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Potential pathobionts in vaginal microbiota are affected by fish oil and/or probiotics intervention in overweight and obese pregnant women☆

Noora Houttu [a,*], Kati Mokkala [a], Wisam Tariq Saleem [b], Seppo Virtanen [c,d], Juuso Juhila [e,1], Ella Koivuniemi [a], Outi Pellonperä [f], Kristiina Tertti [f], Paula Luokola [e], Timo Sorsa [g,h], Anne Salonen [c], Leo Lahti [b], Kirsi Laitinen [a]

[a] Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, University of Turku, Turku, Finland
[b] Department of Computing, Faculty of Technology, University of Turku, Turku, Finland
[c] Human Microbiome Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland
[d] Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[e] Actim Oy, Espoo, Finland
[f] Department of Obstetrics and Gynecology, University of Turku and Turku University Hospital, Turku, Finland
[g] Department of Oral and Maxillofacial Disease, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[h] Department of Oral Diseases, Karolinska Institutet, Huddinge, Sweden

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ABSTRACT

New means to stabilize the microbial balance during pregnancy could benefit maternal health. Our objectives were to investigate in overweight/obese pregnant women 1) the impact of long-chain polyunsaturated fatty acids (fish oil) and/or probiotics on the vaginal microbiota, 2) its relation to gestational diabetes mellitus (GDM) and 3) its interaction with vaginal active matrix metalloproteinase-8 (aMMP-8) and serum high sensitivity C-reactive protein (hsCRP) and phosphorylated insulin-like growth factor-binding protein-1 (phIGFBP-1), IGFBP-1 and aMMP-8.

The women were allocated to fish oil + placebo, probiotics + placebo, fish oil + probiotics and placebo + placebo-groups, from early pregnancy onwards (fish oil: 1.9 g docosahexaenoic acid and 0.22 g eicosapentaenoic acid; probiotics: *Lacticaseibacillus rhamnosus* HN001 (formerly *Lactobacillus rhamnosus* HN001) and *Bifidobacterium animalis* ssp. *lactis* 420, 10^{10} colony-forming units each). Vaginal and serum samples (early pregnancy, n = 112; late pregnancy, n = 116), were analyzed for vaginal microbiota using 16S rRNA gene amplicon sequencing and vaginal aMMP-8 and serum hsCRP, aMMP-8, phIGFBP-1 and IGFBP-1 by immunoassays. GDM was diagnosed from a 2-h 75 g OGTT. ClinicalTrials.gov, NCT01922791.

The intervention exerted effects on many low-abundant bacteria. Compared to the placebo-group, there was a lower abundance of potential pathobionts, namely *Ureaplasma urealyticum* in the fish oil-group, *Ureaplasma, U. urealyticum* and *Prevotella disiens* in the probiotics-group, *Dialister invisus* and *Prevotella timonensis* in the fish oil + probiotics-group. Moreover, probiotics decreased the abundance of a few potential pathobionts during pregnancy. Many bacteria were related to GDM. The vaginal aMMP-8 level correlated significantly with α-diversity and inversely with two *Lactobacillus* species.

Dietary interventions, especially probiotics, may have beneficial effects on the vaginal microbiota during pregnancy.

1. Introduction

The vaginal microbiota plays an important role as the first line of defense against pathogenic bacteria [1]. Normally, the vaginal microbiota composition is stable, low in diversity and dominated by beneficial lactic acid producing bacteria, with *Lactobacillus* being the most

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* Corresponding author.
E-mail address: nhmhou@utu.fi (N. Houttu).

[1] Affiliation at the time of the study.

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predominant genus. Previously, five vaginal community state types (CST) have been observed; CST I dominated by *Lactobacillus crispatus*, CST II by *Lactobacillus gasseri*, CST III by *Lactobacillus iners*, CST IV with diverse species including anaerobic bacteria and CST V dominated by *Lactobacillus jensenii* [2]. Compositional changes occur in the vaginal microbiota, mainly as a result of changes in the regulation of sex hormones; these are present throughout a woman's whole lifespan. It seems that when she is pregnant, the stability of her vaginal microbiota as well as the abundances of vaginal *Lactobacillus* species are elevated [3], whilst the overall bacterial diversity is lower than that present in a non-pregnant woman [4–6]. Furthermore, lifestyle related factors, such as diet and particularly a vegetarian diet [7] and obesity [8], may affect the vaginal microbiota by increasing its diversity. Interestingly, some investigators have postulated that the composition of the vaginal microbiota differs according to the gestational diabetes mellitus (GDM) status [9,10].

Due to the importance of a balanced vaginal microbiota for maternal and neonatal health, it would be advantageous to devise novel approaches to seek an optimal equilibrium within the vaginal microbiota, particularly in certain high-risk groups, such as overweight and obese pregnant women. Based on previous studies, probiotics and long-chain polyunsaturated fatty acids (LC-PUFA) could act as such modifiers since they have been shown to exert a beneficial impact on enteric microbiota and further favorably influence the metabolic health of the host via their immunomodulatory function [11]. It has been reported that orally administered probiotics, with time, can modulate the vaginal microbiota [12] and alter the interaction network present in the vaginal bacterium in pregnant women [13] although not all investigators agree with this proposal [14–16]. Currently, there is only one previous study which has evaluated the impact of fish oil; it could detect no effect [17] and certainly there are no studies which have examined the combination of probiotics and fish oil supplements.

Vaginal secretions contain compounds that can be utilized as potential biomarkers for adverse pregnancy outcomes, e.g. an elevated level of phosphorylated insulin-like growth factor-binding protein-1 (phIGFBP-1) has been associated with preterm birth [18] and furthermore, the level of matrix metalloproteinase-8 (MMP-8) has been linked with bacterial vaginosis [19]. Reduced levels of circulating IGFBP-1 at week 20 of gestation have been linked with the appearance of GDM [20]. It is still not completely clear whether these vaginal secretions or circulating biomarkers reflect the actual vaginal microbiota.

We hypothesized that overweight pregnant women would benefit from the consumption of probiotics and fish oil via an improved vaginal microbiota composition and furthermore we examined whether the consumption of these two ingredients would be related to dampened inflammatory responses in the circulation. The first objective of this study was to investigate the impact of LC-PUFA (fish oil) and probiotics separately or in combination on the vaginal microbiota, secondly to determine whether the vaginal microbiota would be related to GDM. Thirdly, we clarified if the interactions of vaginal microbiota and vaginal level of active MMP-8 (aMMP-8) would be reflected in circulating levels of aMMP-8, IGFBP-1 and phIGFBP-1 and a marker of inflammation (high sensitivity C-reactive protein, hsCRP) in overweight and obese pregnant women by utilizing data from an on-going trial.

2. Materials and methods

2.1. Study design and subjects

We examined vaginal and serum samples collected in a placebo controlled randomized trial on the effects of fish oil and/or probiotic dietary supplements on maternal and child health [21]; the secondary outcomes of the trial are reported here. This study was executed in the Turku University Hospital and University of Turku in Finland and the study participants were recruited between October 2013 and July 2017 (ClinicalTrials.gov, NCT01922791). The study complied with the Declaration of Helsinki as revised in 2000. The Ethics Committee of the Hospital District of Southwest Finland approved the study protocol, and all participants provided written informed consent. The study design has been described in more detail previously [21]. Briefly, eligible women were randomly assigned to one of the four parallel groups on the first study visit during early pregnancy: fish oil + placebo (i.e., placebo for probiotics), probiotics + placebo (i.e., placebo for fish oil), fish oil + probiotics, or placebo + placebo (placebo for probiotics and placebo for fish oil, i.e. pregnancy induced changes).

Supplements were provided on the first study visit to be consumed throughout the pregnancy. The fish oil capsules (Croda Europe Ltd., Leek, U.K.) contained a total of 2.4 g of n-3 fatty acids, of which 1.9 g was docosahexaenoic acid (22:6 n-3, DHA) and 0.22 g eicosapentaenoic acid (20:5 n-3, EPA), the rest being other n-3 fatty acids. Placebo capsules for fish oil contained an equal amount of medium-chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%). Probiotic capsules contained *Lacticaseibacillus rhamnosus* HN001 (formerly *Lactobacillus rhamnosus* HN001) (ATCC SD5675; DuPont, Niebüll, Germany) and *Bifidobacterium animalis* ssp. *lactis* 420 (DSM 22089; DuPont), each 10^{10} colony-forming units per capsule. The placebo for the probiotics consisted of microcrystalline cellulose. The compliance to the intervention was 88.4%, as determined by interviewing and 91.8 ± 15.9% as calculated from the returned fish oil capsules [21].

Women made two visits to the study center during gestation, in early (mean 13.8 ± 2.1 gestational weeks) and late (mean of 35.2 ± 1.0 gestational weeks) pregnancy. From 439 pregnant women, vaginal samples were available from 113 women in early pregnancy and 120 in late pregnancy (early-late pairs n = 83). One woman in early pregnancy and four women in late pregnancy were excluded from the analyzes since those women had been treated with vaginal antibiotics, resulting in 112 women in early pregnancy (mean 13.9 ± 1.8 gestational weeks) and 116 in late pregnancy (mean 35.2 ± 0.8 gestational weeks) (early-late pairs n = 82).

2.2. Clinical characteristics

The participants' heights were measured by a wall stadiometer to the nearest 0.1 cm in early pregnancy. Self-reported weight in kilograms was collected from welfare women clinic records. Prepregnancy body mass index (BMI) (kg/m^2) was calculated by dividing the weight in kilograms by height as meters squared. Overweight was defined as BMI ≥ 25 < 30 kg/m^2 while obesity was considered as BMI ≥ 30 kg/m^2.

GDM was diagnosed with a 2-h 75 g oral glucose tolerance test (OGTT) if one or more values were at or above the threshold levels: 0 h ≥ 5.3, 1 h ≥ 10.0, 2 h ≥ 8.6 mmol/l, according to the Finnish Current Care guidelines [22], between 24 and 28 weeks of gestation. An OGTT was offered also to high-risk women (BMI ≥ 35 kg/m^2, previous GDM, glucosuria, polycystic ovarian syndrome, or family risk of diabetes) in early pregnancy, 12–16 weeks of gestation. The women who were diagnosed with GDM in early pregnancy (n = 11) were excluded from the analysis relating to the vaginal microbiome with respect to the onset of GDM in mid-pregnancy; subsequently comparisons were made between these women who are called here women developing GDM in later pregnancy and those not developing GDM. We also analyzed those women who were diagnosed with GDM at any stage of their pregnancy, and compared them against those who remained GDM-free – those are called women with and without GDM, respectively.

The information about systemic and vaginal antibiotic usage during pregnancy was collected from the participants' diaries and by interviewing the mothers during their study visits.

2.3. Vaginal microbiota samples

Vaginal samples for microbiota analyses were taken by a research coordinator using sterile flocked swabs (FLOQSwab® 520CS01, Copan Italia s.p.a., Italy) [23]. The swabs were kept in the vagina for 10–15 s

and then the tips of the swabs were transferred into sterile 1.5 ml microfuge tubes and were frozen at − 20 °C immediately after sampling and then transferred to − 80 °C within one week and kept there until further analysis.

Bacterial DNA was extracted from the vaginal swab samples using a bead beating method as previously described [24]. DNA was quantified using Quanti-iT Pico Green dsDNA Assay (Invitrogen, San Diego, CA, USA). Sample preparation and Illumina MiSeq sequencing of the V3-V4 6S rRNA gene amplicons were performed as previously described [24] using 2 × 300 bp reads and a MiSeq v3 reagent kit at the Biomedicum Functional Genomics Unit (FuGU), Helsinki, Finland.

The amplicon sequencing produced 14,195,964 paired end reads for 234 samples. The five samples of women who did not fall into the inclusion criteria and one duplicated sample were excluded, resulting in 8,448,002 sequenced reads for 228 samples. The median read count per sample was 56,205 (range from 180 to 244,519 reads per sample). The sequencing data was pre-processed in QIIME 2 2019.10 [25]. Raw sequencing data was trimmed for sequencing primers with cutadapt [26] (via cutadapt trim-paired) and denoised with DADA2 [27] (via denoise-paired). The denoising produced 318 amplicon sequence variants (ASV) with a frequency above 100. The ASVs were annotated with BLAST + 2.9.0 [28] megablast against NCBI 16SMicrobial database (date accessed 09-12-2019) to acquire the 100 best matches for every ASV. These results were filtered in R 3.6.1 [29] with the comprehensive listing of vaginal bacteria from Diop et al. [30] to obtain the correct annotations for species that had several equally good annotations and thus could not be annotated with megablast alone. The filtering was conducted in three steps: 1) Selecting the best hits among the Diop species list, 2) If there was a tie in step 1., we chose the species name with the highest abundance in step 1., 3) Some very low abundance (*Anaerococcus*) species that had ambiguous species annotations after steps 1–2. were annotated as [genus name] sp.

2.4. Serum and vaginal low-grade inflammatory and metabolic marker analyses

A blood sample was drawn from the antecubital vein of the mothers after at least 9 h overnight fasting. The serum was separated and analyzed for hsCRP and the rest of the samples were kept in − 80 °C until analyzed for phIGFBP-1, IGFBP-1 and aMMP-8 (Actim, Espoo, Finland). Vaginal samples were obtained by the research coordinator using sterile swabs (Puritan Sterile Polyester swabs, Puritan Medical Products Company Co. LLC, Guilford, USA). They were dissolved in PROM/Partus Specimen Extraction (Actim, Espoo, Finland) and MMP-8 buffer (Actim, Espoo, Finland) solutions and were kept at − 20 °C until subsequent analysis. The PROM/Partus Specimen Extraction Solution consists of phosphate-buffered solution which contains bovine serum albumin (BSA), protease inhibitors and preservatives. Briefly, the level of serum hsCRP was analyzed by using an automated colorimetric immunoassay on the Dade Behring Dimension RXL autoanalyzer (Siemens Healthcare, Camberley, Surrey, UK) and the levels of serum and vaginal MMP-8 were quantified with a solid-phase immunoenzymometric assay (MMP-8 IEMA, Actim, Espoo, Finland) [31–33]. The immunoassay used for MMP-8 analysis was selective for the analysis of active forms of MMP-8 (aMMP-8) [34,35]. Concentrations of serum IGFBP-1 and phIGFBP-1 were measured by two immunoenzymometric assays using monoclonal antibodies (Actim, Espoo, Finland) [36].

2.5. Statistical analyses

The principal outcomes of the main trial were the effects of fish oil and/or probiotic intervention on glycemic status and GDM prevention in which the power calculations were based on Pellonperä et al. [21]. There was no a priori data on the impact of a combination of fish oil and probiotics on the vaginal microbiota. Thus, power was not calculated for this study due to the lack of previous data and the previously calculated main outcome power. However, we assumed that our sample size would be sufficient to detect differences with regard to probiotics as published studies with relatively low sample sizes (n = 12–15 per group [12]; n = 8 per group [37]) have reported significant effects in vaginal microbiota after consumption of probiotics in pregnant women. Our trial consisted of 27–39 pregnant women per intervention group.

The statistical analyses were performed with R 3.6.3 [29] and SPSS Statistics 24.0 (IBM, Chicago, IL, USA). The differences in clinical characteristics between the intervention groups were tested with Chi-Square, Fisher's exact and Kruskal-Wallis test for discrete and continuous variables, respectively. The α-diversity (Shannon index) was calculated with the microbiome R package [38]. We constructed the R data objects with the phyloseq R package [39].

Differences in α-diversity (diversity within community/sample) in pairwise comparisons between groups were performed with Wilcoxon test, and in multi-group comparisons with Kruskal-Wallis test followed by Conover post-hoc test for subsequent pairwise comparisons. Differences in β-diversity (diversity between communities/samples) between sample groups were quantified with PERMANOVA (*adonis* function from vegan R package; [40]) based on the Aitchison distance, i.e. the Euclidean distance between CLR-transformed species abundances. In addition, we identified CSTs of the vaginal microbiota, following the procedure outlined in DiGiulio et al. [41]. For the heatmap visualizations of the CSTs, we chose those taxonomic groups that exhibited the greatest systematic difference between the CSTs based on Kruskal-Wallis test adjusted p-values ($p < 0.05$). Differential abundance analyses in all two-group comparisons were performed with DESeq2 (R package *DESeq2,* [42]).

The systemic antibiotic use during pregnancy was controlled as a covariate in the differential abundance analyses exploring the effect of the intervention at the microbiota's genus and species levels and the effect of the GDM also at the microbiota's genus and species levels. The systemic antibiotic use was not considered as a covariate in the α-diversity analyses since we did not observe any significant association between antibiotic use and α-diversity. The four intervention groups were pooled for the comparisons between the GDM and Healthy groups due to the small sample size per intervention group.

P-values were FDR-adjusted for multiple testing by the Benjamini-Hochberg method (R function p.adjust). Microbiota results are shown with FDR < 0.25. P-values and adjusted P-values < 0.05 are considered significant.

3. Results

3.1. Clinical characteristics

The clinical characteristics of the women are presented in Table 1. The majority of the women were well-educated with a university or college degree and every third woman was obese. GDM was diagnosed in 33.6% of the women; 14.4% of the women reported using systemic antibiotics during pregnancy (NS between the intervention groups).

3.2. Characteristics of the vaginal microbiota

In 228 vaginal samples (early and late pregnancy samples together) the total numbers of sequenced reads were 8,448,002 (min 92, max 101,080; three samples, corresponding to 1.3% of all samples, had fewer than 1000 reads). The average read count was 37,053. Each of the three low-read count samples was in a different intervention group. All samples were included in the analyzes but we confirmed that discarding the three low-read count samples did not change the main outcomes. A total of 97 taxonomic groups were identified (kingdom 1, phylum 7, class 12, order 17, family 24, genus 43, species 97). At the phylum level, Firmicutes (mean relative abundance 89.6%) was the most abundant followed by Actinobacteria (relative abundance 9.5%) and Bacteroidetes (relative abundance 0.8%). The core microbiota were defined based on the

Table 1
Clinical characteristics of the overweight and obese pregnant women.

Characteristics	Fish oil + placebo	Probiotics + placebo	Fish oil + probiotics	Placebo + placebo	All	n	P-value
Age (median (IQR)) (y)	29.9 (27.8–33.0)	30.5 (27.7–35.3)	30.6 (29.0–35.1)	30.4 (28.0–32.8)	30.4 (28.2–33.9)	35/39/36/36/146	0.69[a]
Education (university or college degree) (%)	64.7	62.2	64.7	63.3	63.7	34/37/34/30/135	1.00[b]
Obese (%)[d]	51.4	30.8	30.6	25.0	34.2	35/39/36/36/146	0.09[b]
Prepregnancy BMI (median (IQR) (kg/m^2)	30.6 (27.4–32.9)	28.0 (26.5–30.3)	28.5 (25.7–30.5)	29.2 (26.2–30.4)	28.4 (26.5–30.9)	35/39/36/36/146	0.05[a]
Women developing GDM in later pregnancy (%)[e]	25.0	36.1	25.8	22.2	27.8	32/36/31/27/126	0.60[b]
Women with GDM (%)[f]	31.4	39.5	30.3	32.3	33.6	35/38/33/31/137	0.83[b]
Smoking before pregnancy (%)	8.8	32.4	14.7	30.0	21.5	34/37/34/30/135	0.04[b]
Smoking during pregnancy (%)	0	2.7	6.1	6.7	3.7	34/37/33/30/134	0.44[c]
Systemic antibiotic treatments during pregnancy (%)	12.5	10.8	12.1	23.3	14.4	32/37/33/30/132	0.51[c]

[a] Kruskal-Wallis test.
[b] Chi-square test.
[c] Fisher's exact test.
[d] Obesity was defined as BMI $\geq$ 30 kg/m^2.
[e] GDM diagnosed in late pregnancy i.e. women developing GDM in later pregnancy (women with GDM diagnosed in early pregnancy excluded from the analyzes).
[f] GDM diagnosed at any stage of the pregnancy.

detection threshold (relative abundance) 0.1% i.e. including microbes with a relative abundance $\geq$ 0.1% and a prevalence threshold (above the detection threshold in the population) of 50%, i.e. including microbes that were detected in $\geq$ 50% of the samples, which consisted of Firmicutes (100% prevalence) and Actinobacteria (62.3% prevalence). At the genus level, the most prevalent bacterium was *Lactobacillus* (100%) followed by *Gardnerella* (47.8%) and *Finegoldia* (29.8%) while at the species level, the most prevalent was *L. crispatus* (99.6%), the second most prevalent was *L. iners* (98.7%) with the third most prevalent being *Gardnerella vaginalis* (47.8%) (Suppl. Table 1). Fig. 1 illustrates the microbiota composition at the species level in the four dietary intervention groups in early and late pregnancy. We observed five CSTs which were characterized as follows: the first CST was high in *Alloscardovia omnicolens* and *Streptococcus anginosus* and low in *Lactobacillus* species (named here after CST *A. omnicolens*), the second was high in *L. crispatus* (CST *L. crispatus*), the third was high in *L. iners* (CST *L. iners*), the fourth was moderately high in several *Lactobacillus* species (CST *Lactobacillus*) and the fifth was high in *G. vaginalis* and *Fannyhessea vaginae* (formerly *Atopobium vaginae*) and low in *Lactobacillus* species (CST *G. vaginalis*). The combinations of species, which are separated into distinctive clusters from each other, are presented in heatmaps (Fig. 2a–e). Most of the participants (60.9%) belonged to CST *G. vaginalis* in early pregnancy and CST *A. omnicolens* in late pregnancy (55.6%) (Suppl. Table 2). Among the five clusters, the highest α-diversity was found in CST *G. vaginalis* at both the early and late pregnancy visits (Kruskal-Wallis, $p < 0.001$) (Suppl. Table 3, Suppl. Fig. 1).

3.3. Impact of the dietary intervention on the vaginal microbiota

In the group of women who consumed fish oil, α-diversity increased significantly from early to late pregnancy ($p = 0.03$, Fig. 3). This change, which occurred during the pregnancy, was not observed in the

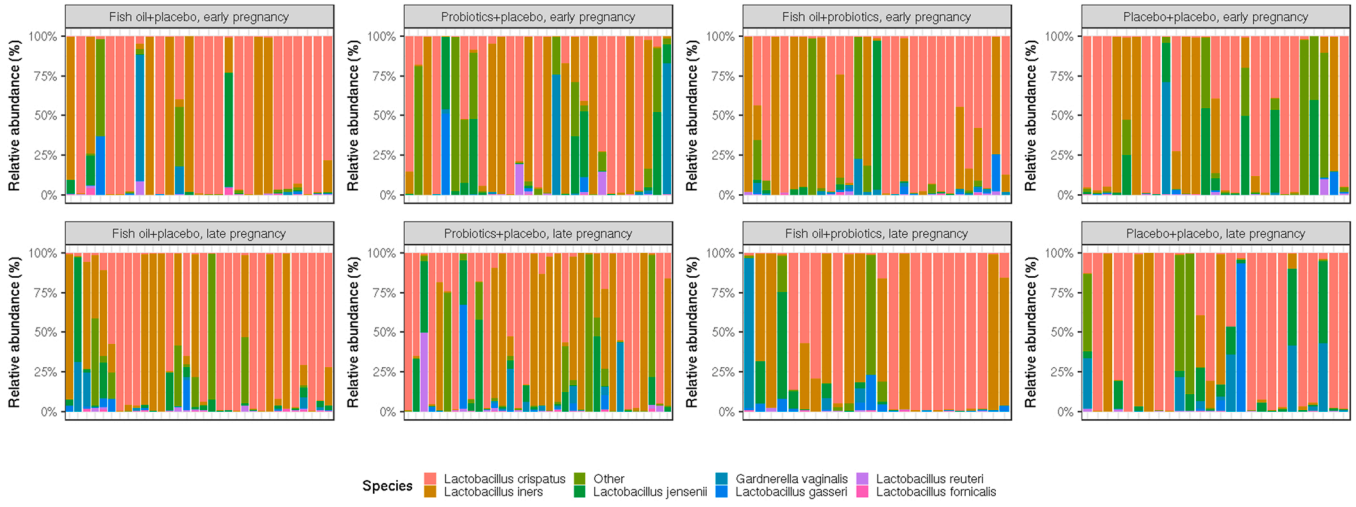

Fig. 1. Community composition at the species level in early (mean 13.9 ± 1.8 gestational weeks) and late pregnancy (mean 35.2 ± 0.8 gestational weeks) in the four intervention groups. Each bar represents a single pregnant woman. The most prevalent species that were detected in over 33% of all samples at 0.1% relative abundance; the 7 most abundant species are presented with the remaining species included in "other". The samples have been sorted according to the abundance of *Lactobacillus crispatus* in each figure.

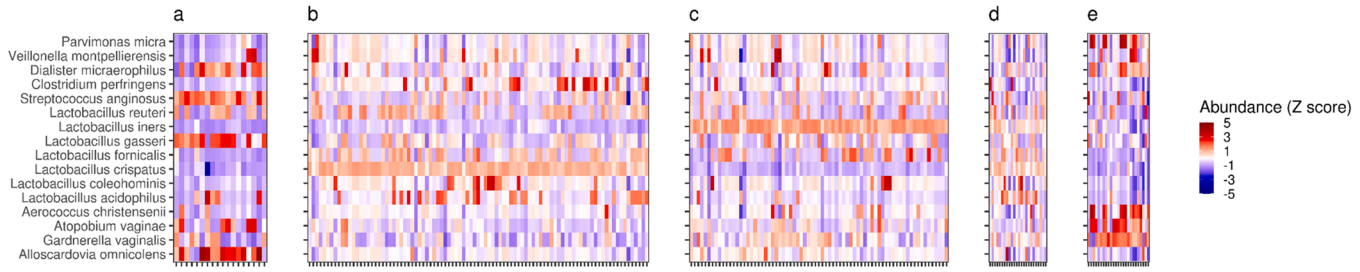

Fig. 2. a–e. Heatmaps describing the abundances of specific species within the observed community state types (CST): a) CST *A. omnicolens*, b) CST *L. crispatus*, c) CST *L. iners*, d) CST *Lactobacillus*, e) CST *G. vaginalis*. The clr-transformed abundances have been shifted to zero mean and scaled to unit variance in order to highlight the relative differences between the observed community types in the taxonomic groups with different absolute abundance levels. Taxonomic groups exhibiting the strongest systematic differences between the community types are shown (Kruskal-Wallis test; adjusted $p < 0.05$).

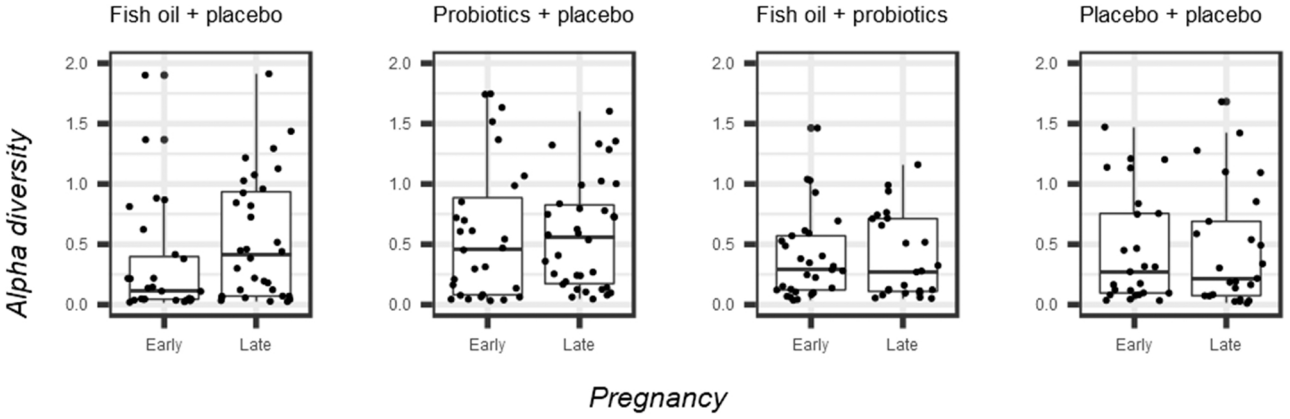

Fig. 3. α-Diversity in early (mean 13.9 ± 1.8 gestational weeks) and late (mean 35.2 ± 0.8 gestational weeks) pregnancy in all four groups. Fish oil + placebo group is $p < 0.05$, Kruskal-Wallis test.

other intervention groups, nor was there any difference between the intervention groups in late pregnancy ($p = 0.44$). We did not observe changes in β-diversity between early and late pregnancy or in late pregnancy in any of the intervention groups ($p > 0.05$ in all comparisons). When evaluating all the bacteria at the genus level, changes from early to late pregnancy were detected in fish oil, probiotics and placebo groups but not in the combination (fish oil + probiotics) group. In the fish oil group the relative abundance of *Corynebacterium*

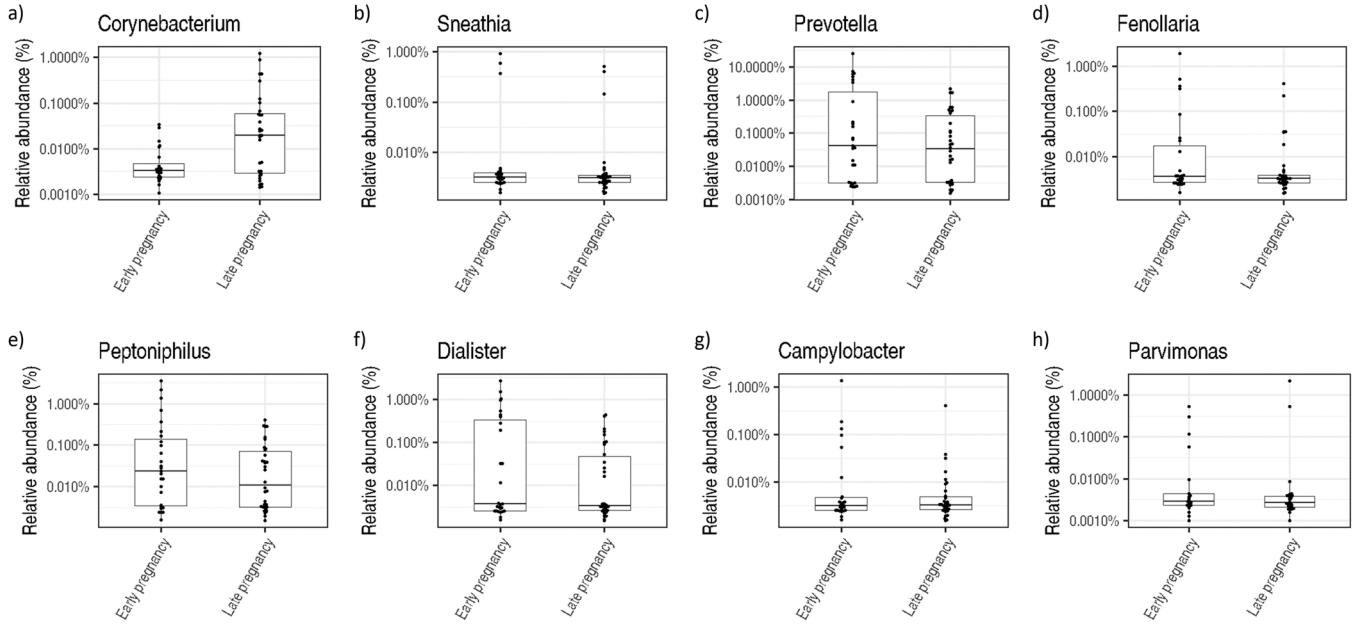

Fig. 4. Significantly abundant genera in a) fish oil group, b), c), d), e), f) and g) probiotic group and h) placebo group from early (mean 13.9 ± 1.8 gestational weeks) to late (mean 35.2 ± 0.8 gestational weeks) pregnancy. Note that the Y axis is shown on a log10 scale, and a pseudocount 1 has been added to the read counts before estimation of the relative abundance.

increased ($p < 0.001$), in the probiotics group the abundances of *Sneathia, Prevotella, Fenollaria, Peptoniphilus*, *Dialister* and *Campylobacter* decreased ($p < 0.05$ in all comparisons) and in the placebo group *Parvimonas* increased ($p < 0.001$) over the course of pregnancy (Fig. 4a–h, Table 2). When the intervention groups were compared to the placebo group in late pregnancy, the abundances of *Ureaplasma* in the probiotics group and *Corynebacterium* and *Anaerococcus* in the fish oil + probiotics group were lower as compared to the placebo group ($p < 0.05$ in all comparisons, Suppl. Table 4), while the fish oil group did not differ from the placebo group. Regarding the difference in abundances of *Corynebacterium* and *Anaerococcus* between the fish oil + probiotics and the placebo group, this may have been due to the differences already present at baseline (Suppl. Table 4).

Changes throughout the course of pregnancy were also detected at the species levels: the abundance of *Parvimonas micra* in placebo group increased, while that of *Lactobacillus acidophilus* in the fish oil + probiotics group decreased ($p < 0.05$). No changes occurred in the fish oil or probiotics groups at the species level. (Suppl. Fig. 2a–b, Suppl. Table 5). When comparing the intervention groups to placebo in late pregnancy, the abundances of *G. vaginalis* and *Ureaplasma urealyticum* in the fish oil group, *U. urealyticum* and *Prevotella disiens* in the probiotics group, *Dialister invisus* and *Prevotella timonensis* in the fish oil + probiotics group were lower than those present in the placebo group ($p < 0.05$ in all comparisons, Suppl. Table 4). It is noteworthy that the difference in *G. vaginalis* in fish oil versus placebo group comparison was evident already at baseline ($p < 0.001$, Suppl. Table 4).

Considering the CSTs, no differences were observed in the proportion of the women in the CSTs in the four intervention groups (Fisher-exact test, late pregnancy $p = 0.19$).

3.4. Vaginal microbiota in relation to GDM

α-Diversity in early pregnancy did not differ between women developing GDM in later pregnancy compared to those not developing GDM ($p = 0.6$, Suppl. Fig. 3a). A similar observation was made when women diagnosed with GDM and those without were compared at the late pregnancy sampling point ($p = 0.6$, Suppl. Fig. 3b).

At the genus level, the abundances of *Megasphaera* and *Corynebacterium*, *Ureaplasma* were lower ($p < 0.05$ in all comparisons, Fig. 5a–c, Table 3) and at the species level, the abundances of *Megasphaera elsdenii*, *Veillonella montpellierensis* and *Bifidobacterium dentium* were lower ($p < 0.001$ in all comparisons, Suppl. Fig. 4a–c, Suppl. Table 6) in early pregnancy in women developing GDM later in pregnancy when compared to those not developing GDM.

Although the genus level comparison revealed no differences in the abundance of any genera in late pregnancy between women with GDM compared to those without GDM (Table 3), at the species level, *V. montpellierensis* in late pregnancy was lower in women with GDM as compared to women without GDM ($p < 0.001$, Suppl. Table 6). No differences were observed in the proportion of the women with or without GDM in the different CSTs in late pregnancy (Fisher-exact test, $p = 0.52$).

3.5. Interaction of vaginal microbiota and vaginal and systemic metabolic and inflammatory markers during pregnancy

Vaginal aMMP-8 correlated significantly with α-diversity in late pregnancy ($p < 0.001$, Table 4). The other serum inflammatory or serum and vaginal metabolic markers did not correlate significantly either in the α-diversity (Table 4) or with any of the genera in early or late pregnancy (Fig. 6a–b). Instead at the species level, *Lactobacillus fornicalis* (correlation coefficient − 0.38, $p = 0.02$) correlated inversely with the level of vaginal aMMP-8 in early pregnancy while *L. crispatus* (correlation coefficient − 0.40, $p = 0.005$) correlated inversely with the vaginal aMMP-8 level in late pregnancy (Suppl. Fig. 5a–b).

Interestingly, we observed the highest levels of vaginal aMMP-8 in CST *G. vaginalis* in early and late pregnancy (Table 5), Kruskal-Wallis $p < 0.001$, pairwise comparison (Conover post-hoc test). The differences were significant ($p < 0.001$) for clusters CST *A. omnicolens,* CST *L. crispatus,* CST *L. iners,* CST *Lactobacillus*, when compared to the cluster CST *G. vaginalis*.

4. Discussion

We observed differences in the vaginal microbiota, mainly in the low-abundant potential pathobionts, as a response to our dietary intervention. These included a lower abundance of potential pathobionts, namely *U. urealyticum* in the fish oil group, *Ureaplasma, U. urealyticum*

Table 2
The genera with FDR < 0.25 in the intervention groups from early (mean 13.9 ± 1.8 gestational weeks, n = 112) to late pregnancy (mean 35.2 ± 0.8 gestational weeks, n = 116).

Intervention group	Taxon	Mean/median relative abundance (5/95% quantile) (%), early pregnancy	Mean/median relative abundance (5/95% quantile) (%), late pregnancy	Effect size (Log2FC)	Adj. P
Fish oil + placebo	*Corynebacterium*	<0.01/<0.01 (<0.01/0.02)	0.11/0.02 (<0.01/0.63)	-4.9	< 0.001
Probiotics + placebo	*Sneathia*	0.07/<0.01 (<0.01/0.52)	0.02/<0.01 (<0.01/0.05)	24.0	< 0.001
	Prevotella	4.59/0.04 (<0.01/6.97)	0.26/0.02 (<0.01/1.70)	4.6	< 0.001
	Fenollaria	0.12/<0.01 (<0.01/0.46)	0.01/<0.01 (<0.01/0.03)	7.6	0.001
	Peptoniphilus	0.33/0.02 (<0.01/1.93)	0.05/0.01 (<0.01/0.28)	3.2	0.01
	Dialister	0.30/<0.01 (<0.01/1.37)	0.04/<0.01 (<0.01/0.18)	4.1	0.02
	Campylobacter	0.07/<0.01 (<0.01/0.16)	<0.01/<0.01 (<0.01/0.02)	5.1	0.04
	Mageeibacillus	0.08/<0.01 (<0.01/0.43)	0.02/<0.01 (<0.01/<0.01)	6.5	0.12
	Anaerococcus	0.30/0.01 (<0.01/1.74)	0.11/<0.01 (<0.01/0.51)	2.8	0.12
	Finegoldia	0.26/0.05 (<0.01/1.41)	0.11/0.04 (<0.01/0.25)	1.6	0.19
Fish oil + probiotics	(no significant differences)				
Placebo + placebo	*Parvimonas*	0.05/<0.01 (<0.01/0.30)	0.10/<0.01 (<0.01/0.39)	12.1	< 0.001

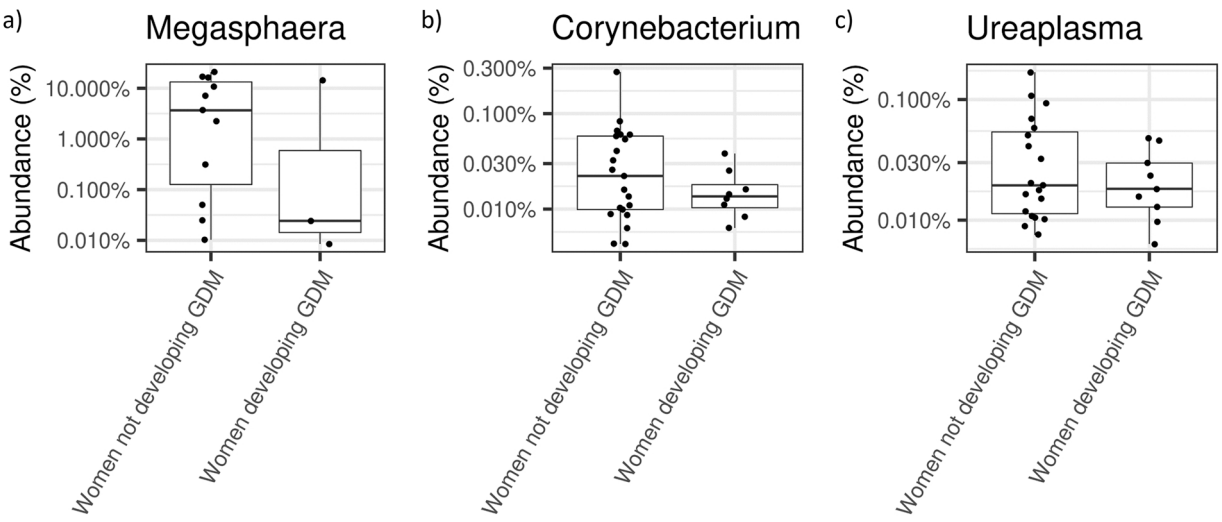

Fig. 5. a–c. The significantly differentially abundant genera in early pregnancy (mean 14.1 ± 1.8 gestational weeks) between women developing GDM in later pregnancy and those not developing GDM ($p < 0.05$).

Table 3
The genera with FDR < 0.25 in early pregnancy (mean 14.0 ± 1.8 gestational weeks) in those women developing GDM in later pregnancy and those not developing GDM, and in late pregnancy (mean 35.2 ± 0.8 gestational weeks) between women with and without GDM.

Stage of pregnancy	Taxon	Mean/median relative abundance (5/95% quantile) (%)	Mean/median relative abundance (5/95% quantile) (%)	Effect size (Log2FC)	Adj. P
		Women developing GDM	**Women not developing GDM**		
Early pregnancy	*Megasphaera*	0.54/<0.01 (<0.01/0.02)	1.23/<0.01 (<0.01/10.31)	-23.3	< 0.001
	Corynebacterium	<0.01/<0.01 (<0.01/0.02)	0.01/<0.01 (<0.01/0.06)	5.1	0.01
	Ureaplasma	0.01/<0.01 (<0.01/0.04)	0.01/<0.01 (<0.01/0.07)	5.0	0.01
	Gardnerella	7.80/0.11 (<0.01/48.83)	7.32/0.05 (<0.01/47.26)	3.2	0.22
Late pregnancy		**Women with GDM**	**Women without GDM**		
	Gardnerella	6.00/0.09 (<0.01/42.90)	5.07/0.07 (<0.01/31.75)	3.0	0.15
	Peptoniphilus	0.08/0.01 (<0.01/0.43)	0.19/0.01 (<0.01/0.86)	-2.6	0.15

Table 4
Spearman correlation between α-diversity and levels of systemic inflammatory and metabolic markers in early (mean 13.9 ± 1.8 gestational weeks) and late pregnancy (mean 35.2 ± 0.8 gestational weeks).

Systemic inflammatory & metabolic markers	Diversity (Shannon index), early pregnancy, correlation coefficient	Adj. P	Diversity (Shannon index), late pregnancy, correlation coefficient	Adj. P
Vaginal aMMP-8 (ng/ml)	0.21	0.16	0.38	< 0.001
Serum aMMP-8 (ng/ml)	0.08	0.53	0.01	0.98
Serum phIGFBP-1 (ng/ml)	0.07	0.53	0.002	0.98
Serum IGFBP-1 (ng/ml)	-0.06	0.53	0.05	0.27
Serum hsCRP (mg/ml)	-0.12	0.53	-0.15	0.98

and *P. disiens* in the probiotics group and *D. invisus* and *P. timonensis* in the fish oil + probiotics group. The beneficial impact of probiotics on vaginal microbiota composition was strengthened by the finding of a reduced abundance of potential pathobionts, namely *Prevotella, Peptoniphilus*, *Dialister* and *Campylobacter* from early to late pregnancy. In the placebo group i.e. reflecting pregnancy-induced changes, there were clear increases in the abundance of *Parvimonas* and *P. micra*. Finally, the level of vaginal aMMP-8 correlated directly with vaginal α-diversity in late pregnancy and indirectly with two *Lactobacillus* species. Interestingly, the clinical benefit of the microbial changes during pregnancy was demonstrated as the vaginal microbiota was related to the onset of GDM; the abundances of *Megasphaera* and *Corynebacterium*, *Ureaplasma*, *M. elsdenii*, *V. montpelliernsis* and *B. dentium* were lower in those women developing GDM in comparison to those not developing this disorder. Further, the vaginal microbiota differed in women with and without GDM, with *V. montpelliernsis* being lower in women with GDM. In summary, the vaginal microbiota seems to play a role in pregnant women's health and to some extent it may be modulated with a dietary intervention consisting of fish oil and probiotics.

We detected several alterations in bacterial genera and species due to the dietary intervention with fish oil and probiotics. The consumption of probiotics induced beneficial alterations in the composition of the vaginal microbiota i.e. a decline in potential pathobionts (*Peptoniphilus*, *Dialister*, and *Prevotella*) linked with preterm birth [41,43] and also a decrease in *Sneathia*, a genus related to bacterial vaginosis and preterm birth [44]. One possible mechanism to explain how probiotics can alter the vaginal microbiota may be attributable to their vaginal pH lowering properties and the production of anti-inflammatory mediators (review [45]). Previous studies have indicated that the consumption of

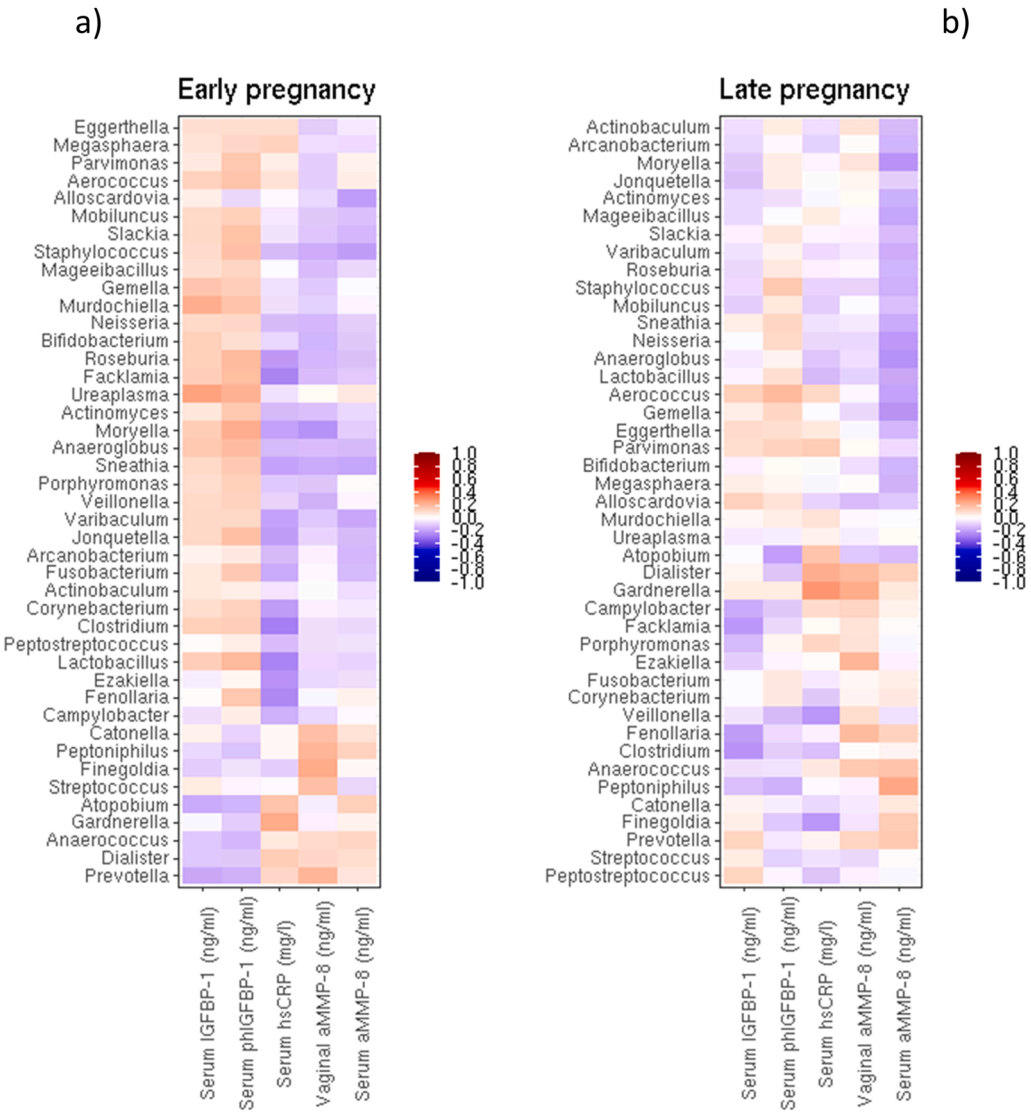

Fig. 6. Spearman correlation between the inflammatory and metabolic markers and the 43 genera in a) early (mean 13.9 ± 1.8 gestational weeks) and b) late (mean 35.2 ± 0.8 gestational weeks) pregnancy (NS).

Table 5
Mean of the vaginal aMMP-8 concentration in 5 CSTs in early (mean 13.9 ± 1.8 gestational weeks) and late pregnancy (mean 35.2 ± 0.8 gestational weeks). The vaginal microbiota composition of the CSTs can be found in Fig. 2.

Vaginal aMMP-8	CST *A. omnicolens*	CST *L. crispatus*	CST *L. iners*	CST *Lactobacillus*	CST *G. vaginalis*	P-value
Vaginal aMMP-8 (ng/ml), early pregnancy	195.6 ± 434.0	36.6 ± 70.3[a]	87.1 ± 221.7[a]	49.5 ± 67.2[a]	393.4 ± 579.4	< 0.001
Vaginal aMMP-8 (ng/ml), late pregnancy	181.6 ± 393.1[b]	68.1 ± 218.8	112.8 ± 319.7[b,c]	203.2 ± 506.3[c]	491.2 ± 1031.5	< 0.001

[a] Significant differences between CST *G. vaginalis* and CST *L. crispatus* ($p < 0.001$), CST *G. vaginalis* and CST *L. iners* ($p < 0.001$), CST *G. vaginalis* and CST *Lactobacillus* ($p < 0.001$) in early pregnancy.
[b] Significant differences between CST *L. crispatus* and CST *A. omnicolens* ($p = 0.01$), CST *L. crispatus* and CST *L. iners* ($p < 0.001$) in late pregnancy.
[c] Significant differences between CST *G. vaginalis* and CS *L. iners* ($p = 0.008$) and CST *G. vaginalis* and CST *Lactobacillus* ($p = 0.003$) in late pregnancy.

probiotics can exert many beneficial effects in the vagina, since they increase antimicrobial activity, lower pH, produce H_2O_2 and they may reduce the adherence of potential pathogens in the vagina (reviews [1, 46]). Furthermore, probiotics may also bind to the extracellular matrix to inhibit tissue processing by MMPs as discussed by Basavaprabhu et al. [46]. The route of oral probiotics to vagina occurs via the gastrointestinal tract, i.e. the probiotics descend down the gut to the rectum and from there to vagina. However, with respect to the two probiotics used in this trial, *L. rhamnosus* was detected in only a few vaginal samples while *B. animalis* in none, suggesting that the probiotics did not reach the vagina, although we are not able to confirm this finding due to the limits of our sequencing method to detect strain-specific bacteria. In contrast, two previous studies have reported colonization of *L. rhamnosus* HN001 in vaginal microbiota after 2 or 3 weeks of oral consumption [47,48]. Both probiotics used in the study, *L. rhamnosus* HN001 and *B. animalis* ssp. *lactis* 420, have been shown to colonize the gut [49,50].

On the contrary, while the consumption of fish oil increased the α-diversity and the abundance of genus *Corynebacterium,* this did not occur in the women receiving the probiotic supplementation. These findings are surprising as these increases have been observed previously

to be linked with pathological complications e.g. bacterial vaginosis [51, 52] and with a failure of bacterial vaginosis treatment [53]. The only alteration evident during pregnancy in the fish oil + probiotics group was a decrease in *L. acidophilus* species, which has conferred benefits when administered either orally or vaginally e.g. an increase in vaginal lactobacilli and a normalization of the vaginal microbiota [54]. One possible mechanism to explain how fish oil can shape the vaginal microbiota may be linked to changes in the estrogen-microbiome axis; in our study it is possible that the consumption of fish oil alone and the combination of fish oil + probiotic decreased the numbers of estrogen-metabolizing bacteria, which secrete enzymes that deconjugate estrogen into an active form in the gut [55]. The reduction in the numbers of estrogen metabolizing bacteria in the gut would subsequently affect the levels of circulating estrogen, which would be translocated to vagina. This would explain why there were increases in the abundances of the pathobionts and decreases in those of *L. acidophilus* in the vagina in the fish oil and fish oil + probiotics groups, respectively. Another potential mechanism could be the ability of PUFA to be incorporated into the probiotic cell lipids [56] which could possibly affect the adhesion properties of probiotics. Moreover, DHA and EPA have recognized anti-inflammatory effects, therefore they may dampen the inflammatory processes locally in vagina and affect the vaginal milieu.

When comparing the active groups to the placebo groups in late pregnancy, a lower abundance of several potential pathobionts was revealed; lower abundances of *P. disiens and D. invisus, P. timonensis, Ureaplasma and U. urealyticum,* the last mentioned is a species which may cause genital and urinary tract infections [57]. Thus, our findings indicate that the intervention with probiotics alone was beneficial while the outcomes with respect to fish oil and fish oil + probiotics supplementation are more controversial. For example, although the co-supplementation of probiotics and fish oil evoked a beneficial effect i.e. a decrease in *D. invisus* and *P. timonensis,* a potentially adverse effect was evident i.e. the decrease in *L. acidophilus.* Fish oil alone seemed also to be associated with some negative effects i.e. diversity was increased and the abundances of some potential pathobionts (*Corynebacterium)* increased although there was one beneficial effect when compared to placebo, i.e. the abundance of *U. urealyticum* was lower in the fish oil group. Interestingly, the effect of the combination of fish oil and probiotics on the vaginal microbiota was different from the effect of either fish oil or probiotics on their own. It is possible that probiotics counteract the effect of fish oil, since probiotics i.e living microbes, can have a direct impact on dietary fatty acids either via the level of saturation (review [11]) or the ability to incorporate the dietary lipids into their cell walls [56]. Moreover, when probiotics and n-3 LC-PUFA were encapsulated, it was found that the n-3 LC-PUFA had increased the survival of probiotics in vitro [59] and fish oil enhanced the adhesion of probiotics to gut mucosal cells (review [60]). However, it is clear that more studies on probiotics and fish oil are needed – determining the optimal probiotic strain and if it should be used in some kind of combination as well as the timing and duration of the intervention will need to be carefully considered before initiating any new studies.

Previous studies investigating probiotics have mainly used different mixtures of probiotics and focused on treating bacterial vaginosis in non-pregnant women; the outcomes have been far from unanimous (meta-analysis [61], review [54,62,63]). A recent meta-analysis including 27 studies found no benefit of consuming probiotics orally in different infections including bacterial vaginosis [64]. Our study participants were supplemented with *L. rhamnosus* HN001 which belongs to same species as *L. rhamnosus* GR-1 which is a commonly used vaginal probiotic. Previously, in one report *L. rhamnosus* GR-1 mixed with *Limosilactobacillus reuteri* RC-14 (formerly *Lactobacillus reuteri* RC-14) alleviated vaginal microbiota in pregnant women [65] but other investigators have been unable to replicate this finding [14,15,66]. When vaginal microbial health was measured with either the Nugent score or purely with vaginal symptoms, Russo et al. found that consumption of oral *L. rhamnosus* HN001 and *L. acidophilus* GLA-14 improved the diversity of the vaginal microbiota in non-pregnant women [48,67]. We demonstrated that probiotics could exert beneficial effects by decreasing the abundances of potential pathobionts in the vaginal microbiota in pregnant women, as has been shown previously in non-pregnant women suffering from bacterial vaginosis (reviewed by Basavaprabhu et al. [46]). This finding is clinically important since a predominance of pathogenic bacteria may lead to adverse pregnancy outcomes, such as preterm birth. According to van de Wijgert [68] vaginal microbiota studies usually report results about the dominant species (*Lactobacillus* species) and at the same time under-report results about pathobionts. Pathobionts exist in lesser numbers than *Lactobacillus* but should be reported equally if one seeks clarification about their clinical relevance.

To our knowledge, only one study has investigated the effect of n-3 LC-PUFA on vaginal microbiota (a daily dose of 2.4 g, 55% EPA and 37% DHA [17]) in pregnant or non-pregnant women. In that study, in contrast to our results, a 12-week supplementation of fish oil was not associated with α-diversity (Shannon index) or any specific genera among the 344 pregnant women and they did not report any effect on β-diversity, which is in line with our observations. In a study conducted in experimental animals, Wang et al. [69] utilized an animal model of the polycystic ovary syndrome and showed that administration of flaxseed oil which contains plant-derived α-linolenic acid increased the vaginal abundances of *Lactobacillus*, *Faecalibacterium*, and *Parabacteroides* as well as decreasing that of *Streptococcus*. Overall, there have been very few trials conducted in pregnant women and even less is known about the pros and cons of interventions involving fish oil and especially the combination of fish oil and probiotics, in their abilities to modify the vaginal microbiota in these women. Thus, there is a clear need for larger randomized placebo controlled trials as well as experimental studies which are planned to examine the potential synergetic effects of the combination of fish oil and probiotics focusing on the mechanisms behind the changes occurring in the vaginal microbiota in pregnant women. It is recommended that these should exploit the advanced techniques now becoming available in microbial analytics i.e. metagenomics.

Changes also occurred in the placebo group, these are considered here to be pregnancy-induced changes in the vaginal microbiota, i.e. increases in genus *Parvimonas* and species *P. micra,* a species that has been associated with genital inflammation in young Africans [70]. These findings are evidence that the vaginal microbiota becomes altered during pregnancy. In contrast to our results, one study reported a decrease in the abundance of *P. micra* [3]. Nonetheless, our observations are to some extent in agreement with a previous report in which an extensive microbial variation has been detected between the first and the second and the third trimester [71]. Some investigators have claimed that the vaginal microbiota is rather stable throughout the pregnancy [41] while others have detected an increase in *Lactobacillus* species [3,41,72] or a reduction in microbial diversity towards the end of pregnancy [73]. In contrast, one publication reported an increase in α-diversity from weeks 24 to 36 of gestation [74]. In our study, one may argue that the placebo group did not reflect the pregnancy-induced changes since the placebo for fish oil consisted of medium-chain fatty acids which according to two recent reviews may have been able to alter the gut microbiota, at least in experimental animals [75,76]. However, one human study did not find an effect on gut microbiota when coconut oil (rich in medium chain fatty acids) was supplemented to overweight women [77] but the effect of medium chain fatty acids on the vaginal microbiota has not been evaluated.

When we assessed GDM, then differences in the abundances of potential pathogenic bacteria were seen: a lower abundance of *Ureaplasma*, a genus with pathological species, and species *M. elsdenii*, which has been associated with bacterial vaginosis in West African women [78]. Furthermore, lower abundances of *V. montpellierensis*, a species belonging to a *Veillonella* genus, and *Corynebacterium* genus, both genera linked with failures in the treatment of bacterial vaginosis [53], were

detected in women developing GDM when compared to those not developing this disorder. In addition, a lower abundance of species *V. montpelliernsis* was detected in those women with GDM. In contrast to our findings, it was recently reported that there is a higher abundance of *Veillonella* with GDM in the third trimester [10]. Also at odds with our finding of no association between microbiota diversity and richness as assessed with GDM, Cortez et al. [10] reported lower diversity indices (Shannon and Simpson) and richness in association with GDM; this was possibly attributable to the differences in BMI between the control group and women with GDM. It is noteworthy that it may well be the GDM which drives the changes in microbiota, not necessarily the microbiota which triggers the pathogenesis of GDM.

Interestingly, the level of aMMP-8 correlated directly with α-diversity and inversely with the abundances of two *Lactobacillus* species, i.e. *L. fornicalis* and *L. crispatus*. This finding clearly shows that there is a relationship between aMMP-8 and the marker commonly used to detect the presence of bacterial vaginosis and with an unbalanced vaginal microbiota, i.e. a higher diversity and a lower abundance of *Lactobacillus* species, suggesting that aMMP-8 could be exploited as a marker for the presence of vaginal microbiota dysbiosis. The immunoassay for MMP-8 utilizes an antibody selective for the active form(s) of MMP-8 [33,79]. It is recognized that vaginal dysbiosis may increase the risk for vaginal infections and preterm birth (review [80]). Interestingly, the highest levels of vaginal aMMP-8 were found in the CST characterized with high *G. vaginalis* and *F. vaginae* (formerly *A. vaginae* [81]) which also had the highest diversity. Our analysis is also in line with the CSTs previously reported by Digiulio et al. [41] and others [3,6,72,82]. Although there were some minor variations as would be expected in an independent data set, we identified a total of 5 CSTs as did Digiulio et al. [41]. The three CSTs characterized by *L. crispatus, L. iners* and *G. vaginalis*, respectively, correspond to the same CSTs described by Digiulio et al. [41]. Romero et al. [72] have also reported similar findings, with three CSTs characterized by *L. crispatus*, *L. iners* and *G. vaginalis*. Hence, our analysis can be seen to support the broad community type characteristics that have been reported in the previous independent analyses of the vaginal microbiota.

The strength of this study lies in its novelty; this is the first trial to have investigated the effect of a combination of fish oil and probiotics on the vaginal microbiota in pregnant women. Another strength is also the detailed collection of data within a clinical study setting. Information about local and systemic antibiotic treatments during pregnancy was collected from all participants which allowed us to control for the effect of antibiotic exposure on the microbiota. Antibiotic use is a well-known factor contributing to the shaping of the microbiota present in the human body, including the vagina [83]. The reported compliance determined by interviewing the women and by calculating the returned capsules was good and this is consistent with the levels of lipids in serum, which reflected the intake of fish oil as reported in our previous study [84]. Due to the small sample size, we could not assess the effect of the intervention on the vaginal microbiota exclusively in GDM cases; in this respect, this may be considered a pilot study. Many genera and species ($> 0.1\%$ abundance threshold) were detected in less than 20% of the samples and further many bacteria were of low-abundance ($< 0.1\%$); we identified 43 genera and 97 species. These results are in agreement with Romero et al. (in total 143 genera and species) [3], in contrast, Freitas et al. detected fewer species (22) [4]. These discrepancies may be due to different prevalence thresholds. We acknowledge that the resolution of V3-V4 region of 16S rRNA for species level analysis is not optimal. However, previous studies focusing on the vaginal microbiota at the species level have utilized the V3–V4 region [24,85] and for that reason, we are confident in being able to report at the species level.

In conclusion, our study showed that an intervention with fish oil and/or probiotics affected the diversity of the vaginal microbiota as well as the vaginal composition at the genus and species levels in comparison to placebo. The probiotics exerted a beneficial effect to decrease the abundances of potential pathogens in pregnant women when compared to placebo and also during the course of gestation. This is an important finding since pregnancy itself predisposes women to vaginal dysbiosis and thus the consumption of probiotics may prevent pregnant women from developing this condition and further adverse pregnancy outcomes, such as preterm birth. Interestingly, we found that the concentration of aMMP-8 and microbial diversity was highest in the CST in which the major species were anaerobic and potential pathogens suggesting that aMMP-8 could be used as a marker of vaginal dysbiosis.

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CRediT authorship contribution statement

KL, NH, KM: Conceptualization. **KL:** Supervision, Project administration, Funding acquisition. **LL, WTS, SV, NH:** Formal analysis. **NH, EK, OP:** Investigation. **KL, AS, JJ, LL:** Resources. **NH, KL, KM:** Writing – original draft. **All authors:** Writing – review & editing.

Disclosure statement

TS and JJ are inventors of aMMP-8 US-patents: Sorsa US-patent-19: aMMP-8 POCT oral fluid test: 2019/0023572A1. Pussinen, Juhila, Sorsa et al. US-patent: 2017/0023671A1/a-MMP-8 serum test and patent (FIN) 127416. The other authors report no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112841.

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Further reading

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