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Probiotic ice cream influences gut and vaginal microbiota in women at high risk of preterm birth: a randomized controlled study

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Abstract

Background Research into probiotic use in pregnancy typically focuses on general probiotic strains. We instead investigated the relation between intake of ice cream with vaginal commensal probiotics (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. rhamnosus GR-1*; these may govern a stable microbiota and may carry beneficial functions in the vagina), throughout pregnancy, and the impact on gut and vaginal microbiomes, in women at high risk of preterm birth.

Methods This was a randomised controlled feasibility trial where the impact on gut and vaginal microbiomes was assessed by using 16 S rRNA gene sequencing and qPCR. In total 43 pregnant women were randomized, with 29 assigned to the intervention group and 14 to the control group. Both groups provided vaginal and rectal swabs by self-sampling at gestational time points. Pregnancy outcomes were registered through hospital records, and ice cream adherence and study experience was recorded.

Results We observed statistically significant gut and vaginal *Lactobacillus* increase during first half of pregnancy in all women with a continued increase in the second half in women compliant with the intervention. *L. crispatus* was found more often in the intervention group, and *L. gasseri*, *L. jensenii* and *L. rhamnosus GR-1* in the ice cream could be recovered in both rectal and vaginal samples. Finally, vaginal *Prevotella spp*, as well as gut *Gardnerella* and *Atopobium spp*, significantly decreased upon intervention. Adherence to the intervention varied but gradually decreased throughout the study with 30.4% displaying excellent adherence in the first time period.

Conclusions We conclude that vaginal commensal probiotics administered in ice cream can be an effective method of optimizing the vaginal and intestinal health in pregnant women at high risk of preterm birth when administered regularly. We give recommendations for future studies.

Trial registration Clinicaltrials.gov registration number 18/27209. Date of registration 03/25/2019. Date of first enrolment 04/08/2019.

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Keywords 16S, Food fortification, Ice cream, Lactobacillus, Longitudinal sampling, Microbiome, Pregnancy, Preterm birth, Probiotics, Vaginal microbiota

Background

Preterm birth (PTB; birth before 37 completed weeks of gestation) accounts for ~ 15 million births annually [1–3] and is the major cause of neonatal mortality and morbidity worldwide [4, 5] even if occurring later in pregnancy [6]. Historically, PTB was thought to be idiopathic since ~ 50% of PTBs occurred in women without risk factors. However, following thorough investigation of fetomaternal and placental factors, it is now recognised that in 96% of cases of PTB there is one recognized risk factor and in 58% of cases, two or more recognized risk factors responsible. Overall, infection accounted for ~ 40% of PTBs [7].

The vaginal and intestinal microbiota in pregnant women is essential in establishing and maintaining a healthy pregnancy including prevention of PTB [8, 9].

The microbiome maintains important functions throughout the human body. It comprises complex communities of bacteria, archaea, fungi, and viruses and is found in large quantities especially in the gut and on the skin [10]. The microbial composition found in the various microbial communities is distinguishable by body site and holds intra- and interindividual variation [11]. As such, the vaginal microbiota is disparate in composition from the gut or skin microbiota and hold variations on its own, from the menstruation cycle, during pregnancy, contraceptive use, antibiotic use, and douching [12]. In healthy women of reproductive age, *Lactobacillus* species are the dominant microorganisms in the vagina, with *Lactobacillus crispatus*, *L. gasseri*, *L. iners* or *L. jensenii* as the most common and abundant strains [13]. A reduction in lactobacilli has been associated with bacterial vaginosis (BV), typically with an elevated relative abundance of *Gardnerella vaginalis*, *Mobiluncus spp.* and *Atopobium vaginae* [14, 15]. BV is often associated with adverse pregnancy outcomes (such as PTB), even mild or asymptomatic cases, making it difficult to predict which pregnancies are most at risk [16]. Accordingly, probiotics have been proposed to beneficially modify the vaginal microbiome in a manner that protects against PTB [9, 17, 18].

The overall aim of this randomized controlled feasibility study among pregnant women with a high risk of spontaneous PTB, was to assess the feasibility and adherence of vaginal commensal probiotics, given as daily ice cream throughout pregnancy. The primary aim was to characterize gut and vaginal microbiota from swabs taken throughout pregnancy with respect to composition, compartmentalization, progression, and colonization of the probiotic strains within and between groups throughout pregnancy. The secondary aims were to examine uptake, participant experience and satisfaction, and to register pregnancy outcomes in both groups.

Methods

Study design and cohort

This was a pilot randomized controlled feasibility study based at Odense University Hospital (OUH) in Denmark between April 2019 and August 2020. Forty-five pregnant women with a high risk of PTB were recruited in the study during their first antenatal midwife session at OUH, and 43 agreed to participate. Recruited women were in their first trimester and had previously a PTB (before gestational age (GA) 37 + 0), and/or a late spontaneous miscarriage (from week GA 16 + 0), and/or had a history of cervical conization. Exclusion criteria were women < 18 years old, current or previous gestational diabetes, pre-gestational diabetes, twin pregnancy, allergy to milk protein, previous bariatric surgery, and non-Danish language skills. All participants provided written informed consent and the study was reviewed by the ethics committee (REC nr: S-20180157). The women were randomized by a computer through OPEN RedCap into two groups at 2:1, an intervention group ($n = 29$), and a control group ($n = 14$). The intervention group followed the intervention protocol with daily ice cream and provided microbiome swab samples. The control group did not ingest the ice cream during their pregnancies but followed the usual care protocol and provided microbiome swab samples. All women were followed up from inclusion until after delivery.

Intervention

The intervention group was instructed to ingest two custom-designed ice creams of 100 ml beakers from Skarø is (a manufacturer of organic ice cream and functional foods) daily from inclusion until labour. The ice cream contained the probiotic strains *Lactobacillus crispatus*, *L. jensenii*, *L. gasseri*, and *L. rhamnosus GR-1* (Astarte™ on the market since 2015 and sold in stores worldwide as a dietary supplement). All strains were added in the same amounts: 0.000125 g/100 g ice cream, 1×10^7 cfu/ml bacteria with a bacteria concentration of 100 billion cfu/g (cfu = colony-forming unit). Prebiotics, protein, 14 g carbohydrates with low glycaemic index and omega-3 fatty acid from fish were also added. Each ice cream contained around 56 calories. The ice cream varied in five flavours and the women could decide which flavours they wanted. Ice cream was chosen to best preserve the pre- and probiotics.

Data collection

All 43 women attended three appointments at the antenatal care units during their pregnancies. The first appointment was an inclusion, randomization, and baseline meeting where all women completed a questionnaire and provided vaginal and rectal swabs. In addition, the intervention group also received forms to register their daily ice cream intake throughout their pregnancy. Second appointment (time point 2) and third appointment (time point 3) involved registration of pregnancy complications as well as vaginal and rectal swabbing for all women. Descriptive data (Table 1) were collected from all included women through interview together with electronic health records and documented in OPEN RedCap database at time point 1. Body Mass Index (BMI) was calculated. Outcome variables (Table 2) were collected postpartum through electronic health records. Feedback from the intervention group, and their own perception of their participation in the study, were registered by a survey post-partum. Based on adherence to ice cream ingestion, participants were divided into the following groups: excellent (ingested 67–100% of the ice cream), good (34–66%), poor (10–33%), no adherence (< 10%) or no answer (N/A). Only women with excellent or good adherence were included in the metagenomic analysis as intervention group. Women with no to poor adherence operationalized as extra controls. This way group comparison was based on effective exposure to the probiotic, reflecting intervention status and adherence.

Vaginal and rectal microbiome samples

The swabs were performed by self-sampling with standard swabbing kits containing 2 ml liquid Amies medium, where the women would swab themselves from the vagina and rectum in the hospital bathroom. If the women were uncomfortable with self-sampling, the midwife would perform these instead. The women were given both oral and written information on how to swab correctly and were handed two separate swabbing kits marked V (vaginal) and R (rectal). The swabs were performed within the gestational time windows of week 9–18, week 16–32, and week 27–40. Because of the COVID-19 pandemic, it was necessary to reschedule some consultations and swab collections. However, the goal was to keep 8–10 weeks between each sample. Swab samples were stored at −80 °C within 15 min of collection. All samples were pseudonymized. Further analysis was conducted at the Experimental and Clinical Research Centre (ECRC), Berlin, Germany.

DNA isolation, protocol accuracy and 16 S rRNA gene sequencing

Laboratory procedures were conducted under a hood with laminar flow (LabGarda ES Energy Sever Classe II Laminar Flow, NuAire Inc., Plymouth, MN, USA) to limit environmental contamination. Following the manufacturer's procedures, total DNA was extracted from all swab samples with the ZymoBIOMICS DNA Miniprep Kit (ZYMO Research Europe GmbH, Freiburg, Germany). 250 ml aliquots from the swab solution were processed. DNA extraction followed the manufacturer's recommendations with slight modifications for better mechanical disruption [19]. Isolated DNA-samples were stored at −20 °C before being shipped to LGC Genomics Berlin for 16 S sequencing. All the samples were shipped in dry ice. To account for inter-person variations in laboratory workflow, the same person performed the DNA extraction on all swabs from the same subject on the same day. DNA concentrations were determined using a Qubit assay kit. V3-V4 hypervariable region of the 16 S rRNA gene was PCR amplified using 16 S rRNA-specific Primers:341 F "Klindworth" (CCTACGGGNGGCWGCAG) – 785R "Klindworth" (GACTACHVGGGTATCTAAKCC). Bacterial 16 S rRNA gene amplicons were sequenced targeting the V3-V4 (300 bp paired-end sequencing) using Illumina MiSeq platform performed by LGC Genomics GmbH (Berlin, Germany).

Real-time quantitative PCR

For all samples collected for each time point, quantitative PCR (qPCR) was performed using an Applied Biosystems QuantStudio 3 system (Thermo Fisher Scientific, Darmstadt, Germany). Amplification and detection were performed in 96-well optical plates (Applied Biosystems) with SYBR-Green qPCR assay (Applied Biosystems). All amplifications were performed in duplicates in a final volume of 5 µL containing 13.8 µL of a 2xSYBR Green PCR Master Mix including ROX as a passive reference (Applied Biosystems), 500 nM of each primer *L. crispatus* (Lcrisp_CbsA2F: GTACCAAGCCAAAGCAAGAC - Crisp_CbsA2R: GTTTGAAGCCTTTACGTAAGTC), *L jensenii* (L_jensenii-Fw: AGTTCTTCGGAATGGACA TAG - L_jensenii-Rev: GCCGCCTTTTAAACTTCT), *L. gasseri* (L_gasseri-Fw: TCAAGAGCTGTTAAGGCTGT - L_gasseri-Rev: CTATCGCTTCAAGTGCTTT), *L. iners* (L_iners-Fw: GTCTGCCTTGAAGATCGG - L_iners-Rev: ACAGTTGATAGGCATCATC), and *L. rhamnosus* (L_rhamnosus-Fw: TGCTTGCATCTTGATTTAATTT TG - L_rhamnosus-Rev: GTCCATTGTGGAAGATTCC C) and 1.2 µL of template DNA (0.5 mg/mL). For amplification, the standard protocol of the Applied Biosystems QuantStudio 3 system was followed, i.e., an initial cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 1 min at 60 °C. PCR product specificity was tested by melting curve (Tm) analysis. Standard curves for quantification consisted of 10-fold serial dilutions in the range of 10^8–10^0 copies of the 16 S rRNA gene of the *E. coli* (Invitrogen, C404010) amplified with primers 27 F (50-G

TTTGATCCTGGCTCAG-30) and 1492R (50-CGGCTACCTTGTTACGAC-30). The total amount of bacterial 16 S in rectal swabs was quantified with universal primers, Univ 337 F 50-ACTCCTACGGGAGGCAGCAGT-30 and Univ 518R 50-GTATTACCGCGGCTGCTGGCAC-30.

Data management and statistical analysis

Continuous variables were presented as means and standard deviations (normally distributed variables) or medians and ranges if not normally distributed. Categorical variables were presented as frequencies and proportions. The characteristics and outcomes between the two groups were compared using Mann-Whitney U test for continuous variables and Chi^2-tests and Fisher's exact tests for categorical variables.

A value of $p < 0.05$ or a value of $q < 0.1$ (Benjamini-Hochberg false discovery rate control (FDR) corrected p values) were considered significant for all analyses. Stata (v.17) was used for statistical data analysis and R was used for the bioinformatic data analysis. The raw sequences obtained were processed using LotuS (1.62) [20]. Poisson binomial model-based read filtering was applied [21]. Operational taxonomic unit (OTU) clustering (UPARSE) [22] was based on sequence similarity of 97%, while SILVA version 138 [23] was used for taxonomic profiling. A total number of 4854 OTUs were detected. To account for the difference in sampling depth and avoid bias in alpha diversity metrics, rarefaction of the data was performed using the rtk package [24]. OTU counts were rarefied to the smallest retained sample size (i.e., 16230 and 16560 raw reads for the vaginal and rectal swabs respectively) to obtain relative microbiota abundances in each sample, accounting for read depth in each sample. Since the data were not normally distributed, nonparametric tests were used for all association tests. The Mann-Whitney-U test was used for discrete predictors. For pairs of continuous variables, a nonparametric Spearman correlation test was used. FDR was applied in all multiple testing situations to control the family-wise error rate at 10%. Hierarchical clustering was used to establish grouping patterns of the different study samples, including an updated adaptation of the approach used to define "enterotypes" in the human gut using the "Dirichlet multinomial" R package (v. 1.36.0) [25]. The Chi^2-test implemented in base R was used to test for significant differences in the resulting community-type distribution between samples grouped by time point and intervention, respectively. Alpha diversity (Shannon index) and beta diversity (Bray–Curtis dissimilarity) [26] were calculated from OTU-level taxonomic profiles. Baseline comparisons between groups were performed using the Mann–Whitney U test. To determine the impact of intervention, time point, and swab site on the taxonomic composition of the microbiome, permutational multivariate analysis of variance (PERMANOVA) was performed. Bray-Curtis distances were used for all analyses. PERMANOVA test was performed using the "adonis" package in R (v. 0.4). Mantel test was performed using the "ape" package in R (v. 5.3). Gestational age was compared between groups using Wilcoxon tests and Levene's tests. Models were compared using likelihood ratio tests (LRTs). Interaction terms were included and retained only when they significantly improved model fit. All bioinformatic analyses were conducted in R.

Results

Characteristics of the study population

Of $n = 43$ recruited women at high risk of PTB, $n = 6$ withdrew from the intervention group within the first two weeks, leaving $n = 37$ women to be followed throughout pregnancy. Accordingly, $n = 23$ were randomized to ingest daily synbiotic ice cream containing vaginally commensal lactobacilli (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. rhamnosus GR-1*), prebiotic, omega-3 fatty acid, carbohydrates with low glycaemic index and protein, and $n = 14$ were in the control group ingesting no such ice cream. Gut and vaginal microbiota composition were determined in all women through qPCR and 16 S sequence analysis of swab samples taken at three time points during pregnancy, with trial satisfaction and outcomes assessed postpartum. Adherence was self-tracked throughout, and relevant demographic and clinical covariates were collected at enrolment. Patient characteristics are displayed in Table 1. Controls had fewer previous pregnancies ($p = 0.015$), and more conizations ($p = 0.015$) than the intervention group, but no other significant differences were found in the registered parameters (all $p > 0.05$) (Table 1). No significant differences were seen when comparing characteristics of the intervention group ($n = 23$) with the women who withdrew from the study ($n = 6$).

Adherence to the fortified daily ice cream with vaginal commensal strains

Each woman in the intervention group had variable adherence from time point to time point (Fig.1 and supplementary data Table S1). Adherence to ice cream gradually decreased throughout the study, with 30.4% ($n = 7$) displaying excellent adherence in period 1 and 4.35% ($n = 1$) in period 3. A total of 13% ($n = 3$) of the intervention group had good or excellent adherence throughout their pregnancy, while 43.5% ($n = 10$) had good or excellent adherence in one or more periods. Additionally, 43% had no adherence during the last time period before birth. No participants in either the control or intervention group reported taking other probiotic supplements. No statistically significant connection was seen between parity and adherence. The women without conization

Table 1 Patient characteristics among 37 pregnant women with high risk of preterm birth

	Intervention group, $n=23$	Control group, $n=14$	*P*-value*
Maternal age at inclusion in years, median (IQR)	32.0 (28.0–33.0)	30.0 (29.0–32.0)	0.777
BMI kg/m^2, median (IQR)	23.1 (21.8–25.8)	24.6 (19.9–26.9)	0.876
Gestation at inclusion in weeks, median (IQR)	12 + 6 (12 + 1–14 + 0)	13 + 1 (12 + 5–14 + 6)	0.293
Gravidity, *n* (%)			
Primigravida	0 (0)	4 (28.6)	0.015
Multigravida	23 (100)	10 (71.4)	
Parity, *n* (%)			
Nulliparous	1 (4.35)	4 (28.6)	0.057
Multiparous	22 (95.65)	10 (71.4)	
Smokers, *n* (%)	5 (21.7)	0 (0)	0.135
Risk of PTB, *n* (%):			
Previous preterm birth < GA 37	14 (60.9)	5 (35.7)	0.184
Previous late miscarriage > GA 16	2 (8.7)	0 (0)	0.517
Conization	5 (21.7)	9 (64.3)	0.015
Previous preterm birth < GA 37 & conization	1 (4.3)	0 (0)	1.000
Previous late miscarriage > GA 16 & conization	1 (4.3)	0 (0)	1.000
Gestation at swab samples in weeks, median (IQR)			
1. Samples (Baseline)	12 + 6 (12 + 1–14 + 0)	13 + 1 (12 + 5–14 + 6)	0.293
2. Samples (Time point 2)	20 + 1 (19 + 4–20 + 5)	20 + 4 (20 + 0–21 + 6)	0.212
3. Samples (Time point 3)	32 + 0 (31 + 0–32 + 6)	31 + 2 (29 + 3–34 + 0)	0.719

PTB Preterm birth, *BMI* Body mass index, *GA* Gestational age, *IQR* Interquartile range

*The *P*-value was calculated with the Mann-Whitney U-test/Wilcoxon rank sum test if the median was used and with the Chi^2-test and Fisher's exact in case of frequencies and proportions.

as their PTB risk factor had significantly lower adherence in time period 2 than the women with conization ($p=0.014$).

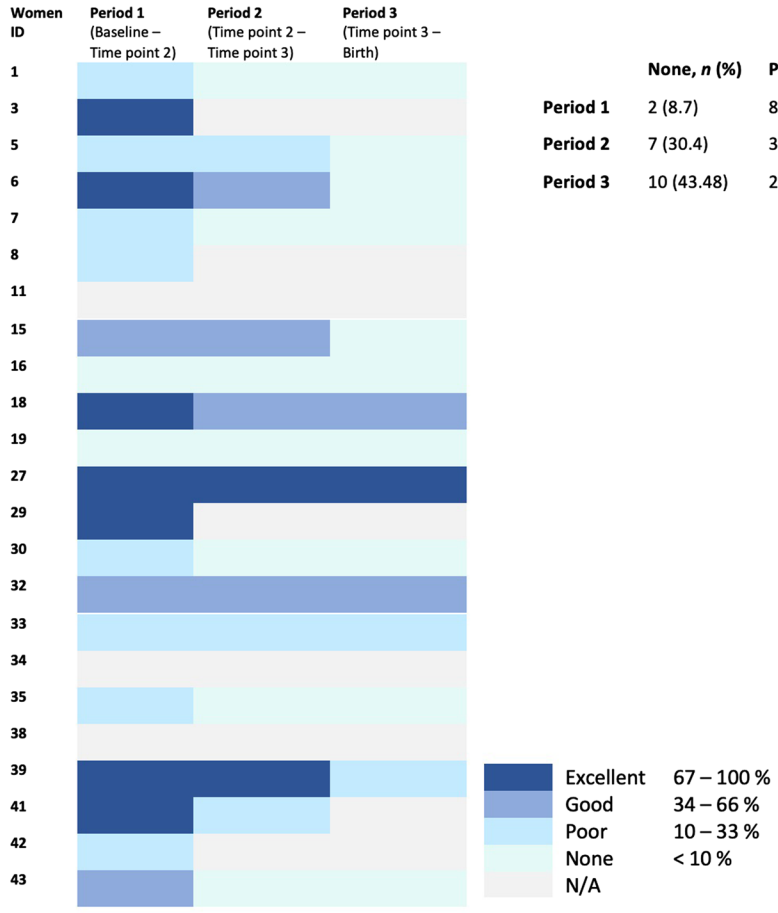
Feasibility of the intervention

Of the 23 women in the intervention group, 17 (74%) participated in the survey regarding their study experience. With respect to adherence, not being in the mood (53%), disliking the taste (53%) and nausea (41%) were the most common reasons for non- or poor adherence, followed by satiety (29%) and too large ice cream portions (29%). 59% ($n=10$) of the intervention group found the ice cream portions too large in general. 59% of all included women who answered the survey ($n=29$, $n=17$ intervention group, $n=12$ control group) reported that the overall experience of the study was not time-consuming, while 24% ($n=7$) found it to be a burden and 17% ($n=5$) were unsure. Finally, nearly all women (93.1%, $n=27$) found self-sampling to be easy and efficient.

Diversity and composition of vaginal and gut microbiome during pregnancy

The following analysis was done with control group and no to poor adherence versus women in the intervention group with good or excellent adherence to the intervention, in order to compare exposure to non-exposure. To avoid bias all analyses are accompanied by checks for whether any bias exists at baseline with regards to each measurement with potential confounding from this, described, and accounted for throughout. As expected, the vaginal microbiome was significantly less diverse than and compositionally different from the gut microbiome (MWU test, $q < 0.001$) (Fig. 2a). There was no significant impact of time point or intervention on alpha diversity or multivariate measures of microbiome composition. Furthermore, microbial communities from vaginal swabs were significantly different in microbial community composition than the microbial communities from rectal swabs (Mantel statistic R: 0.5974, $p < 0.001$) (Fig. 2c, d). Comparison between the control and intervention group was based on their daily ice cream intake adherence. A PERMANOVA test at baseline showed no significant difference between groups in vaginal or gut microbiome composition before the intervention, implying no major discrepancies in host-microbiome interactions prior to the intervention resulting from the analytical group separation. We further assessed baseline comparability, by analyzing alpha diversity (Shannon index) and beta diversity (Bray–Curtis dissimilarity) between adherence groups using Wilcoxon rank-sum tests and PERMANOVA, respectively, stratified by swab site. No significant differences were detected in either metric (all $q>0.1$), indicating that microbiome diversity and composition were similar across groups before the intervention (Supplementary Fig. S1). Gestational age at baseline was also comparable between groups (Wilcoxon $p = 0.23$), although a difference in GA variance was observed at swab sample time point 2 (Levene's test $p = 0.039$), suggesting that GA should be included as a covariate in downstream analyses. There was no significant effect of intervention or time point on overall alpha or beta diversity at swab sample time point 2 or 3 (Supplementary Fig. S2). Thus, baseline comparisons confirmed no pre-existing group differences in microbiome composition, supporting the conclusion that any downstream variation was not attributable to initial imbalances between groups. To determine whether distinct vaginal microbial community state types (CSTs), previously reported [27, 28], were visible in our cohort, we employed Dirichlet multinomial mixtures (DMM) modelling. This revealed three distinct vaginal microbial CSTs (CST I, CST II, and

a)



b)

	None, *n* (%)	Poor, *n* (%)	Good, *n* (%)	Excellent, *n* (%)	N/A, *n* (%)
Period 1	2 (8.7)	8 (34.8)	3 (13.04)	7 (30.4)	3 (13.04)
Period 2	7 (30.4)	3 (13.04)	4 (17.4)	2 (8.7)	7 (30.4)
Period 3	10 (43.48)	2 (8.7)	2 (8.7)	1 (4.35)	8 (34.8)

Fig. 1 Adherence with the ice cream during pregnancy in the intervention group. **a)** Colour chart of the ingested amount of the fortified ice cream in percentage during each period for each of the 23 women in the intervention group. **b)** Allocation of women by their adherence each period. The table shows both the number of women and the percentage concerning the entire intervention group

CST III), and two distinct gut enterotypes (community 1 and community 2) (Fig. 2b). We could not replicate all previously described vaginal CST variations, possibly because our cohort was relatively homogeneous.

Genus level site overlap and directional shift analyses

To investigate body site-specificity, we evaluated genus-level overlap between rectal and vaginal swabs at the three time points (TP1, TP2, and TP3) in both the intervention group with good to excellent adherence and in the control and no to poor adherence group (Fig. 3).

After applying a prevalence filter to include only genera detected in at least 10% of samples at each time point, we calculated the observed number of shared genera between sites and the Jaccard similarity index, a test of difference in compartmentalization between analytical groups. A permutation-based null model (1,000 label permutations) was used to estimate the expected degree of overlap under the assumption of random site assignment, allowing for the calculation of empirical *p*-values and 95% confidence intervals.

In the intervention group, the number of shared genera between rectal and vaginal swabs was consistently low across all time points, despite high overall richness. At time point 1 (baseline), 68 genera were shared, 74 at time point 2, and 51 at time point 3. Jaccard indices ranged from 0.226 to 0.374, and all *p*-values were effectively 1.0. A similar pattern was observed in the control group: 94 genera overlapped at time point 1, 72 at time point 2, and 77 at time point 3. Again, all empirical *p*-values equalled 1.0. Furthermore, in the control group time point 1 and 2 and showed no vaginal-unique genera, indicating that the vaginal community was entirely nested within the rectal community, to the resolution afforded under 16 S analysis.

Overall, these findings demonstrate that the two sites overlap. The intervention does not change the overall degree of compartmentalization, but there remains in

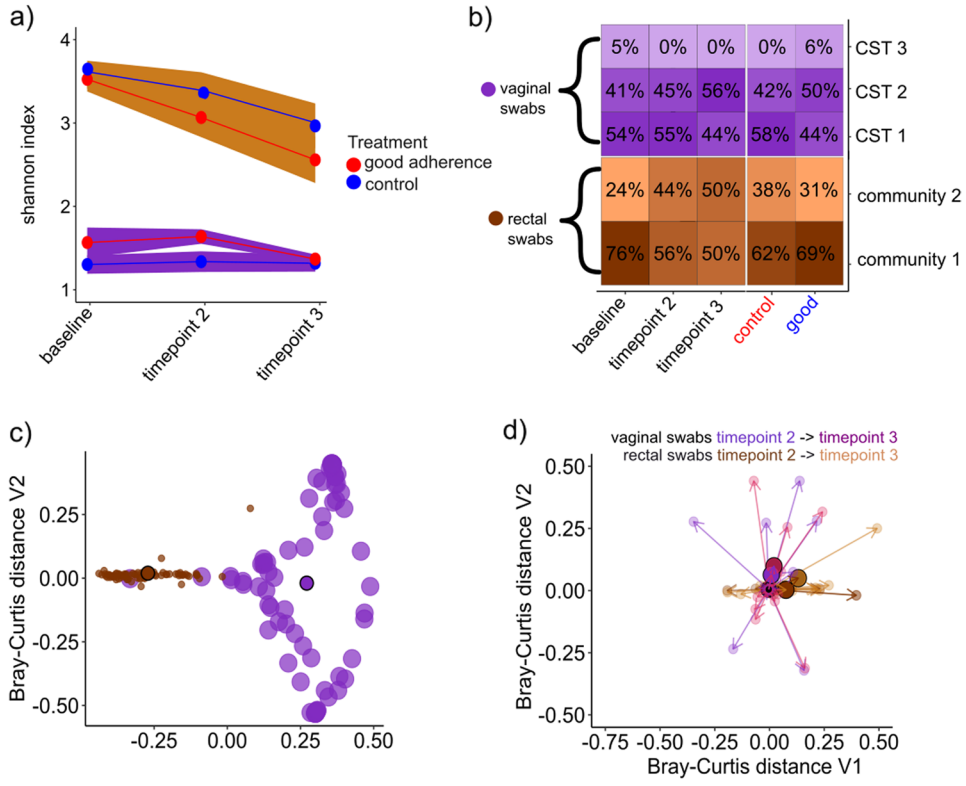


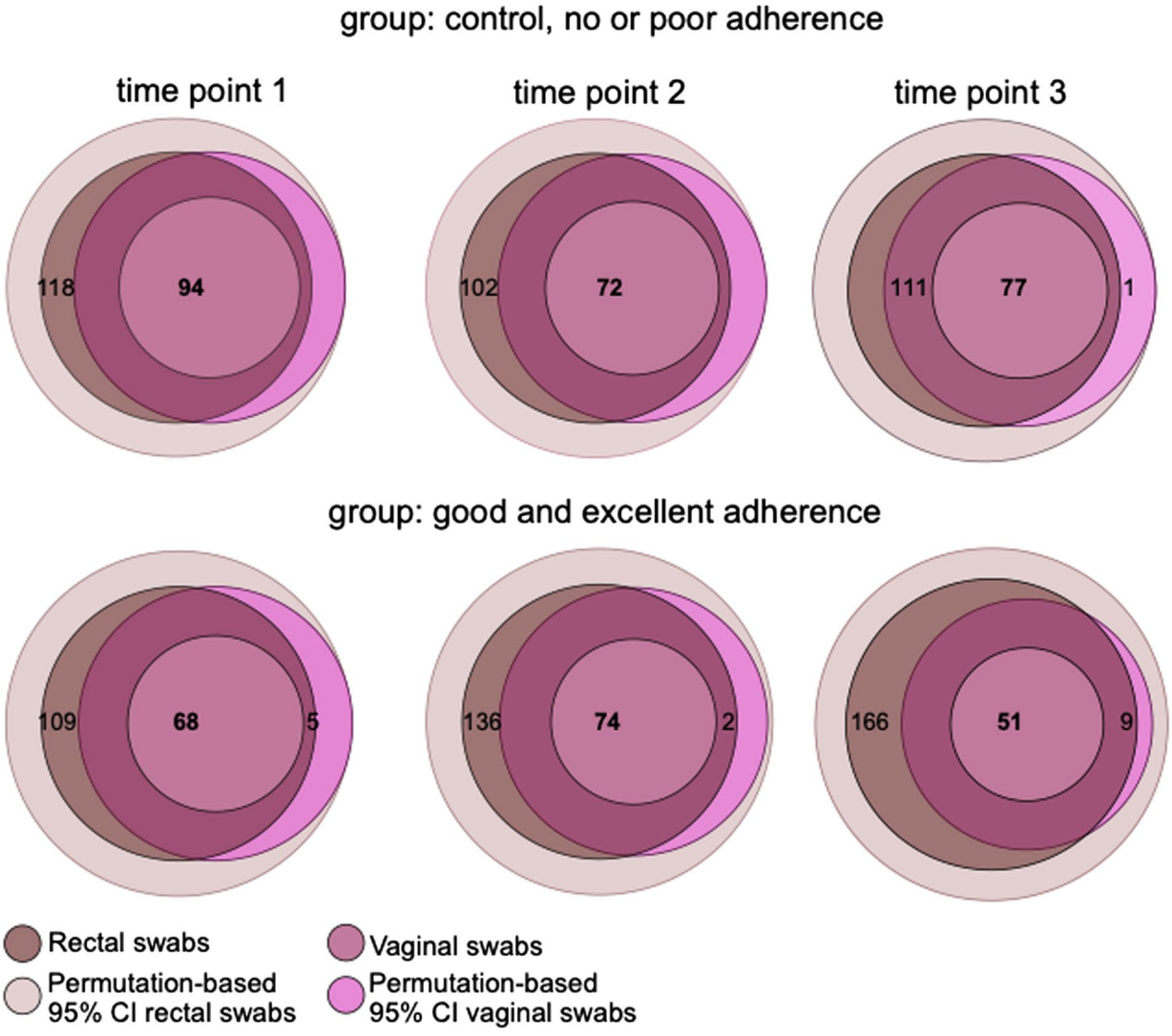
Fig. 2 Multivariate analysis of gut and vaginal microbiome stratified by intervention and time point. Intervention is stratified control and no to poor adherence vs. intervention (women with excellent or good adherence). **a)** Vertical axis shows Shannon diversity in rectal vs. vaginal swabs over time stratified by intervention. Rectal swabs show a significantly higher Shannon index compared to the vaginal (MWU test, $q < 0.001$, FDR-BH). **b)** Clustering (community typing) of all samples based on a Dirichlet multinomial model stratified by swab site. Vaginal and gut community type distribution (vertical axis) stratified by time point and intervention in all samples. Testing for significant differences between groups was done using the chi-square test. **c)** Projection of genus-level microbiome composition of all samples stratified by swab site. Principal coordinates analysis (PcoA) plots of Bray–Curtis dissimilarity showing a significant difference between the two swab sites (Mantel statistic R: 0.5974, $p < 0.001$). **d)** Principal coordinates analysis (PcoA) plots of Bray–Curtis distance trajectory over time stratified by swab site and shows fluctuation in the microbiome over time

either case transfer between the sites. Thus, changing gut composition can credibly change vaginal composition, and therefore analysis proceeds to further characterize any such changes. To further explore potential microbial transfer between sites, we examined directional shifts from rectal to vaginal swabs (Supplementary data Fig. S5). Genera that were present only in rectal swabs at time point 1 and absent in vaginal swabs were tracked at subsequent time points to determine whether they appeared in vaginal swabs at time point 2 or 3. It showed transition of some taxa over time from the rectal environment to the vaginal, however not significant in regard to intervention.

Impact of pregnancy progression and synbiotic intervention on key vaginal microbiota

We next focused on a subset of bacterial taxa either linked previously to reproductive health such as BV or PTB, or that were vaginal commensals present within our tested synbiotic ice cream (*L. crispatus*, *L. gasseri*, *L. jensenii*, *L. rhamnosus GR-1*). Using linear mixed-effects models we assessed whether their distribution and presence in our cohort was impacted by either the course of pregnancy or by the course of the intervention, representing the latter through interaction terms between intervention status and the passage of time (Fig. 4). Figure 4a, and 4c denotes that the ice cream intervention significantly increases vaginal carriage of *L. jensenii* (LRT) $q < 0.1$) and significantly depletes a subset of *Prevotella* OTUs from baseline to time point 3. Furthermore, we observed a significant enrichment of a subset of *Prevotella* OTUs from baseline to time point 2, followed by a depletion from time points 2 to 3, over time. More surprising was the significant enrichment of *Lactobacillus* in the control and no to poor adherence group from time points 2 to 3 and the significant depletion of *L. iners* and *L. gasseri* during the same period. Otherwise, we saw no significant shifts in the abundance of any other selected taxa across time points or groups.

Figure 4b, and 4d displays the rectal distribution and presence over time and intervention of the specific lactobacilli species. There was a significant depletion of



intervention group	time point	shared	only rectal	only vag	total	Jaccard distance	null mean	95% CI	p-value
good adherence	TP1	68	109	5	182	0.374	154.7	86–172	0.991
good adherence	TP2	74	136	2	212	0.349	180.3	139–198	0.999
good adherence	TP3	51	166	9	226	0.226	132.7	85–152	1.000
control	TP1	94	118	0	212	0.443	208.5	204–212	1.000
control	TP2	72	102	0	174	0.414	165.1	145–172	1.000
control	TP3	77	111	1	189	0.407	179.8	164–186	1.000

Fig. 3 Genus-level overlap between rectal and vaginal swabs across time points and groups. Each panel shows observed genus sharing between rectal and vaginal swabs at time point 1, time point 2, and time point 3 for the intervention group with good to excellent adherence and control and no to poor adherence group. Numbers within circles indicate observed counts: Center: Shared genera. Left ring: Genera unique to rectal swabs. Right ring: Genera unique to vaginal swabs. Outer shell size reflects the 95% CI upper bound of the overlap expected under random swab site assignment (permutation test, $n = 1{,}000$). In all cases, observed overlaps were substantially lower than the null expectation, indicating strong anatomical separation of the two microbial niches

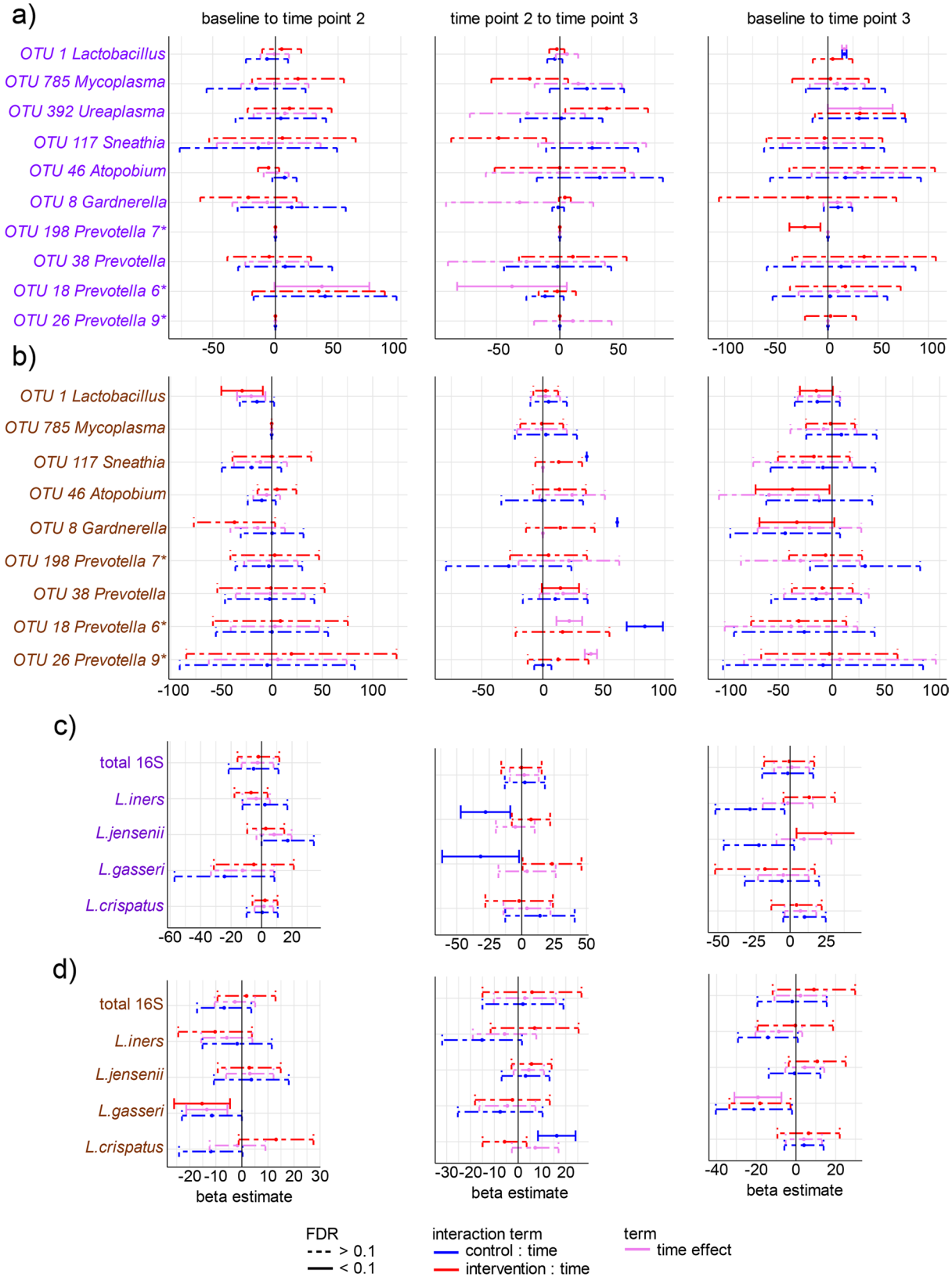


Fig. 4 (See legend on next page.)

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Fig. 4 Univariate analysis of intervention and time effect on bacterial taxa. Linear mixed-effects model showing time and interaction (time point: intervention) effect on specific genera from 16 s (**a**, **b**) and specific *Lactobacillus* species copy numbers from qPCR (**c**, **d**), stratified by time trajectory. Forest plots showing models beta estimate and 95% CI (x-axis) of taxa for the interaction term (control: time (blue), intervention: time (red)) and time term (purple). Solid lines indicating significant effects on the respective taxa (likelihood ratio test, FDR-BH), for the vaginal microbiota (**a**, **c**) and gut microbiota (**b**, **d**), respectively

Lactobacillus from baseline to time point 2 in the intervention group. This effect was accelerated from baseline to time point 3. In the qPCR model a significant depletion in *L. gasseri* from baseline to time point 2 in the intervention group was seen. Furthermore, a significant enrichment in *Prevotella* was seen in the intervention group from time point 2 to time point 3 and an enrichment in *L. crispatus* from time point 2 to 3 in the control group. Finally, a significant depletion of *Atopobium* and *Gardnerella* was evident in association with the treatment from baseline to time point 3.

Assessing probiotic penetrance and colonization through qPCR

Going beyond the limitations of 16 S amplicon sequencing, we used qPCR to test for the presence of the probiotic strains specifically in each sample and *L. iners*. This later species has been associated with adverse pregnancy outcomes such as PTB or intra-uterine infection [27–29]. Although the intervention group tended to have lower lactobacilli prevalence at baseline than the control and no to poor adherence group, this difference was not statistically significant (Supplementary data Fig. S6). There was an upward shift of the specific *Lactobacillus* species in the entire cohort from baseline to time point 2 (Fig. 5). In the control and no to poor adherence group the *Lactobacillus* enrichment then largely ceased before time point 2, whereas the enrichment continued in the intervention group until time point 3. Thus, all probands gained lactobacilli in the first half of pregnancy, but those who adhered to the intervention also continued to gain lactobacilli in the latter half of pregnancy. Figure 5 shows a significant increase in vaginal *L. rhamnosus GR-1* in the intervention group from baseline to time point 3 ($q < 0.001$). In addition, rectal amount increased significantly from baseline to time point 2 and between time point 2 and 3 ($q < 0.1$). Moreover, both vaginal and rectal *L. jensenii* abundance increased significantly between time point 2 and 3 in the intervention group compared to the control group ($q < 0.01$). *L. gasseri* abundance decreased significantly in rectal samples in the intervention group from baseline to time point 2 ($q < 0.001$) but increased significantly from baseline to time point 3 compared to the control group ($q < 0.001$). There was also an increasing trend of both vaginal and rectal *L. crispatus* and *L. iners* in the intervention group throughout the study, though neither was statistically significant ($q > 0.1$). Since the different *Lactobacillus* species exist as a part of the normal vaginal microbiota, it was expected to also find these species in the control group vaginal swabs albeit a lower abundance.

Registered pregnancy and neonatal outcomes

Observations during pregnancy and birth from 37 pregnant women are presented in Table 2. There were no significant differences between the two groups with respect to pregnancy or birth outcomes, including GA at birth, number of PTBs, mode of delivery, live or perinatal death, birth weight, bleeding during delivery, admission to neonatal care, 5-minute APGAR score or asphyxia (all $p > 0.05$). Consistent with background rates, two women in the intervention group developed GDM during their pregnancies. Both had low adherence with the intervention and had no other adverse pregnancy outcome. Gestational hypertension was observed in one woman in the control group. The groups had no significant differences in hospitalizations, tocolysis, or treatment with progesterone, antenatal corticosteroids, or antibiotics during pregnancy (all $p > 0.05$). Seven women (intervention group $n = 4$; control group $n = 3$) were prescribed antibiotics because of urinary tract infections (UTI) ($n = 6$) or as Lyme disease prevention ($n = 1$) during their pregnancies. Subgroup analysis for multigravidity and conization showed no significance in any of the outcome measurements.

Discussion

Summary of findings

In this feasibility study comparing exposure and non-exposure to an intervention, we investigated the relationship between intake of ice cream containing vaginal commensal probiotics and the effect on the vaginal and gut microbiome in pregnant women at high risk of PTB. We demonstrated that: (i) while a small amount of daily probiotics did not shift the overall diversity in either community, all women showed enrichment of vaginal lactobacilli during the first half of pregnancy, with a continued increase in the latter half of pregnancy in compliant women from the intervention group; (ii) *L. gasseri*, *L. jensenii* and *L. rhamnosus GR-1* from the ice cream could be recovered in both rectal and vaginal samples; (iii) *L. crispatus* was found slightly more often in the intervention group, though this was not statistically significant and (iv) vaginal *Prevotella*, as well as gut *Gardnerella* and *Atopobium* (all unfavourable bacteria when present in the urogenital tract), significantly decreased in

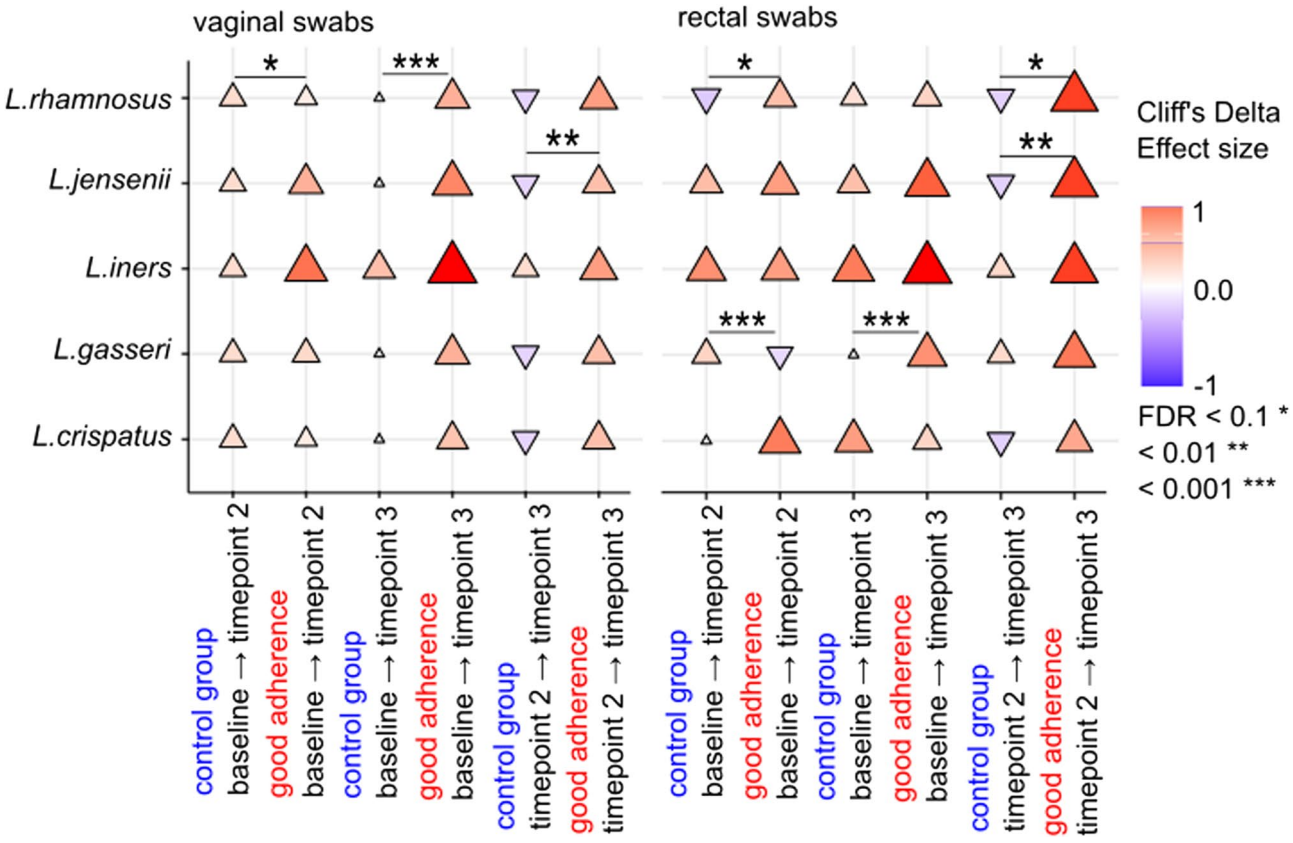

Fig. 5 Effect of intervention on changes in Lactobacillus prevalence (qPCR) from baseline to subsequent time points, shown separately for control and no to poor adherence versus good adherence (treatment) groups. For each species, upward triangles indicate enrichment and downward triangles indicate depletion; triangle size is proportional to the Cliff's delta effect size. Comparisons show the change in each group from baseline to time point 2, baseline to time point 3, and time point 2 to time point 3 stratified by treatment. Stars indicate taxa for which the change over time in the treatment group was significantly different from the change in the control group (time × intervention interaction, FDR-BH corrected). Significance levels: *FDR < 0.1*, *FDR < 0.01*, *FDR < 0.001*

association with the intervention. To our knowledge, this study is novel and original regarding the use of ice cream to deliver and preserve probiotics and nutritional supplements during pregnancy.

Probiotics

Probiotic bacteria adapted to colonize the vagina can migrate from the intestine to the vagina and influence the vaginal microbiota by what we eat or drink [30, 31]. Probiotics can be administered as lyophilized (drying by freezing in a high vacuum) pills, or live bacteria in refrigerated capsules, exhibiting best shelf life and effectiveness if stored cold until intake [32]. Other synbiotic dietary factors such as prebiotics [33] (indigestible fibres that have immunoregulatory functions [34]), fish oil [35] and high levels of protein [36] can be added to support probiotic growth and activity [37]. A daily intake of the vaginal commensal strains (*L. crispatus*, *L. jensenii*, *L. gasseri*, *L. rhamnosus GR-1*) that promote a stable microbiota and carry out beneficial functions in the vagina, along with one or more of the above-mentioned dietary beneficial supplements, may be a practical and efficient way to promote a healthy and stable microbiota composition throughout pregnancy. Several studies have demonstrated that probiotics are safe and effective for the treatment or prevention of inflammatory conditions such as necrotizing enterocolitis and inflammatory bowel disease [38]. Current research on the effect of probiotics in pregnancy is contradictory and this may be due to the use of nonspecific probiotics [39]. Among probiotic studies, there remains heterogeneity with respect to population, trial methodology, choice of probiotic microorganism *(*whether *Bifidobacteria* or *Lactobacillus)*, and whether the probiotic is used alone or as an adjunct to antibiotic therapy (mainly metronidazole but increasingly clindamycin). The species of lactobacilli and whether single or multiple strains are used are also important. In general, non-vaginal species of lactobacilli like *L. rhamnosus* and *L. reuteri* or *L. rhamnosus* and *L. gasseri* in combination have been used. In addition, the length of treatment is important as well as the use of different non-standardized BV outcome endpoints with

Table 2 Pregnancy and neonatal outcomes among 37 pregnant women with high risk of preterm birth.

	Intervention group, *n* = 23	**Control group, *n* = 14**	***P*-value***
Gestation at birth in weeks, median (IQR)	39 + 4 (37 + 6–40 + 4)	40 + 1 (39 + 1–41 + 1)	0.222
Preterm birth < GA 37, *n* (%)	5 (21.7)	1 (7.1)	0.376
GA, median (IQR)	25 + 2 (16 + 4–35 + 4)	34 + 4 (34 + 4–34 + 4)	0.770
Delivery, *n* (%)			0.474
Vaginal	17 (73.9)	13 (92.9)	0.217
C-section	5 (21.7)	1 (7.1)	0.376
Vacuum	1 (4.4)	0 (0)	1.000
Spontaneous	18 (78.3)	11 (78.6)	1.000
Induced	5 (21.7)	3 (21.4)	1.000
Alive/perinatal death, *n* (%)			
Alive	21 (91.3)	14 (100)	0.517
Perinatal death	0 (0)	0 (0)	
Late miscarriage, *n* (%)	2 (8.7)[#]	0 (0)	
Birth weight, g, median (IQR)	3400 (3240–3740)	3627.5 (3230–4105)	0.449
Bleeding, ml, median (IQR)	320 (250–650)	250 (200–350)	0.180
Admittance to neonatal care, *n* (%)			
Yes	4 (17.4)	1 (7.1)	0.627
No	17 (73.9)	13 (92.9)	
N/A	2 (8.7)	0 (0)	
5-minute Apgar score, *n* (%)			
0–3	1 (4.35)	0 (0)	1.000
4–6	0 (0)	0 (0)	
7–10	19 (82.61)	14 (100)	
N/A	3 (13.04))	0 (0)	
Asphyxia, *n* (%)			
No	18 (78.3)	13 (92.9)	0.777
Little	2 (8.7)	0 (0)	
Moderate	2 (8.7)	1 (7.1)	
N/A	1 (4.3)	0 (0)	
Hospitalization during pregnancy, *n* (%)			
Yes	4 (17.4)	2 (14.3)	1.000
No	19 (82.6)	12 (85.7)	
N/A	0 (0)	0 (0)	
Gestational hypertension, *n* (%)			
Yes	0 (0)	1 (7.1)	0.378
No	23 (100)	13 (92.9)	
N/A	0 (0)	0 (0)	
GDM, *n* (%)			
Yes	2 (8.7)	0 (0)	0.517
No	21 (91.3)	14 (100)	
N/A	0 (0)	0 (0)	
Tocolysis, *n* (%)			
Yes	2 (8.7)	2 (14.3)	0.625
No	21 (91.3)	12 (85.7)	
N/A	0 (0)	0 (0)	
Progesterone, *n* (%)			
Yes	5 (21.7)	3 (21.4)	1.000
No	18 (78.3)	11 (78.6)	
N/A	0 (0)	0 (0)	
Antenatal steroids, *n* (%)			
Yes	3 (13)	2 (14.3)	1.000
No	20 (87)	12 (85.7)	
N/A	0 (0)	0 (0)	

Table 2 (continued)

	Intervention group, *n* = 23	Control group, *n* = 14	*P*-value*
Antibiotics during pregnancy, *n* (%)			
Yes	4 (17.4)	3 (21.4)	0.667
No	17 (73.9)	8 (57.2)	
N/A	2 (8.7)	3 (21.4)	

GA Gestational age, *IQR* Interquartile range, *GDM* Gestational diabetes mellitus

**P*-value calculated with Mann-Whitney U-test/Wilcoxon rank sum test if median was used and Chi^2-test and Fischer exact test if frequencies and proportions

#Late miscarriages in GA 16+4 and 15+6 respectively

respect to the treatment of and the prevention or recurrence of BV. Finally, the route of administration, whether vaginal (direct via capsules, tampons, applicators) or oral is likely to be of more importance.

Microbiology of vaginal eubiosis and dysbiosis

The healthy gut has a very diverse microbiota. In contrast, vaginal eubiosis is characterized by a significantly lower diversity than gut eubiosis. In the vagina, eubiosis is provided by a beneficial lactic-acid-producing microbiota, predominantly but not uniquely, from the genus *Lactobacillus.* Vaginal eubiosis is provided by such organisms through numerical dominance, prevention of biofilm formation through vaginal epithelial cell adhesion, by lactic acid production that decreases the vaginal pH, and hydrogen peroxide (H_2O_2) production, which is toxic to other microorganisms and pathogens [13, 40]. In contrast, vaginal dysbiosis constitutes a prolonged deviation from a low-diversity, *Lactobacillus*-abundant/-dominant vaginal microbiota to a microbiota that has a high diversity and high abundance of potentially pathogenic organisms [41]. By identifying organisms such as *L. iners* and *Atopobium vaginae* (previously under-detected and hence under-appreciated) using cultivation-independent techniques, we now know that there are different subtypes of vaginal eubiosis and dysbiosis, which may influence the response to therapy and phenotypic outcome and cannot be discerned using vaginal Gram stain microscopy, currently the gold standard for the diagnosis of BV. Using culture techniques, lactobacilli can only be identified to the genus level, so it is impossible to comment on species-specific properties such as production of lactic acid, H_2O_2 among other factors that promote eubiosis. New information from molecular-based techniques [42] demonstrates that worldwide, the eubiotic microbiota of the healthy vagina is dominated by one, or at the most two species of lactobacilli from a shortlist of four species: *L. crispatus*; *L. gasseri*; *L. iners* and *L. jensenii* corresponding to CSTs I, II, III and V proposed by Ravel et al. [13].

Biochemistry of vaginal eubiosis and dysbiosis

The antimicrobial, antiviral and immunomodulatory properties of lactic acid, the major organic acid metabolite produced by lactobacilli has been demonstrated eloquently by Tachedjian et al. [43]. Acidity levels as well as H_2O_2 production differ between *Lactobacillus* species. In women with a *Lactobacillus* dominated vaginal microbiota lactic acid concentrations are inversely proportional to vaginal pH, and lactic acid is primarily responsible for acidification of the vagina. The eubiotic vagina has a pH of no higher than 4.5 while the dysbiotic vagina has a pH of >4.5. Production of L- and D-isomers of lactic acid differs between lactobacilli species and emphasizes the D-isomer's possible protective role. Lactic acid also exists as a protonated (non-dissociated H^+; neutrally charged ion) or a lactate anion (dissociated H^+; negatively charged ion). The protonated form has antimicrobial and immunomodulatory properties compared to the lactate anion, and the protonated form of lactic acid predominates at a pH < 3.9. Finally, the concentration of lactic acid in the eubiotic vagina is ~ 110mM compared to < 20mM in vaginal dysbiosis. This contrasts with the concentrations of short chain fatty acids like acetate, succinate, butyrate, and propionate that barely reach a concentration of 1mM in the eubiotic vagina but in vaginal dysbiosis, acetic acid concentrations may reach 120mM. These findings led other researchers to conclude: "The association of high lactic acid levels with a *Lactobacillus* dominated microbiota suggests that this organic acid metabolite contributes to the beneficial properties ascribed to lactobacilli, such as decreased susceptibility of the human host to urogenital pathogens, which would be a desirable characteristic for a vaginal probiotic." [43].

The vaginal microbiome and preterm birth

Approximately 95% of strains of *L. crispatus* and 94% of strains of *L. jensenii* produce H_2O_2, and 9% and 7% of such women respectively had BV. In contrast, approximately 71% of strains of *L. gasseri* and only 9% of strains of *L. iners* produced H_2O_2 of which 43% and 36% of such women respectively had BV in a previous study [42]. A vaginal microbiota with *L. iners* dominance or low lactobacilli in general, especially in the first trimester of pregnancy, is associated with adverse pregnancy outcomes such as PTB or intra-uterine infection [27, 28]. *L. iners* co-exists well with unfavourable bacteria such as *Gardnerella* and *Prevotella* [16, 44] that significantly

increase the risk of short cervix, PTB, late miscarriage [43] through BV [17, 45, 46]. Conversely, dominance by *L. crispatus* in the first half of pregnancy produces a stable microbiome and suggests protective properties that decrease the risk of PTB [47]. Accordingly, there is increasing interest in the role that *L. iners* may play in the aetiology of PTB [17, 29, 47]. We included *L. iners* in our qPCR analysis, because *L. iners* was found to dominate vaginal microbiota in 67% of women who gave birth before 34 completed weeks of pregnancy, compared to 31% and 29% of women who gave birth between 34 and 37 weeks of gestation, and at term respectively (p = 0.003). In contrast, *L. crispatus* was associated with term birth (p = 0.009) [17, 47]. The presence or absence of *L. iners* however does not distinguish vaginal eubiosis from dysbiosis. While *L. iners* is commonly found in association with BV as well as vaginal eubiosis, it is rare to find one of the other three common vaginal lactobacilli, *(L. crispatus*, *L. gasseri*, and *L. jensenii*) in abundant numbers in the presence of BV and, conversely, it is rare to find BV when these three species of lactobacilli are found in abundance in the presence of minimal diversity of other potentially pathogenic microorganisms [17, 42, 48]. This reflects why recent advances in cultivation-independent techniques for the diagnosis of BV and characterization of vaginal eubiosis and dysbiosis do not include *L. iners* in their choice of candidate organisms [41].

The association between the rectal and vaginal microbiota

A certain degree of correspondence between the vaginal and rectal microbiota exists. In a genotyping study by Aila et al. of bacteria from paired vaginal and rectal samples from pregnant women 63 species were identified. 14.3% were present in the vagina, 41.3% present rectally and 44.4% present in both locations [49]. In our study, we identified on average 200 species and found that most bacteria found in the rectum were also found in the vagina, but not vice versa. This would suggest a bacterial transit from the gastrointestinal tract to the vagina, with bacteria that can exist in both environments, but also another transit to the vagina possibly from the perineum or through sexual transmission [50]. Furthermore, Kim et al. reported that the vaginal microbiota changes spatially throughout the vaginal tract [51], which may indicate that bacteria found in the lower part of the vagina might be in closer relation to the gut bacteria. These findings support our hypothesis that the vaginal microbiota can be improved through oral intake of probiotics [52]. Pregnancy is associated with hormonal changes and immune response changes, changes in the mucosa of the vagina and cervix, and most often also dietary and behavioural changes that predispose to a less rich and less diverse vaginal microbiota compared to non-pregnancy microbiota [53–55]. Under normal circumstances, the vaginal microbiota changes during pregnancy as well, with high amounts of lactobacilli in the early stages of pregnancy, caused by the placental estrogen peak [56]. This shifts to a lower diversity mid pregnancy, with a more simplified community of bacteria that persists throughout pregnancy [53–55]. Lactobacilli not only protect against BV, but also against several other urogenital diseases such as bacterial and yeast infections, sexually transmitted diseases [57], UTIs [58], and HIV infection [59–61]. In a trial by Reid et al., 2001 where 42 women were randomized to ingest one of three different encapsulated probiotics (*L. rhamnosus GR-1*, *L. fermentum* or *L. rhamnosus GG*) for 28 days, a favourable change in vaginal microbiota in women who ingested *L. rhamnosus GR-1* and *L. fermentum*, indicated that the probiotics ingested orally, affected the vaginal microbiota [62]. Another study showed a significant association between frequent intake of probiotic dairy products and a reduced risk of PTB [63]. Antonio et al. found co-existence of *Lactobacillus spp.* in both the vagina and the rectum are protective against BV, in contrast to exclusive presence in either location or absence in both. They further suggested that the rectum contributes as a *Lactobacillus* reservoir for the vagina [50], emphasizing that in contrast to vaginal administration oral intake of lactobacilli would be the preferred route.

Vaginal commensal *Lactobacillus* strains from the probiotic ice cream used in this study could provide a protective mechanism to the vagina that is beneficial to a larger demographic beyond pregnant women at high risk of PTB. The hypothesis of a protective effect strengthens with the decrease in vaginal *Prevotella*, as well as of gut *Gardnerella* and *Atopobium*, in our intervention group, since all three bacteria are associated with BV and linked to PTB [13, 64]. Besides decreasing BV, the intervention could also prevent bacteria ascending from the vagina to the intrauterine space, which is a mechanism for spontaneous preterm labour leading to PTB [65]. Similarly, vaginal probiotics may reduce vaginal inflammation. Pregnancy causes physiological changes which influence the immunoprotective effect of cervical mucus and stimulation of IgA synthesis in the gut by lactobacilli, and such lactobacilli might be both anti-infective and anti-inflammatory systemically and locally [66, 67]. *Sneathia* is a BV related bacterium also involved in unfavourable pregnancy outcomes. We found that the presence of *Sneathia* in the vagina was directly associated with its presence in the gut, which merits further investigation [68].

Strengths, limitations, and feasibility

Although adverse pregnancy outcome frequencies in the two groups were not significantly different, this feasibility trial was not powered to assess this because of their rarity. Nevertheless, this study establishes the vaginal

microbiome baseline in pregnancies at high-risk of PTB. Intervention with ice cream was selected as an effective method for preserving the pre- and probiotics. Ice cream provides a protective barrier for the probiotic during its passage through the gastrointestinal tract, and increases probiotic survival by permitting addition of prebiotics, fat, and sugar [69]. Of note is that some loss of viability is unavoidable when adding probiotics to ice cream, because of freezing, storage, and melting [70], which is important when calculating the concentration of probiotics. Many *Lactobacillus* strains are resistant to freezing, though it is possible that *L. crispatus* is one of the sensitive strains [71, 72]. The recommended concentration of probiotics is 10^7 bacteria/gram product, but 10^8–10^9 viable bacteria/gram product is suggested for more certain therapeutic effect [73] and future research involving *L. crispatus*, should consider increasing the amount of this specific probiotic.

Recruiting pregnant women for this study was easy due to the use of ice cream for administration, with only two women declining participation. However, adherence presented a challenge, mainly because of the large portions of ice cream used twice a day and dislike of the taste. In future studies, women should be informed of the ice cream's healthy properties as a small meal, and that it also works as a yogurt if defrosted, which might improve adherence albeit viability may decrease when melting the ice cream [70]. Limiting the intervention to a shorter period might also improve adherence to the intervention. Ice cream was safe to use, convenient, non-invasive with no reported adverse events related to the study intervention were reported. Self-sampling was effective and might promote adherence to testing [74]. In this study 93.1% of the women found it easy to self-sample, and Fig. 2 confirms the legitimacy of the swab location origin. The longitudinal design of the study enabled a comprehensive view of both the vaginal and gut microbiota changes throughout pregnancy. In addition, we included women of different PTB risk profiles ranging from moderately high-risk pregnancies (with a history of conization) to very high-risk pregnancies (with multiple late abortions, previous PTB, and more than one risk factor). This allowed us to generalize our findings to this patient group, although the benefits of a synbiotic might be greater in very high-risk pregnancies. Another strength of our investigation was the unique idea of using ice cream for administration, which not only appeals to a wider audience and preserves the synbiotic well but is also beneficial to pregnant women experiencing nausea or reduced appetite [75]. The probiotic we used is easily available commercially. One notable advantage is that we accounted for antibiotic use as a potential confounding variable. Finally, the feasibility of future studies is supported by our choice of candidate *Lactobacillus* as a probiotic particularly the use of *L. crispatus.* This study was conducted at strain-species level, which previous research have urged [17].

Since this was a feasibility study, it involved a small study population, which limits our ability to conclude significance of differences or associations, and the limited variation in data decreases the general applicability of the findings. We acknowledge that all statistical analyses performed on outcomes in this study should be regarded as exploratory and not proof of causality. The analytical groups combine two distinct causal factors, intervention mechanism and intervention adherence, which can pose a major limitation if unaddressed. In this study, we conducted extensive microbiome analyses to identify potential biases and confirmed that the groups showed no significant baseline differences for the tested outcomes.

Gestational age at sampling was evaluated across groups and time points. While GA was well balanced at baseline, there was a significant difference at swab sample time point 2 and a trend at swab sample time point 3, which supports adjusting for GA in downstream models. This might have been due to a lack of a more specific time interval for the second samples. However, when adjusting for all possible confounders, we still demonstrate that the intervention has an effect on the microbiome. An additional limitation is the lack of information on dietary intake that contain probiotics (milk, yogurt etc.) apart from the intervention, which could act as an important confounding variable. In addition, the study did not consider racial diversity. Such racial variation in microbiota have previously been noted with Hispanic and African women harbouring more of anaerobic microbiota than *Lactobacillus* with dominant CST IV, while most of the Asian and Caucasian women have *Lactobacillus* as the dominant member of the microbiota [53]. Although self-reporting of ice cream adherence in studies with regular hospital follow-ups have shown to be a reliable method [76], it must be considered as a possible limitation in this study. All six women who withdrew from the study early were from the intervention group, which may have influenced the results, though the extent of this impact is uncertain.

Due to the small population in this study, we were restricted in our choice of women at risk of PTB. In any future study, we would recommend using as an entry criterion, a previous PTB following spontaneous preterm labour prior to 34 completed weeks gestation. This is likely to identify a group of women at high risk of infection related PTB in whom the use of probiotics is most likely to be of benefit. We should also be more stringent in future studies to exclude women who took antibiotics for other reasons in pregnancy after randomisation. In addition, the safety of the ice-cream delivery from

this study and feedback from participants should help recruitment in future.

Conclusion

In conclusion, PTB is a significant cause of neonatal and perinatal mortality and morbidity. This randomized controlled feasibility study indicates that vaginal commensal probiotics can be effective in optimizing vaginal and intestinal health in pregnant women at high risk of PTB if taken regularly. The use of ice cream to deliver and preserve probiotics and nutritional supplements during pregnancy is a novel and unique approach, that could present an acceptable mode of administration for this patient group, if adjusted by our recommendations. Further research on the effect of well-chosen probiotics on PTB is merited.

Abbreviations

PTB	Preterm birth
BV	Bacterial vaginosis
OUH	Odense University Hospital
GA	Gestational age
Cfu	Colony–forming unit
BMI	Body mass index
qPCR	quantitative PCR
OTU	Operational taxonomic unit
CSTs	Community state types
DMM	Dirichlet multinomial mixtures
LRT	Likelihood ratio test
UTI	Urinary tract infection
H_2O_2	Hydrogen peroxide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40748-025-00238-3.

Supplementary Material 1.

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Authors' contributions

LSB and TUPB contributed equally to this work. LSB collected data, coordinated the project, contributed to the metagenomic and statistical analyses, performed PCR analysis, and wrote the manuscript. TUPB processed the metagenomic data, performed the bioinformatics analyses and contributed to the manuscript. RFL contributed to the manuscript, supervised, and reviewed the study. JRB participated in the design of the study, organized the database, and collected data. LM contributed to the metagenomic analyses and to the statistical analyses. UL pre-processed the metagenomic data. CAV and RD supervised and reviewed the study. SKFS contributed to the metagenomic and statistical analyses and supervised the study. JSJ conceived the study, participated in its design and coordination, and supervised the study. All authors have read and approved the final manuscript.

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Data availability

Sequence files for the 16S rRNA gene sequencing and metadata used in this study have been deposited in Figshare ([https://doi.org/10.6084/m9.figshare.25213607](https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.6084%2Fm9.figshare.25213607&data=05%7C02%7Clebor17%40student.sdu.dk%7Cfa4b819f69234544c1d108dc2d4f0966%7C9a97c27db83e4694b35354bdbf18ab5b%7C0%7C0%7C638435064692683410%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=igYuCn9TscTZbjB5LG5GGLVGkOLMTEBNIV2k8bVMv2g%3D&reserved=0)). Sequence files for the qPCR analysis have also been deposited in Figshare ([https://doi.org/10.6084/m9.figshare.25213604](https://eur03.safelinks.protection.outlook.com/url=https%3A%2F%2Fdoi.org%2F10.6084%2Fm9.figshare.25213604&data=05%7C02%7Clebor17%40student.sdu.dk%7Cfa4b819f69234544c1d108dc2d4f0966%7C9a97c27db83e4694b35354bdbf18ab5b%7C0%7C0%7C638435064692693372%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=HyhBize8C9LrZaHNn6IjcpQF8ulqFXyvg5lhe361pPs%3D&reserved=0)). The original script performed in R is available in GitHub ([https://github.com/Theda-sys/PIG-VAMP/tree/main](https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fgithub.com%2FTheda-sys%2FPIG-VAMP%2Ftree%2Fmain&data=05%7C02%7Clebor17%40student.sdu.dk%7Cfa4b819f69234544c1d108dc2d4f0966%7C9a97c27db83e4694b35354bdbf18ab5b%7C0%7C0%7C638435064692706795%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=Kvddo8KxzlSKPEQXWkQDmh1r%2F6gwCqNb2bjRUMEgCy4%3D&reserved=0)).

Declarations

Ethics approval and consent to participate

This study followed all regulations by, as well as was submitted to and reviewed by the Ethics Committee for the region of Southern Denmark (REC nr: S-20180157). The committee decided that the study did not need approval from their institution, since the study was performed with ordinary foods and the vaginal and rectal samples were done by self-sampling. All participants provided informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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