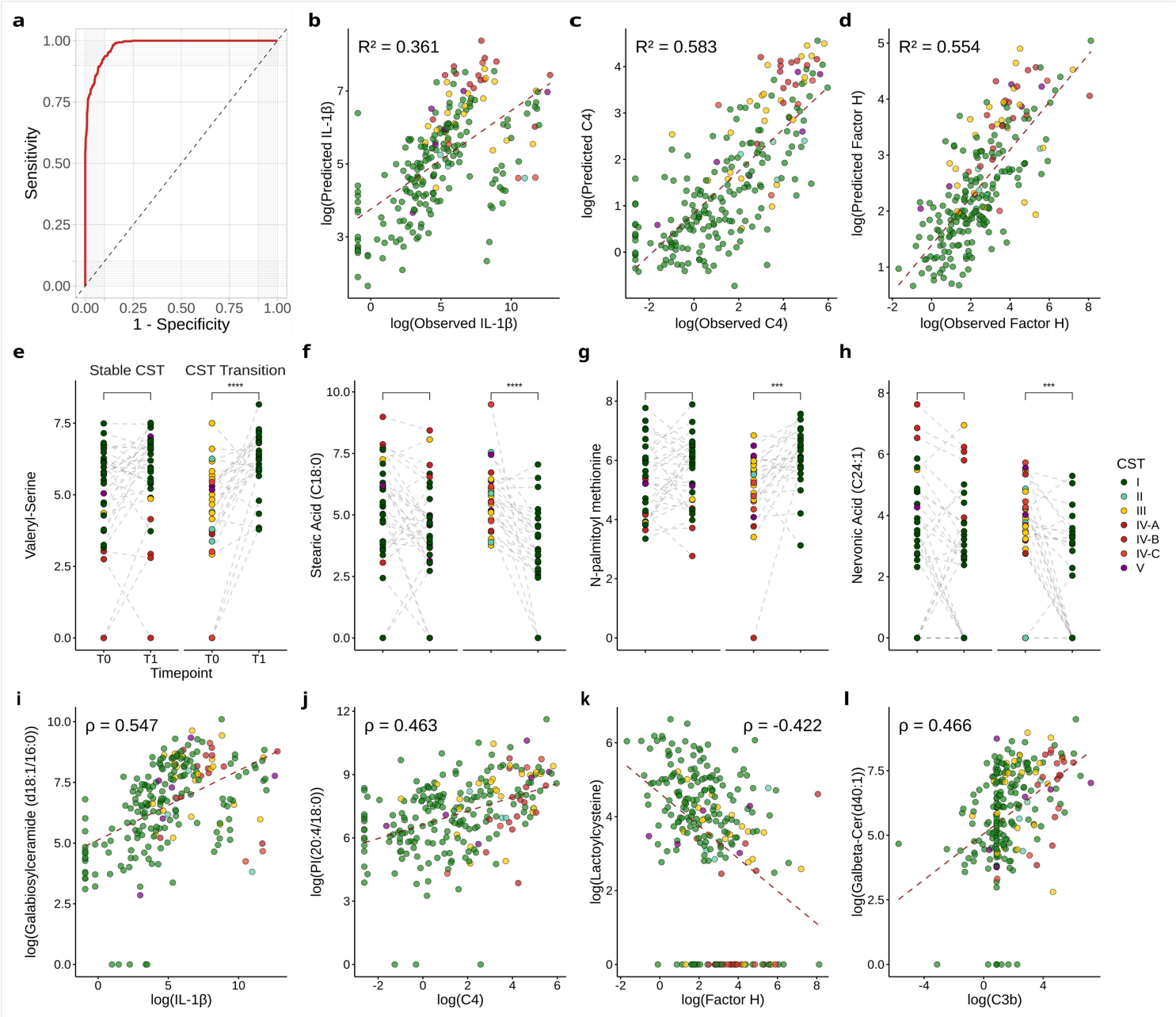


Figure 5

Metabolite changes associated with compositional shifts and mucosal inflammation.

a, Cross-validated receiver operating characteristic (ROC) curve (stratified 7-fold with 15 repeats) for classification of *Lactobacillus*-dominant microbiota (CST I, II, III and V) versus *Lactobacillus*-deplete microbiota (CST IV), using a random forest classifier trained on DESI-MS features (AUC = 0.977). **b-d**, Cross-validated predictions (7-fold; results from a single repeat shown) from random forest regression models trained to predict log-transformed concentrations of IL-1 β , C4 and factor H from cervicovaginal metabolomic profiles. Points are coloured by CST assignment. Dashed lines indicate linear fits between observed and predicted values, with corresponding R^2 values shown. **e-h**, Paired comparisons of four metabolites between baseline (T0) and post-treatment (T1) timepoints, stratified by CST stability. Left panels show participants whose CST remained stable after initiation of LACTIN-V, while right panels show participants who shifted to another CST. Paired measurements from the same pregnancy are connected by grey lines. Exemplar metabolites shown are valyl-serine ($P = 3.28 \times 10^{-6}$), stearic acid (C18:0) ($P = 5.91 \times 10^{-4}$), *N*-palmitoyl methionine ($P = 1.26 \times 10^{-6}$) and nervonic acid (C24:1) ($P = 1.20 \times 10^{-3}$). Points are coloured by CST assignment. Statistical significance was assessed using the paired Wilcoxon signed-rank test (ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). All metabolites shown remained significant after Benjamini–Hochberg false discovery rate correction ($q < 0.05$). **i-l**, Spearman correlations between metabolite abundances and immune mediator concentrations for galabiosylceramide (d18:1/16:0) (IL-1 β), PI(20:4/18:0) (C4), lactoylcysteine (Factor H), and gal β -Cer(d40:1) (C3b). Dashed lines show linear regression fits.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Extendeddata20.3.26.docx](#)