Metagenomics analysis of gut microbiota in response to diet intervention and gestational diabetes in overweight and obese women: a randomized, double-blind, placebo-controlled clinical trial

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Objective. Gut microbiota and diet are known to contribute to human metabolism. We investigated whether the metagenomic gut microbiota composition and function change over pregnancy, are related to gestational diabetes, GDM, and can be modified by dietary supplements, fish oil and/or probiotics.
Design. The gut microbiota of 270 overweight/obese women participating in a mother-infant clinical study were analyzed with metagenomics approach in early (mean gestational weeks 13.9) and late (gestational weeks 35.2) pregnancy. GDM was diagnosed with a 2h 75g oral glucose tolerance test.
Results. Unlike women with GDM, women without GDM manifested changes in relative abundance of bacterial species over the pregnancy, particularly those receiving the fish oil+probiotics combination. The specific bacterial species or function did not predict the onset of GDM nor did it differ according to GDM status, except for the higher abundance of *Ruminococcus obeum* in late pregnancy in the combination group in women with GDM compared to women without GDM. In the combination group, weak decreases over the pregnancy were observed in basic bacterial housekeeping functions.
Conclusions. The specific gut microbiota species do not contribute to GDM in overweight/obese women. Nevertheless, the GDM status may disturb maternal gut microbiota flexibility and thus limit the capacity of women with GDM to respond to diet, as evidenced by alterations in gut microbiota observed only in women without GDM. These findings may be important when considering the metabolic complications during pregnancy, but further studies with larger populations are called for to verify the findings.

Data availability statement

Data are stored on the Turku university server; anonymised data are available upon reasonable request. There are restrictions to the availability of the results due to patient confidentiality reasons.

Significance of this study

What is already known on this study?

- gut microbiota has been associated with metabolic diseases
- probiotics and fish oil have the capacity to modify gut microbiota

What are the new findings?

- Metagenomics gut microbiota, i.e. the composition and function of gut bacteria, is not involved in the incidence of GDM in overweight and obese women
- fish oil and probiotics as a combination can modulate the composition of the gut microbiota, particularly in pregnant women with overweight or obesity but without GDM

How might it impact on clinical practice in the foreseeable future?

- the less flexible gut microbiota of overweight and obese women with GDM may limit the capacity of these women to respond to dietary modulation of gut microbiota, which might be of importance for the metabolic health of the women as the potential benefits of the gut microbiota are being increasingly discovered
- overweight and obese women without GDM may benefit from dietary modulation through gut microbiota modulation

Introduction

There is increasing evidence that the gut microbiota acts as a regulator of metabolic health, which during pregnancy may influence the health of both the mother and the child. Gestational diabetes (GDM) is the most common metabolic disorder that women may suffer during pregnancy, affecting one in every seven live births worldwide[1]. Since there are inadequate means of prevention and management of GDM by diet, a modification of the gut microbiota has been proposed as one feasible and novel non-pharmacological solution.

There are a few studies pointing to an involvement of the gut microbiota in the pathogenesis of GDM[2-7] but not all studies agree[8]. Variations in the methods used to assess the modification may explain these heterogeneous findings. Most importantly, gut microbiota analytics based on 16SrRNA gene sequencing, have recently been criticized for not providing accurate information at the species level[9]. In addition to more in-depth information on bacterial taxonomy, metagenomics can provide data from gut microbiota genes and thus reveal the functional potential of the gut microbiota.

This is the first study to have investigated the gut microbiota-GDM interaction throughout the course of pregnancy using a deep sequencing metagenomics approach within a clinical study context, i.e. a detailed prospective recording and evaluation of the potential factors contributing to the microbiota-GDM interaction. Furthermore, we investigated the potential benefit of two food supplements in our trial, probiotics and long-chain polyunsaturated fatty acids (fish oil), in modifying the composition of the gut microbiota during pregnancy. Probiotics have been shown to support a healthy gut microbiota composition[10] and both probiotics and long-chain polyunsaturated fatty acids (fish oil) are known to possess gut microbiota modulating effects as well as anti-inflammatory and glucose metabolism modulating properties[11-15].

Subjects & design

The study population included overweight and obese pregnant women participating in a mother-infant dietary single-center intervention trial (ClinicalTrials.gov, NCT01922791) being conducted in Southwest Finland. The study protocol in detail and the primary outcomes of the food intervention during pregnancy have been previously reported in Pellonperä et al. 2019[16]. In the report we demonstrated no impacts of consuming the supplements on glucose metabolism or the incidence of GDM.

Briefly, the inclusion criteria for the study were overweight (prepregnancy BMI ≥25) and early pregnancy (<18 weeks of gestation). The exclusion criteria were GDM diagnosed before the first study visit, multifetal pregnancy, the presence of metabolic or inflammatory diseases. Furthermore, prior entering the study, the women were tested by HbA1c to exclude the possible type 2 diabetes. Of the 439 women recruited in the clinical trial, those women who did not provide fecal samples at both time points of their pregnancy, had used antibiotics within 8 weeks before the stool sampling or had used medication (insulin, metformin or both) for treatment of GDM were excluded. This resulted in a total of 270 women with fecal samples at early (gestational weeks mean 13.9 (SD14.1)) and late (gestational weeks 35.2 (1.0)) pregnancy.

GDM was diagnosed either in early (gestational weeks 14.6 (1.9)) or mid-pregnancy (gestational weeks 26.3 (2.0)) according to national guidelines, as previously described[16]. The early pregnancy OGTT was offered to high-risk women (BMI ≥35 kg/m2, previous GDM, glucosuria, polycystic ovarian syndrome, or family risk of diabetes).

In addition to national criteria for GDM diagnosis, we also used the international guidelines (International Association of the Diabetes and Pregnancy Study Groups (IADPSG)). Subsequently, we

formed four GDM groups (Table 1). Of the women who developed GDM in mid-pregnancy, 81% had early pregnancy fasting blood glucose below the 0 hour - OGTT threshold for GDM diagnosis (≥5.3 mmol/l), suggesting that most of these women had developed GDM later in their pregnancy.

Table 1. Definitions and numbers of the women in each GDM diagnosis groups.

GDM criteria	GDM diagnosed in early pregnancy = early onset GDM	GDM diagnosed in mid-pregnancy = mid-pregnancy onset GDM	Tested negative in early pregnancy, and GDM positive in mid-pregnancy = confirmed mid-pregnancy onset GDM	Tested GDM positive in early or mid-pregnancy = all GDM
National guidelines[1], number of GDM positive/negative women (% positive)	14/60 (18.9%)	53/203 (20.7%)	16	67/203 (24.8%)
International (IADPSG) guidelines[2], number of GDM positive/negative women	31/43 (42.0%)	72/167 (30.1%)	11	103/203 (38.1%)

[1]National guidelines: one or more values were at or above the threshold level: 0 h ≥5.3, 1 h ≥10.0, and 2 h ≥8.6 mmol/L;

[2] International (IADPSG) guidelines: one or more values were at or above the threshold level: 0 h ≥5.1, 1 h ≥10.0, and 2 h ≥8.5 mmol/L

The women were randomized into four intervention groups: fish oil+placebo (n=68), probiotics+ placebo (n=72) , fish oil+probiotics (n=69) or placebo+placebo (61) from the first study visit throughout the pregnancy, and until 6 months postpartum. Good compliance was reported by 88.4% of the women and when calculated from the returned fish oil capsules, a mean of 91.8% (SD 15.9) of the capsules had been consumed.The composition of the capsules are described in table 2.

Table 2. The composition and dose of the probiotics and fish oil capsules.

	Probiotics/fish oil	**Placebo for probiotics/fish oil**
Probiotics	*Bifidobacterium animalis ssp. lactis* 420 (DSM 22089; Dupont, Niebuell, Germany) and *Lactobacillus rhamnosus* HN001 (ATCC SD5675; Dupont, Niebuell, Germany), 10^10 cfu of each bacteria in 1 capsule consumed daily	Microcrystalline cellulose
Fish oil	n-3 LC-PUFA capsule (Croda Europe Ltd, Leek, England) consisted of 1.2 g of n-3 LC-PUFA (79.6% DHA and 9.7% EPA) in two capsules consumed daily to give a total daily dose of 2.4 g.	Medium chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%)

Patient and public involvement

The patients were engaged via advertisements distributed in maternal welfare clinics. In addition, media and social media were used to inform about the study. Patients were not involved in the design of the study. The study protocol was described to study participants on the first study visit in early pregnancy.

Outcomes

The primary endpoint of this study was to investigate whether the gut microbiota composition and function change over pregnancy and are related to GDM in overweight and obese women. The secondary endpoint was to investigate whether the gut microbiota can be modified by dietary supplements, fish oil and/or probiotics.

Metagenomic sequencing & bioinformatics analyses

Fecal samples were collected in sterile plastic pots the morning of the study visit or the previous evening and kept at -20°C until DNA extraction. DNA was extracted by using a GTX stool extraction kit and a fully automated GenoXTract machine (Hain Lifescience). Before extraction, mechanical lysis was performed by bead-beating the samples in ceramic bead tubes with a MOBIO PowerLyzer 24 Bench Top Bead-Based Homogenizer (MO BIO Laboratories, Inc., Carlsbad, CA).

Metagenomic sequencing was performed by Clinical Microbiomics (Denmark). The genomic DNA was randomly sheared into fragments of approximately 350 bp, which were used for library construction using NEBNext Ultra II Library Prep Kit for Illumina (New England Biolabs). The prepared DNA libraries were evaluated using Qubit 2.0 fluorometer quantitation and Agilent 2100

Bioanalyzer for the fragment size distribution. Quantitative real-time PCR was used to determine the concentration of the final library before sequencing. The library was sequenced using 2x150 bp paired-end sequencing on an Illumina HiSeq-platform.

Quality control and pre-processing of raw FASTQ reads were performed using KneadData [17]. These steps included read trimming, adapter removal and separation of both rRNA sequences (SILVA version 128) and human host DNA (Hg38). KneadData was run with default settings except for specifying the above database versions.

The analysis of the microbial composition was performed using MetaPhlAn2[18] version 2.6.0 with the default settings for paired-end reads. Pathway profiling was performed using the HUMAnN2 pipeline[19] version 0.11.1 in two steps. First, HUMAnN2 was run with ChocoPhlAn database version 0.1.1 and UniRef90 (created 09/2016). Next, the files were renormalized to relative abundances using the helper script "humann2_renorm_table.py" included in the HUMAnN2 distribution. The ChocoPhlAn and UniRef90 databases were downloaded using the supplied method ("humann2_database --download uniref uniref90_diamond", "humann2_database --download chocophlan full").

Statistical analysis

In order to gain an overall understanding of the gut microbiota composition and its relation to GDM status, we performed Kruskall Wallis-test followed by Mann–Whitney U-tests (M-W-test) using Bonferroni corrections to compare gut microbiota composition among the intervention groups, M-W-tests to compare differences according to GDM status and Wilcoxon Signed Ranks-Test when the change from early to late pregnancy were evaluated. The P-values for these analyses were corrected for

multiple testing with Benjamini-Hochberg-method, with FDR<0.25 considered as a statistically significant finding.

The output abundances of both MetaPhlAn2 and HUMAnN2 were associated with clinical metadata using MaAsLin[20] version 0.0.5, a multivariate statistical framework utilizing generalized linear models, which is useful when assessing the association between gut microbiota and GDM along with a simultaneous evaluation of any possible confounding factors[20]. The relative abundance data were transformed by MaAsLin with an arcsine square root transformation before testing for associations. The FDR threshold was set at q<=0.25 for all MaAsLin tests, with boosting and quality control steps enabled. As this threshold is rather high, we also present the findings with a lower q-value, i.e. q=0.05. The possible confounding factors were prepregnancy BMI, which may contribute both to gut microbiota and GDM status and previous GDM, a strong risk factor for GDM (Table 3). Age was not considered as a potential confounding factor in the MaAslin analyses, as in our previous[16] and current report, age did not differ according to GDM status (Table 4). As probiotics and fish oil and GDM status may influence the composition of the gut microbiota, these were included in the adjusted MaAslin also as confounding factors when needed in the analysis (Table 3).

Table 3. Confounding factors used in MaAslin analysis.

	Outcome	**Confounding factors in MaAslin**
Model 1	Difference in early pregnancy gut microbiota according to GDM status at early and late pregnancy*	• prepregnancy BMI • previous GDM
Model 2	Difference in late pregnancy gut microbiota according to GDM status	• prepregnancy BMI • previous GDM • intervention
Model 3	Gut microbiota change from early to late pregnancy	• prepregnancy BMI • previous GDM

		• intervention • with GDM status included and without GDM status
Model 4	Difference in late pregnancy gut microbiota according to GDM status within intervention group Gut microbiota change from early to late pregnancy separately according to GDM status at late pregnancy within intervention group	• prepregnancy BMI • previous GDM

*intervention not included in the analysis, as the women only began to consume the dietary supplements after providing the early pregnancy sample and due to our previous finding that the intervention has no influence on GDM[16].

The confounding factors were forced into the model, unless it contained only a single level in the subset. In that case, it was removed as unnecessary. Multiple time points per subject were taken into account by including the subjects in the model as random effects.

Bacterial community analyses were performed by multidimensional scaling, MDS, which is based on Bray-Curtis distance matrix for bacterial communities. Diversity and richness analyses were done with R[21], version 3.5.3 using the vegan package (version 2.5-4). The indexes used were Shannon index, a marker for species diversity of the samples, which takes into account the evenness of the bacteria in a sample and metagenomic species richness, which is the number of species observed in a sample.

The differences in maternal characteristics were analyzed using chi-square-test for categorical variables and M-w-test for continuous variables. These analyses were performed using SPSS version 25 (IBM Inc.).

Results

The baseline characteristics of the women are presented in table 4 and the numbers of the study subjects including those affected with GDM in each intervention group at early and late pregnancy are presented in the flow chart (Figure 1).

Table 4. Baseline characteristics of the women. GDM diagnoses according to the national guidelines.

	Early pregnancy			Late pregnancy			
	Women with early onset GDM (n=14)	Women without GDM (n=60)	P-value[1]	Women with mid-pregnancy onset GDM (n=53)	Women without GDM (n=203)	P-value[2]	n=270
Prepregnancy BMI	32.5 (5.5)	31.0 (5.1)	0.331	30.1 (4.8)	28.7 (3.5)	0.052	29.2 (4.0)
Obese	8 (57%)	33 (55%)	0.885	24 (45.3%)	63 (31.0%)	0.051	95 (35.2%)
Age (years)	31.7 (6.2)	31.1 (4.4)	0.679	31.3 (4.4)	30.7 (4.3)	0.421	30.9 (30.5)
University degree	7/14 (50%)	40/58 (69%)	0.181	30/53 (56.6%)	141/199 (70.9%)	0.048	178 (65.9%)
Previous GDM	6 (43%)	11 (18.3%)	0.050	4 (7.5%)	10 (4.9%)	0.455	20 (7.4%)
Smoked before pregnancy	11/14 (78.6%)	13/59 (22.0%)	0.9618/53	8/53 (15.1%)	39/200 (19.5%)	0.463	50/267 (18.7%)
Smoked during pregnancy	1/14 (0.7%)	4/59 (0.7%)	0.961	2/53 (3.8%)	10/199 (5.0%)	0.704	13/266 (4.9%)
Gestational weeks at early pregnancy visit	12.4 (2.3)	14.0 (2.1)	0.021	14.2 (1.8)	14.0 (2.0)	0.580	13.9 (14.1)
Gestational weeks at late pregnancy visit	34.8 (1.0)	35.1 (0.9)	0.309	35.1 (1.1)	35.2 (0.9)	0.529	35.2 (1.0)
Gestational weeks in early pregnancy OGTT	15.0 (2.0)	14.5 (1.9)	0.379				14.6 (1.9)/74

Gestational weeks in mid-pregnancy OGTT	26.7 (2.8)	26.2 (1.8)	0.093	26.5 (2.3) 26.3 (2.0)/256
OGTT negative in early pregnancy, positive in mid pregnancy	16			

P^1: Pearson Chi-square or Mann Whitney U test between women with and without GDM diagnosed at early pregnancy. P^2: Pearson Chi-square or Mann Whitney U test between women with and without GDM diagnosed in late pregnancy. OGTT:oral glucose tolerance test

The main phyla in the faeces both in early and late pregnancy were Bacteroidetes (56.9% and 57.6% respectively), followed by Firmicutes (37.9% and 36.5%), Proteobacteria (2.5% and 2.7%), Actinobacteria (1.7% and 2.1%) and Verrucomicrobia (0.93% and 1.3%) (Table S1a).

Specific gut microbiota species do not influence GDM onset when adjusting for confounding factors

We observed differences in seven bacteria species in early pregnancy when comparing women with mid-pregnancy onset GDM to those without and four when those with confirmed mid-pregnancy onset GDM were compared to those without this condition (Table S1b). After correcting for multiple testing, only *Megasphaera unclassified* remained statistically significant and only in the confirmed mid-pregnancy onset GDM cases, with the abundance being higher in those women developing GDM (FDR<0.25). In MaAslin (Model 1), no statistically significant differences were detected in the early pregnancy gut microbiota using either of the GDM criteria (Table S2a). In gut microbiota community analysis, the women with and without GDM were found to overlap (Figure S1). No differences were found in gut microbiota diversity and richness. Furthermore, no differences in the Firmicutes to Bacteroidetes ratio were observed according to the GDM status.

Specific gut microbiota species do not differ between women with and without GDM

Several differences at the species level were detected according to the GDM status, but none of these remained significant after correcting for multiple testing or in MaAslin (Model 2) (Table S1c, d, Table S2b, c). Furthermore, no differences were observed between women without and those with GDM in bacterial communities in late pregnancy (Figure S2). The only difference in the Firmicutes/Bacteroidetes-ratio was lower ratio in women with early onset GDM (0.51 (0.42-0.88), P=0.048)) compared to those without (median 0.56 (0.28-1.02)). Furthermore, several bacterial species

differed between the women with early onset GDM compared to mid-pregnancy onset, but the observed differences were no longer significant after correcting for multiple testing (Table S1e) and in MaAslin (Table S2d).

The evolution of the gut microbiota throughout pregnancy is influenced by GDM and diet intervention

We next investigated whether there are changes in the gut microbiota from early to late pregnancy and whether these changes are influenced by GDM. The placebo group represents the change induced by pregnancy alone. Indeed, several alterations in different taxonomic levels were detected due to pregnancy, although the changes were no longer significant when corrected for multiple testing (Table S1f). In all women in the placebo group, *Coprococcus catus* increased (q=0.077), as evaluated in the adjusted MaAslin (Table S2e) (Figure 2 and Figure S3a). When investigating the women without GDM (all the women without GDM, regardless of the intervention group), it was observed that there were changes in the relative abundance of 22 species (FDR<0.25, Table S1g), but in women with mid-pregnancy onset GDM, only one bacterial species. i.e. *Roseburia hominis* changed. In women with early onset GDM, no changes in bacterial species were observed during their pregnancy (Table S1g). Next, we assessed the potential influence of the dietary intervention on the gut microbiota by using MaAslin. When we compared the change in gut microbiota between the four intervention groups of all the women (Model 3), the abundance of *B.animalis* increased in the fish oil+probiotics combination group (q=0.002) (Table S2f). To reveal the possible influence of GDM status, we included GDM as an additional confounding factor in MaAslin (Model 3). In this model, we observed a strong influence of the intervention, which was evident as a change in two species i.e. *B. animalis* (q=0.003, increase in fish oil+probiotics group) and *Bacteroides ovatus* (q=0.132, decrease in probiotics group), but no changes in association with the GDM status (Table S2f). When comparing the difference between the

four intervention groups at late pregnancy, the effect of fish oil+probiotics-combination was also evident in the adjusted MaAsLin, i.e. the highest abundance of *B.animalis* was observed both in the fish oil+probiotics group when we considered all women (q=0.001) and only women without GDM (q=0.006) (Table 5, Table S2g). When those women who had no *B.animalis* at early pregnancy were evaluated (25% of the women), the highest abundance was detected in fish oil/probiotics-group (3.2E-04 (7.2E-0.4), P<0.001 when compared to placebo-group (6.5E-05 (SD1.7E-04), followed by probiotics/placebo-group (1,93E-04 (4.6 E-04), P=0.012). It is noteworthy that also the proportion of women in whom *B.animalis* was detected in faeces was highest in the fish oil+probiotics group (Table 5). Suprisingly, *L.rhamnosus* HN001 was not found in fecal samples of the women in the probiotics or in the fish oil+probiotics-group.

Table 5. Relative abundance of *B.animalis* in intervention groups in late pregnancy. Differences among intervention groups in all women (BH-adjusted Kruskal-Wallis P-value 8.6E-9) and women with mid-pregnancy onset GDM and without GDM (BH-adjusted Kruskal-Wallis P-value 1.1x-7).

Group	**Fish oil+placebo**	**Probiotics+placebo**	**Fish oil+probiotics**	**Placebo+placebo**
All women, n	68	72	69	61
% of subjects with *B.animalis* in feces	29.4%	58.3%	84.1%	21.3%
***B.animalis*, mean (SD)**	$3.25x10^{-4}$ ($1.10x10^{-3}$)	$2.87x10^{-4}$ ($5.87x10^{-4}$)	$5.98x10^{-4}$ ($1,12x10^{-3}$)	$7.02x10^{5}$ ($1.82x10^{-4}$)
***B.animalis*, median (IQR)**	0 ($0-6.32x10^{-5}$)[d: 0.036, e<0.001]	$3.06x10^{-5}$ ($0-2.30x10^{-4}$) [a<0.001, c 0.022, d]	$1.42x10^{-4}$ ($2.29x10^{-5}$-$2.29x10^{-5}$) [b<0.001,c,e]	0 (0-0) [a,b]
women without GDM	49	52	53	49
% of subjects with *B.animalis* in feces	26.5%	59.6%	84.9%	20.4%
***B.animalis*, mean (SD)**	$3.40x10^{-4}$ ($1.25x10^{-3}$)	$3.26x10^{-4}$ ($6.60x10^{-4}$)	$7.11x10^{-4}$ ($1.25x10^{-3}$)	$6.46x10^{-5}$ ($1.65x10^{-4}$)
***B.animalis*, median (IQR)**	0 ($0-2.95x10^{-5}$)[h0.029, i<0.001]	$4.21x10^{-5}$ ($0-2.38x10^{-4}$)[f0.001, h	$1.60x10^{-4}$ ($2.03x10^{-5}$-$6.67x10^{-}$ [4g<0.001, i]	0 (0-0)[f,g]

Women with mid-pregnancy onset GDM	19	20	16	12
% of subjects with *B.animalis* in feces	19 (35.7%)	20 (62.5%)	16 (84.6%)	12 (20%)
***B.animalis*, mean (SD)**	$2.88x10^{-4}$ ($6.12x10^{-4}$)	$1.83x10^{-4}$ ($3.20x10^{-4}$)	$2.25x10^{-4}$ ($2.87x10^{-4}$)	$9.33x10^{-5}$ ($2.49x10^{-4}$)
***B.animalis*, median (IQR)**	0 (0-$3.6x10^{-4}$)	1.84 $x10^{-5}$ (0-$1.91x10^{-4}$)	$1.33x10^{-4}$ ($2.57x10^{-5}$-$2.37x10^{-4}$)	0 (0-$8.60x10^{-5}$)

Superscripts describe the statistically significant differences between the intervention group and the value after the letter represents the Bonferroni corrected P-value.

We detected no change in the Firmicutes/Bacteroidetes ratio when either all the women (P=0.129) or women with mid-pregnancy onset GDM (P=0.919) were included in the analysis. Instead, in the women without GDM, the Firmicutes/Bacteroidetes-ratio decreased (early pregnancy: median 0.653 (IQR 0.349-1.01) vs late pregnancy: 0.578 (IQR 0.311-0.979), P=0.014)), while in women diagnosed with early onset GDM, the ratio increased (median 0.51 (0.42-0.88) vs 0.84 (0.56-1.2), P=0.026). No differences in the bacterial communities were found in late pregnancy among the intervention groups, whether all the women, women with or those without GDM were analysed (Figure S4). The only change in diversity or richness was the decrease in metagenomic species richness in women without GDM (early: 73.0 (67.0-79.0) vs late 72.0 (66.0-77.0), P=0.013).

Dietary intervention modifies the gut microbiota differentially in women with and without GDM

Most of the changes from early to late pregnancy were observed in the fish-oil+probiotics group. We identified a decrease in *Veillonella parvula* and *Veillonella unclassified* and in *Haemophilus parainfluenzae* (FDR<0.25) and an increase in *B.animalis* (Table S1h) in all women. The increase in *B.animalis* (q=4.49e-07) and a decrease in *Veillonella unclassified* (q=0.009), *H.parainfluenzae* (q=0.011) and *V.parvula* (q=0.014) remained significant in MaAslin (Model 4) (Figure 3, Figure S3b) (Table S2e). There were increases in *B.animalis* (q=6.01e-05), along with the decreases in *H. parainfluenzae* (q=0.001), *V. unclassified* (q=0.016), *V.parvula* (q=0.035) and *E.eligens* (q=0.037) (Figure S3b, Figure 4) observed in women without GDM, but not in those with GDM (Table S2e).
In the fish oil group, decreases in a few species were observed when all the women and women without GDM were analysed (FDR<0.25) (Table S1h). None of these changes remained significant in MaAslin (Figure S3c) (Table S2e).

In the probiotics group, changes in several species were observed in all women, and a decrease in one species in women without GDM (FDR<0.25) (Table S1h). In MaAlsin, only the increase in *B.animalis* remained significant when all women were included (q=0.207) (Figure S3d and Figure 5, Table S2e). Significant decreases in the Firmicutes/Bacteroidetes-ratio (0.84 (0.43-1.4) vs. 0.63 (0.38-1.04), P=0.006) were observed in women without GDM, but only in the group receiving fish oil. In bacterial communities, no differences were found in the change of the gut microbiota from early to late pregnancy in the different intervention groups (Figure S4). No differences were found in the change of the gut microbiota diversity and richness.

When investigating the differences in the late pregnancy gut microbiota according to GDM status, most of the differences were detected again in the combined fish oil+probiotics- group (Table S1i). After correcting for multiple testing, higher abundances of *Ruminococcus obeum, Sutterella wadsworthensis, Subdoligralunum unclassified* and *Pseudoflavonifractor capillosus* in women with GDM (mid-pregnancy onset or all GDM) remained statistically significant as compared to women without GDM (FDR<0.25) (Table S1i). In MaAslin (Model 4), the higher abundance of *R.obeum* (q=0.026), *S.wadsworthensis* (q=0.215), and *Subdoligralunum unclassified* (q=0.215), remained significant when women with mid-pregnancy onset GDM were compared to healthy women (Table S2h, Figure 6a-c). When all the women diagnosed with GDM (all GDM) were included in the adjusted MaAslin, only the higher abundance of *R.obeum* was statistically significant (q=0.0892) (Figure 6d). When the differing diagnostic criteria (national or international) for GDM were taken into account, *R. obeum* was higher in women with GDM (all GDM) as compared to women without GDM only when national criteria were applied (Table S3). Furthermore, *S. wadsworthensis* and *Subodoligralunum unclassified* were higher in women with mid-pregnancy onset GDM only when the national criteria were applied (Table S3).

In the fish oil and placebo group, no differences were observed, after correcting for multiple testing (Table S1i) or in the adjusted MaAslin (Table S2h).

The only difference in the Firmicutes/Bacteroidetes- ratio was observed in the fish-oil+probiotics group, i.e. the ratio was lower in women without than in those with GDM (mid-pregnancy onset and all GDM) (median 0.42 (IQR 0.30-0.96) vs 0.89 (0.48-2.4), P=0.010; 2.48 (0.43-3162.27), P=0.007). In the bacterial community analysis, no differences were observed in the intervention groups between women with and without GDM (results not shown). The only difference in diversity or richness was found in Shannon index, which was higher in women with mid-pregnancy onset GDM diagnosed in late pregnancy as compared to women without GDM, but only in the placebo group (3.1 (2.78-3.13) vs 2.83 (2.60-3.03), P=0.048).

Correlations between serum glucose concentrations and gut microbiota

In the MaAsLin analysis, *Holdemania filiformis* abundance correlated positively, (q=0.024), and *Alistipes shahii* (q=0.244) and *Bifidobactrium bifidum* (q=0.244) negatively with early pregnancy glucose concentrations (Table S2i). The late pregnancy glucose concentration did not correlate with any bacterial species.

Gut microbiota and function

As with gut microbiota composition, no association between gut microbiota function and GDM status was detected (Table S4a-c), confirming that gut microbiota is neither involved in the incidence of GDM nor differ according to GDM status. However, an interaction between GDM status and intervention was observed: in women without GDM, a decrease from early to late pregnancy in some

of the bacterial functions were detected, but only in the fish oil+probiotics combination group (Table S4e). In addition, in the combination group, few functions differed according to mid-pregnancy GDM status (Table S4h). All these findings were related to bacterial housekeeping properties e.g. lipid synthesis and energy metabolism (Table S5).

Discussion

The deep-level metagenomics analysis together with the strict bioinformatics analyses applying corrections for the multivariable testing and confounding factors, revealed that neither specific gut microbiota species nor their function is involved in the onset of GDM and they do not differ according to GDM status in overweight and obese women. The novel finding here is that the gut microbiota of women without GDM was amenable to modifications, whilst that of GDM women appeared to be rather inflexible. Whether this is the case needs to be confirmed in further studies.

Similarly to our metagenomic approach, one previous study with a lower number of study subjects using less accurate 16S rRNA sequencing, detected no differences in gut microbiota between women with and without GDM[8]. Compared to those studies reporting associations between gut microbiota and GDM using 16S rRNA sequencing techniques[4-6], our approach, i.e. metagenomic sequencing provides higher resolution of the composition of the microbiota i.e. abundance of bacteria at the species level and also information on the function of the gut microbiota, thus we believe that our findings are likely to be more accurate in clarifying the possible relationship between the gut microbiota and GDM. There are also two studies that have applied a metagenomics approach[2,7] which did find deviations in the gut microbiota according to GDM status in either mid- or late pregnancy. In contrast to our study, these women were of normal weight, and further, only one faecal sample was collected at 21-29 gestational weeks in a rather low number of women, i.e. 43 with GDM and 81 without[2] or in the first

trimester, i.e. 23 women with GDM and 26 without GDM[7]. In addition, we included prepregnancy BMI, known to potentially influence gut microbiota and previous GDM, in the analyses as confounding factors. Furthermore, in this study, when we applied either the national or the international criteria for diagnosis, we did observe some differences, suggesting that these differing criteria may be another reason for the contrasting findings between reports. Importantly, our study also included an intervention, which allowed us to study the potential of diet to modify the gut microbiota of overweight and obese pregnant women.

The observation that the changes due to dietary intervention, i.e. gut microbiota modulation due to the consumption of the combination of fish oil and probiotics, suggests that the gut microbiota of women with GDM is inflexible and thus less readily modifiable by environmental factors, including diet. Consuming the combination of fish oil and probiotics appeared to modify the composition of the gut microbiota, i.e. an increase in the abundance of *B.animalis,* decreases in *H. parainfluenzae*, *V. unclassified, V.parvula* and *E.eligens* in women without GDM. Previous studies have described *H. parainfluenzae* as an opportunistic pathogen e.g. residing in the oral cavity and gastrointestinal tract[22] and in the genital tract of a minority of pregnant women[23]. *V.unclassified* and *V.parvula*, belong to the genus *Veillonella*; its the abundance has been shown to be reduced in children with food allergy, a condition also related to inflammation[24]. The presence of oral *V.parvula* has also been related to a higher blood glucose level in healthy subjects[25]. *E.eligens* may participate in the modulation of inflammation as it can evoke the production of the anti-inflammatory cytokine, IL-10 *in vitro*[26]. Furthermore, in the fish oil+probiotics group, a higher abundance of three species in late pregnancy was found in women with mid-pregnancy onset GDM as compared to women without GDM; *R.obeum,* is a propionate producing bacterial species[27] and associated with obesity, *S.wadsworthensis,* has been found at a lower abundance in patients with asthma[28] and shown to

correlate inversely with overweight in children[29] and *Subodoligralunum unclassified*, is a bacteria belonging to the heterogeneous *Ruminococcae* family. Regarding the functional properties of the bacteria, the only finding was observed in the women in the combination of fish oil and probiotics group in some of the bacterial housekeeping functions, e.g. lipid synthesis and energy metabolism were detected. The reason why these changes in gut microbiota composition and function were observed mostly in the combination group is unclear, but may relate to the capability of fish oil to enhance the adherence potential of the probiotics to the gut epithelial cells and/or to increase the colonization potential of the probiotics[30,31].

Another manifestation of an aberrant metabolism, insulin resistance, has been related to a poorer gut microbiota richness[32]. Unexpectedly, in the current study, the alpha-diversity, as measured by Shannon diversity, was somewhat higher in women with GDM, a group with disturbed glucose metabolism, as compared to women without GDM.

Our findings here are based on several of our study's strengths, e.g. the selection of women not using medication (insulin and/or metformin, antibiotics) and the application of robust statistical methods. Furthermore, the women in this study represent a homogenous study population in relation to their overweight/obese status, as well as the duration of pregnancy. i.e. early and late pregnancy. A further strength of our study is that we used the four different GDM diagnoses. Our study did not include normal weight women; these women may well respond differently both to pregnancy and dietary-induced modification of the gut microbiota and thus this may limit the generalization of our findings. As the women were advised not to consume any foods with probiotics or fish oil, we consider that the changes observed in the gut microbiota composition, particularly in the abundance of *B.animalis*, are due to dietary intervention. However, we did not find *L.rhamnosus* in the fecal samples, this possibly requiring analyses using targeted PCR approach. Further, although we do not expect any effect, we are

not able to rule out that the use of medium chain fatty acids as placebo fatty acids might have gut microbiota modulating effects[33]. Yet another limitation is that although the number of the women in this study was rather large, not so many women were diagnosed with GDM, resulting in a smaller group size as compared to women without GDM suggesting the need for further studies with a higher number of cases. It is noteworthy that in this study, correcting for multiple testing may also lead to false negative results, which may hinder the identification of the specific bacteria related to the outcomes being investigated. To overcome this problem, we have also presented the findings without correcting for multiple testing and in addition, provided the findings also at higher taxonomic levels.

We detected no statistically significant associations between the gut microbiota function and GDM. The intervention effect on the gut microbiota composition was observed only in women without GDM, which indicates that the gut microbiota of the women without GDM is flexible for modifications with food supplements. It still needs to be clarified in other studies, whether our observation is valid and whether the potentially inflexible gut microbiota in women with GDM contributes to the incidence of GDM or the metabolic aberrations detected in GDM. We'd like to speculate that the lack of adaptability of the gut microbiota may also explain why the dietary intervention with fish oil/probiotics conferred no clinical benefits, i.e. no lowering of the risk of GDM[16]. We have previously shown that the metabolic profile of women with GDM deviated from those without GDM already in early pregnancy[34], suggesting that women with GDM have an increased metabolic burden and may thus be less plastic for dietary supplements in relation to metabolic, but possibly also gut microbiota modulation. Further studies integrating the omics data, i.e. metatranscriptomics, proteomics, metabolomics and metagenomics data would provide more insights into the functional potential of the gut microbiota[35] in the regulation of maternal metabolism.

To conclude, the specific gut microbiota species do not contribute to GDM in pregnant women with overweight or obesity. However, the GDM status may disturb the flexibility of the maternal gut microbiota, as the response to this specific dietary intervention was evident only in women without GDM, although the results will need to be confirmed in larger study sets. This finding indicates that in this high risk population of pregnant women, the specific gut microbiota has a negligible role in regulating maternal glucose metabolism, whereas perhaps because of their more flexible gut microbiota, women without GDM may benefit from gut microbiota targeted dietary supplementation aiming to achieve other health outcomes. Furthermore, the inability to react to dietary modulation of gut microbiota in women may indicate that the inflexible gut microbiota may contribute to the incidence and metabolic deviations observed in GDM, calling for further investigations on this topic.

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Declaration of Interest:

The authors declare no competing interests.

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Authors' contributions:

KL designed the original clinical study, directed the project, curated the data and acquired the financial support for the study. KL and KM designed the research and its conceptualization, EK and NH participated in sample and data collection, OP and KT interpreted the GDM diagnoses, NP, SK and SP performed bioinformatics, LE supervised bioinformatics analyses, KM performed the SPSS-analyses. KM and KL interpreted the results, NP, SK SP and EL contributed to the interpretation of the results. KM wrote the manuscript with support from KL and all authors read, critically revised, approved and take responsibility of the final version of the paper.

Figure legends

Figure 1. Flow chart of the study.

Figure 2. Early and late pregnancy relative abundance of *Coprococcus catus* in placebo group when all the women (n=61) were analysed

Figure 3. Early and late pregnancy relative abundances of bacterial species with statistically significant change over the pregnancy in fish oil+probiotics group when all the women (n=69) are analysed.

Figure 4. Early and late pregnancy relative abundances of bacterial species with statistically significant change over the pregnancy in fish oil+probiotics group when women without GDM (n=53) are analysed.

Figure 5. Early and late pregnancy relative abundances of Bifidobacterium animalis in probiotics group when all the women (n=72) were analysed.

Figure 6a-c. Relative abundances of bacterial species according to GDM status in fish oil+probiotics-group between a-c) women without GDM (n=53) and women with mid-pregnancy onset GDM

diagnosis (n=13) d) women without GDM (n=53) and women with GDM (all GDM diagnoses, n=16).

Tables

Supplemental Information

Supplemental tables S1 a-i

Supplemental tables S2 a-i

Supplemental table S3

Supplemental table S4 a-i

Supplemental table S5

Supplemental figures S1- S4:

Figure S1. A principal coordinates analysis (PCoA) (A, B) of the gut microbiota in early pregnancy between women remaining without GDM (n=203) and women with mid-pregnancy onset GDM (A, n=53) or with confirmed mid-pregnancy onset GDM (B, n=16).

Figure S2. A principal coordinates analysis (PCoA) (A, B, C) of the gut microbiota in late pregnancy between women without GDM (n=203) and in women with GDM diagnosed in early and late pregnancy (All GDM, n=67)) (A) or with mid-pregnancy onset GDM (B, n=53) or with confirmed mid-pregnancy onset GDM (C, n=16).

Figure S3a. Changes from early to late pregnancy in bacterial species in the placebo group in all women, in women without GDM and in women with mid-pregnancy onset GDM. All women, n=61, women without GDM, n=49, women with mid-pregnancy onset GDM, n=14.

Figure S3b. Changes from early to late pregnancy in bacterial species in the fish oil+probiotics group in all women, in women without GDM and in women with mid-pregnancy onset GDM. All women, n=69, women without GDM, n=53, women with mid-pregnancy onset GDM, n=13.

Figure S3c. Changes from early to late pregnancy in bacterial species in the probiotics group in all women, in women without GDM and in women with mid-pregnancy onset GDM. All women, n=72, women without GDM, n=52, women with mid-pregnancy onset GDM, n=16.

Figure S3d. Changes from early to late pregnancy in bacterial species in the fish oil group in all women, in women without GDM and in women with mid-pregnancy onset GDM. All women, n=68, women without GDM, n=49, women with mid-pregnancy onset GDM, n=14.

Figure S4. A principal coordinates analysis (PCoA) (A, B, C) of the gut microbiota changes from early to late pregnancy. (A) all women in intervention groups included, (B) women without GDM included, (C) women with mid-pregnancy onset GDM.

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