Changes in the composition and function of the gut microbiome accompany type 1 diabetes in pregnancy

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Research

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Abstract

Background The gut microbiome changes in response to a range of external influences, life events and disease. Pregnancy is a natural life event involving major physiological adaptation but studies of the microbiome across pregnancy are scarce and findings inconsistent. Pregnancy in which the mother has type 1 diabetes (T1D) with disturbed glucose-insulin homeostasis increases maternal and fetal risks and knowledge of the gut microbiome in this context is lacking. To understand how T1D impacts the gut microbiome in pregnancy we used a combination of whole metagenomic sequencing (WMS) and 16S rRNA sequencing to define the composition and function of the gut bacterial microbiome across 145 pregnancies, 94 in women with T1D. Women were participants in the Environmental Determinants of Islet Autoimmunity (ENDIA) study, in which the child has a first-degree relative with T1D.

Results Pregnant women with and without T1D exhibited taxonomic and functional changes in gut microbial composition across pregnancy. Profiles in women with T1D were distinct, with a decrease and increase, respectively, of bacteria with anti- and pro-inflammatory properties, and a decrease in bacteria that synthesize essential vitamins and metabolites. These changes were accentuated in trimester 3, often in opposite directions to women without T1D.

Conclusions The gut microbiome changes in composition and function across pregnancy, but distinctly in women with T1D. The decrease in bacteria that produce anti-inflammatory mediators and essential B-group vitamins suggests that the gut microbiome may contribute to maternal and fetal complications in the T1D pregnancy, potentially opening new avenues for therapeutic intervention.

Background

The gut microbiome provides essential metabolites, vitamins, co-factors and hormones, protects against pathogenic microorganisms and has a key role in the development of the immune and other systems [1, 2]. Adaptations or shifts in the composition of the gut microbiome occur throughout ageing and in association with environmental conditions, life events and disease states [2–4]. In pregnancy, women undergo significant physiological changes, but only recently has the gut microbiome been studied in this context [5, 6]. Koren et al [5] sampled the gut microbiome in the first and third trimesters and found that the taxonomic composition in the first trimester was similar to that of non-pregnant women, but in the third trimester observed an increase in Actinobacteria and Proteobacteria phyla along with an overall decrease in bacterial richness (alpha diversity). Germ-free mice inoculated with third trimester feces had greater weight gain, insulin resistance and gut inflammation than mice inoculated with first trimester feces, which they interpreted as an adaptive proinflammatory response to defend the fetus from pathogens and provide it with nutrients [5, 7]. On the other hand, DiGiulio et al [6] collected weekly fecal samples across pregnancy but found no significant temporal differences in diversity or composition of the gut microbiome. These contrary findings along with a dearth of studies warrant further investigation of the gut microbiome across pregnancy.

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-producing β cells in the islets of the pancreas are destroyed by T lymphocytes leading to insulin deficiency [8]. T1D increases pregnancy risks for mother and fetus [9] but the role of the gut microbiome in this context is unclear. Alterations in the bacterial gut microbiome have been reported in T1D, mainly in children at high risk and at diagnosis (reviewed in [10], [11–16]). They encompass a decrease in the Firmicutes/Bacteroidetes phylum ratio [11, 12], a decrease in richness (alpha diversity) [11–13] an increase in inflammation-associated bacteria [12, 13] and a decrease in bacteria with anti-inflammatory properties, in particular lactate- and butyrate-producing and mucin-degrading species [12–16]. Functionally, this dysbiosis is reflected by a decrease in the abundance of genes that encode related metabolic pathways and enzymes, e.g. butyryl-coenzyme A (CoA)-CoA transferase [14] and butyryl-CoA dehydrogenase [15]. While these changes are not necessarily specific for T1D they nevertheless may have clinical consequences. Gut butyrate is a key determinant of gut health and regulator of gene expression and homeostatic immunity [17–19]. It is the major energy source for the colonic mucosa, induces synthesis of mucin and promotes gut epithelial integrity, preventing 'gut leakiness' and translocation of toxins and dietary antigens into the blood. Such translocation may lead to systemic inflammation and predispose to immune diseases such as T1D. Indeed, dietary butyrate induced regulatory T cell production and decreased the incidence of spontaneous diabetes in the non-obese diabetic (NOD) mouse model of T1D [19]. Increased gut permeability has been described in established T1D [20] and recently by ourselves in association with gut microbiome dysbiosis in children with islet autoimmunity who progressed to T1D [21]. In the T1D pregnancy, gut microbiome dysbiosis could have functional consequences for the mother and the fetus. Consensus about the gut microbiome in pregnancy, even in the absence of T1D, is lacking. To address this knowledge gap, we used a combination of whole metagenomic sequencing (WMS) and 16S rRNA sequencing to analyse the gut microbiome across the three trimesters of pregnancy of women with and without T1D who are participating in the Australia-wide Environmental Determinants of Islet Autoimmunity study (ENDIA).

Results

Study population

Fecal samples were collected from women enrolled in the ENDIA study across five Australian States between February 2013 and October 2017 [22]. ENDIA is a prospective, pregnancy-birth cohort study that follows 1500 Australian children who have a first-degree relative with T1D. Fifteen women (16 pregnancies) without T1D (non-T1D) and 16 with T1D had each provided three fecal samples across pregnancy (total of 96 samples) (Table 1). These were analysed by shotgun WMS (Figure S1A). A second sample set comprising a total of 354 fecal samples collected longitudinally across 145 pregnancies from 51 non-T1D women and 94 T1D women, including the 96 samples analysed by WMS, was analysed by 16S rRNA gene sequencing (Figure S1B). Table 1 summarizes and compares characteristics of the non-T1D and T1D pregnancies.

Whole metagenomic sequencing and 16S sequencing

For the WMS dataset, 43,809,936 ± 9,156,972 (mean ± SD) paired end reads per sample were obtained using an Illumina NovaSeq 6000. Raw reads (SRA accession: PRJNA604850) were pre-processed using KneadData bioBakery tool [23] to eliminate human DNA sequences and filter sequences with poor quality which on average removed 6% of the reads. Reads of 41,013,121 ± 8,739,281 (mean ± SD) were obtained after quality control and read filter steps (Excel file E0). For the 16S rRNA dataset, 42,104 ± 17,296 (mean ± SD) paired end reads per sample were obtained with an Illumina MiSeq. QIIME2 was used to demultiplex and quality-filter the raw sequences (SRA accession: PRJNA604850), generating 24,777 ± 10,749 (mean ± SD) reads per sample (Excel file E0).

Taxonomic diversity and composition of the gut microbiome in non-T1D and T1D women during pregnancy

Metagenomic sequences of the women who provided three fecal samples across trimesters were analysed using MetaPhLan2 implemented within the HUMAnN2 pipeline. Overall, 298 bacterial species were identified with an average of 97 ± 11 (mean ± SD) species per sample.

The top 25 most abundant species accounted for more than 50% of the gut microbiome composition of each subject in any given trimester (Figure 1). The Firmicutes/Bacteroidetes phylum ratio across trimesters was unchanged in non-T1D women but decreased in women with T1D (Figure S2).

Alpha diversity (observed richness or number of species) per sample was calculated (Excel file E1) and generalized estimating equations (GEE) were applied to test for differences between women without and with T1D, between trimesters and to determine if there was an interaction between T1D status and trimester. No significant interactions or differences in richness were found (Figure S3, Excel file E1).

For beta diversity analysis, Bray-Curtis coefficients were calculated between sample pairs, ordinated and plotted by principal coordinate analysis (PCoA; Figure 2). To test for differences in beta diversity, a repeated-measure aware permutational analysis of variance (RMA-PERMANOVA) of the Bray-Curtis coefficients was performed on normalized data. This revealed a significant interaction between T1D status and time at all taxonomic levels. Therefore, differences between T1D and non-T1D women were assessed within trimester. No significant differences were detected in trimester 1. However, differences were significant at the strain level (P=0.02) in trimester 2 and at the strain (P=0.002), species (P=0.001), genus (P=0.015), family (P=0.029) order (P=0.034) and phylum (P=0.032) taxonomic levels in trimester 3 (Excel file E2).

Differences in beta diversity reflect differences in taxonomic composition. To identify differences in taxa between non-T1D and T1D women in pregnancy, differential abundance was analysed in limma; the Benjamini-Hochberg method was used to control the false discovery rate. Taxa were considered differentially abundant if the adjusted P-value expressed as the False Discovery Rate (FDR) was < 0.05; however, borderline significant differences, i.e. FDR from 0.05 to 0.1, were also reported. Note that only taxa for which the prevalence (i.e., proportion of samples with those taxa) was above 50% in at least one group and with a log2 fold-change (logFC) greater than 0.5 or less than -0.5 were considered. T1D status

and trimester were combined into a single factor with six levels and comparisons of interests were defined as contrasts. Across all trimesters, the species *Bacteroides eggerthii* (FDR=0.033), *Bacteroides clarus* (FDR=0.005), *Alistipes sp. AP11* (FDR=0.009), *Escherichia coli* (FDR=0.002), an unclassified *Escherichia* bacterium (FDR=0.042), *Roseburia hominis* (FDR=0.08) and *Bacteroides uniformis* (FDR=0.088), as well as the family *Enterobacteriaceae* (FDR=0.09) and the order *Enterobacteriales* (FDR=0.03), were increased in women with T1D (Figure 3; Excel file E3). On the other hand, the species *Bacteroides massiliensis* (FDR=0.043) and the family *Prevotellaceae* (FDR=0.05) were decreased in T1D (Figure 3; Excel file E3).

Differences between non-T1D and T1D women were also assessed within trimesters. In trimester 1, species *Escherichia coli* (FDR=0.009), *Alistipes sp. AP11* (FDR=0.063), *Bacteroides clarus* (FDR=0.073) and *Bacteroides salyersiae* (FDR=0.073), and the order *Enterobacteriales* (FDR=0.0005) were increased in T1D, while *Bacteroides massiliensis* (FDR=0.016) was decreased in T1D (Figure 3; Excel file E3). In trimester 2, only the family *Prevotellaceae* (FDR=0.065) and the phylum *Firmicutes* were decreased in T1D (Figure 3; Excel file E3).

Most differences between non-T1D and T1D women were in trimester 3, in which species *Bacteroides eggerthi* (FDR=0.012), *B. clarus* (FDR=0.02), *E. coli* (FDR=0.012), an unclassified *Escherichia* bacterium (FDR=0.083), *R. hominis* (FDR=0.007), *B. uniformis* (FDR=0.012), *B. salyersiae* (FDR=0.011), *B. caccae* (FDR=0.012), *Parabacteroides distasonis* (FDR=0.012), *B. faecis* (FDR=0.088), *Sutterella wadsworthensis* (FDR=0.083) and the family *Bacteroidaceae* (FDR=0.078) were increased in T1D, and species *Bifidobacterium adolescentis* (FDR=0.025) and *Ruminococcus bromii* (FDR=0.09) and the order *Bifidobacteriales* (FDR=0.002) were decreased (Figure 3; Excel file E3). Five of the 15 differentially abundant species detected were among the 25 most abundant species in the entire dataset (Figure 1). Differential abundance data are summarised in Excel file E3.

In order to expand on these observations, we sequenced DNA of the V4 region of the 16S rRNA marker gene in a larger dataset comprising 354 samples from 51 non-T1D and 94 T1D women, including the original 96 samples. Sequences were processed with QIIME2 to obtain a feature per sample table and further analysis was performed in R (see Methods). Features were agglomerated based on a phylogenetic tree into an overall total of 349 operational taxonomic units (OTUs) with a mean ± SD of 82 ± 24 (mean ± SD) OTUs per sample (Excel file E0). Fourteen of the 25 most abundant species detected by WMS were also within the 25 most abundant OTUs in the larger 16S rRNA dataset. Moreover, one OTU had 100% sequence identity with both *Bacteroides vulgatus* and *B. dorei* and therefore these two species could not be distinguished by 16S rRNA gene sequencing.

Consistent with the metagenomic analysis, alpha diversity did not differ by T1D status in the larger 16S rRNA dataset in any trimester (Excel file E1). Also, in accord with the WMS analysis, an interaction between T1D status and time was significant for beta diversity at the genus and family taxonomic levels and borderline significant at the OTU taxonomic level (Excel file E2). Therefore, differences in beta diversity were also assessed within trimester. The gut microbiome composition differed significantly

between non-T1D and T1D women within trimester 2 and 3 at the OTU, genus and family taxonomic levels, but not within trimester 1 (Excel file E2). At the order and phylum taxonomic levels, T1D status and the interaction between T1D status and time were not significant.

Only one OTU classified to the species *Bacteroides caccae* was differentially abundant in trimester 3 (FDR=0.02) (Figure 4A; Excel file E3). Generally, the prevalence of taxa was lower with 16S rRNA sequencing than WMS. To confirm the differentially abundant taxa detected between non-T1D and T1D women by WMS, relative abundances by 16S rRNA sequencing were agglomerated at family, order and phylum taxonomic levels. Most of the taxa identified in the 16S rRNA dataset exhibited the same trend in abundance between non-T1D and T1D as observed with WMS (Figures 4B-F).

Effect of time and other factors on the gut microbiome during pregnancy

WMS revealed no significant differences in alpha diversity in non-T1D or T1D women due either to time analysed as days into gestation or by trimester, i.e., as continuous or categorical variables, respectively (Excel file E1). Due to a significant interaction between T1D status and time, differences in beta diversity between trimesters were assessed in non-T1D and T1D women separately (Excel Table E2). No significant differences in beta diversity due to trimester were observed at any taxonomic level in non-T1D or T1D women (Excel Table E2). However, significant differences were present by time analysed as days into gestation at the strain and genus levels (and borderline differences at the species and family levels) in T1D samples and at the strain, species, genus and family levels in non-T1D samples (Excel file E2).

In T1D women, differences in the abundance of specific taxa between trimesters were identified. Thus, the abundance of strain *Collinsella aerofaciens* St. GCF000169035 (FDR=0.087) decreased from trimester 1 to 2 (Figure 5; Excel file E4). *Lachnospiraceae* bacterium 8 1 57FAA St. GCF000185545 (FDR=0.04), *Akkermansia muciniphila* St. GCF000020225 (FDR=0.04), *Ruminococcus bromii* St. GCF000209875 (FDR=0.002) and unclassified strains from the species *Roseburia intestinalis* (FDR=0.04), *Oxalobacter formigenes* (FDR=0.04), *Bifidobacterium adolescentis* (FDR=0.04) and *Parabacteroides goldsteinii* (FDR=0.04) decreased from trimester 1 to 3 (Figure 5; Excel file E4).

Also, in T1D women the abundance of orders *Clostridiales* (FDR=0.011), *Coriobacteriales* (FDR=0.025), *Pasteurellales* (FDR=0.002), *Verrucomicrobiales* (FDR=0.004), *Erysipelotrichales* (FDR=0.002), *Selenomonadales* (FDR=0.002) and *Enterobacteriales* (FDR=0.002) decreased from trimester 1 to 2 (Figure 5; Excel file E4). The orders *Pasteurellales* (FDR=0.025), *Verrucomicrobiales* (FDR=0.004), *Enterobacteriales* (FDR=0.04) and *Bifidobacteriales* (FDR=0.06) decreased from trimester 1 to 3 (Figure 5; Excel file E4). Only the abundance of the order *Lactobacillales* (FDR=0.018) and an unclassified strain from species *B. massiliensis* (FDR=0.087) increased from trimester 1 to 2 (Figure 5; Excel file E4). In non-T1D women, the abundance of an unclassified strain from the species *Haemophilus parainfluenzae* (FDR=0.045) and the family *Pasteurellaceae* (FDR=0.04) decreased from trimester 1 to 3 and from trimester 2 to 3, respectively, while the abundances of the order *Bifidobacteriales* (FDR=0.05) and the phylum *Actinobacteria* (FDR=0.03) increased from trimester 1 to 3 (Figure 5; Excel file E4).

Similar to WMS, 16S rRNA sequencing revealed no differences in alpha diversity by time (Excel file E1) or in beta diversity by trimester (Excel file E2). Differences in beta diversity by time were observed in T1D women only at the family level (Excel file E2). In the differential abundance analysis, within T1D women only, the abundance of an OTU classified to the genus *Lachnospiraceae* (FDR=0.074), the family *Clostridiaceae 1* (FDR=0.047) and the phylum *Bacteroidetes* (FDR=0.005), increased from trimester 1 to 2 (Excel file E4). Family *Peptococcaceae* (FDR=0.037) and the order *Lactobacillales* (FDR=0.028) increased from trimester 2 to 3 (Excel file E4). An OTU classified to the *Oscillibacter* genus (FDR=0.009), the family *Bacteroidaceae* (FDR=0.006) and the phylum *Bacteroidetes* (FDR=0.012) increased from trimester 1 to 3 (Excel file E4). Within non-T1D women only, the orders *Lactobacillales* (FDR=0.011) and *Pasteurellales* (FDR=0.022) increased from trimester 1 to 3 (Excel file E4).

No significant associations were found between beta diversity and other factors included in the models, viz. age at conception, body mass index (BMI), parity and human leukocyte antigen (HLA) class II genotype, for either metagenomic or 16S rRNA data with the exception of parity which was significantly different only in trimester 1 at the order taxonomic level, for the 16S rRNA data (Excel file E2). As expected, non-T1D and T1D women differed in serum 1,5-AG, a marker of short-term glycemic control [24] (Table 1), but in T1D women serum 1,5-anhydroglucitrol (1,5-AG) was not related to beta diversity (Excel file E2). The frequency of pre-eclampsia was higher in the larger group of 145 women (Table 1) but was not related to beta diversity (Excel file E2). The frequency of elective Caesarean section was higher in T1D women (Table 1) but was not related to beta diversity (Excel file E2). Non-T1D and T1D women also differed in total carbohydrate but not fiber intake (Table 1). In the larger group of 145 pregnancies, total carbohydrate intake was lower in those with T1D (P=0.016), most likely reflecting dietary advice. Based on 16S data, interactions were found between carbohydrate and fiber intake and time; therefore, beta diversity was assessed per trimester. Carbohydrate intake was related (borderline P-values greater than 0.05 but less than 0.1) to beta diversity at family, order and phylum levels only in trimester 2. Fiber intake was significantly related to beta diversity at genus and family levels only (borderline significance for order and phylum taxonomic levels) in trimester 3 (Excel file E2). No relationships between either carbohydrate or fiber intake and beta diversity were found with WMS data.

Functional annotation of gut microbiome taxa

For functional profiling, metagenomic sequences were processed with HUMAnN2. Sequences were annotated, gene abundances calculated and regrouped into KO (Kegg Orthology) and MetaCyc reaction functional categories, and complete metabolic pathways were quantified obtaining a total of three functional profiles. A total of 5,480 KO, 3,014 metaCyc reactions and 420 complete pathways, were obtained. No significant differences in richness were detected between T1D and non-T1D women or across pregnancy in any of the three functional profiles (Excel file E1). On the other hand, in the analysis of beta diversity, the interaction between T1D status and time was significant. Therefore, differences between non-T1D and T1D women were assessed in each trimester and were only significant in trimester 3 for pathways, metaCyc reactions and KO functions (Figures 6; Excel file E2).

Differential abundance analysis revealed significant differences between non-T1D and T1D in specific pathways and enzymes only within trimesters 1 and 3 (Figure 7; Excel files E5-7). A comprehensive list of differentially abundant pathways, KOs and MetaCyc reactions is presented in Supplementary Excel files E5-10). Of interest, a pathway (CMP-3-deoxy-D-manno-octulosonate biosynthesis I) and two enzymes (K02852 and K01791) involved in the synthesis of bacterial lipopolysaccharides (LPSs) were enriched in T1D women (Figure 7; Excel files E5 and E7), with contributions from Alistipes shahii, B. thetaiotaomicron, B. ovatus, B. vulgatus, B. cellulosilyticus, Parabacteroides distasonis, Bacteroides uniformis among others (Figure S4). In addition, a KO and a MetaCyc reaction that were increased in T1D women in trimester 3 were involved in biofilm formation (K04334) and the synthesis of the antibiotic mannopeptimycin (K00815), respectively, both being contributed solely by E. coli (Figures 7 and S5; Excel file E5). Table 2 summarizes these and other key pathways and enzymes differentially abundant in T1D women.

Pathways and enzymes related to the biosynthesis and/or transport of branched chain amino acids (BCAAs; two pathways and K00826), short chain fatty acids (SCFAs) acetate, propionate and butyrate; K00171, K13788 and K00209, vitamins B1 (thiamine; K00941, K04487 and K00878), B5 (pantothenate; K00997), B6 (pyridoxine; K00868, K06215 and K08681), B7 (biotin; K16784) and B9 (folate; K13940), as well as the degradation of starch (K01208), were decreased in T1D women relative to the non-T1D (Figure 7; Excel file E5-E7) due to decreased contributions from *Eubacterium rectale, E. eligens, Faecalibacterium prausnitzii, Akkermansia muciniphila, Bifidobacterium adolescentis, Bifidobacterium longum, Bifidobacterium bifidum* and *Lachnospiraceae* bacterium 5 1 63FAA (Table 2; Figures S6-S11).

Functional differences were also present between trimesters (Excel files E8-E10). Most involved a decrease in abundance from trimester 1 to 3. Thus, in T1D women only the abundance of beta-N-acetylhexosaminidase (K01207) involved in the degradation of mucin decreased significantly from trimester 1 to 3, associated with a decrease in *A. muciniphila, R. hominis, B. longum* and *B. adolescentis* (Figures 7 and S12; Excel file E8). Enzymes involved in the synthesis of acetate (K01512), butyrate (K00248 and K00209), acetyl-CoA (K13788; a precursor of acetate and butyrate) and vitamins B1 (K00878) and B5 (K00997) also decreased from trimester 1 to 3 in T1D women only, while pyruvate ferredoxin oxidoreductase (K00171), involved in the conversion of pyruvate into acetyl-CoA, increased from trimester 1 to 3 in non-T1D women only (Figures 7 and S12-S14; Excel file E8).

Discussion

The gut microbiome in pregnancy in healthy women was previously analysed in two studies that reported conflicting findings [5, 6], but has not been studied in pregnancy in the context of T1D. We characterized the taxonomic composition and annotated functions of the gut microbiome across pregnancy in women without and with T1D. Although we found no significant differences in alpha or beta diversity between trimesters, or in alpha diversity between non-T1D and T1D women, we observed changes in the relative abundance of specific taxa across pregnancy that were often opposite in directions in non-T1D and T1D women, particularly in trimester 3. Generally, pregnant women with T1D exhibited a more pro-

inflammatory and catabolic gut microbiome profile. The taxonomic differences between T1D and non-T1D women were reinforced by functional annotation of WMS data, revealing differential abundance in enzymes and pathways as pregnancy progressed. These differences could not be attributed to demographic or other factors including diet. It is important to emphasize that our findings are based on DNA analysis and might not necessarily reflect changes at the RNA or protein level. Nevertheless, the functional consequences of the dysbiosis we observed in women with T1D could contribute to the known increase in maternal and fetal complications in T1D. This has implications for improving the management of women with T1D in pregnancy.

As a discovery dataset, we first employed WMS on 96 fecal samples collected from 15 non-T1D and 16 T1D women in each trimester of pregnancy. To substantiate our observations, we then performed next-generation amplicon sequencing of the 16S rRNA gene V4 region on a larger sample set from 51 non-T1D and 94 T1D women. Koren et al [5] and DiGiulio et al [6] analysed fecal samples from healthy pregnancies by pyrosequencing the 16S rRNA gene V1-V2 and V3-V5 regions, respectively, but arrived at different conclusions. Koren et al [5] compared single samples from trimesters 1 and 3 from 91 pregnancies and reported a decrease in alpha diversity and 'remodelling of the gut microbiome' by the third trimester, specifically a decrease in the abundance of taxa in the genus Faecalibacterium that generate anti-inflammatory butyrate [17] and an increase in taxa in the phylum Proteobacteria recognised to be pro-inflammatory [25]. DiGiulio et al [6], on the other hand, by weekly sampling from 49 women found no significant changes in diversity or composition across pregnancy. Similar to DiGiulio et al [6] we observed no differences in alpha or beta diversity, but detected changes in the relative abundance of specific taxa across pregnancy with progression to a more pro-inflammatory microbiome particularly in women with T1D. In non-T1D women, similar to Koren et al [5], we observed an increase in the phylum Actinobacteria, in particular the order Bifidobacteriales, from trimester 1 to 3. However, in contrast, we observed a decrease in the abundance of taxa in the Proteobacteria.

The ratio of Firmicutes/Bacteroidetes phyla, regarded as an index of gut health in some publications [12], declined between the first and third trimesters in T1D but not non-T1D women. In examining differentially abundant taxa we observed three main patterns: 1) taxa that were differentially abundant between non-T1D and T1D women across all trimesters, 2) taxa that decreased in T1D women only as pregnancy progressed, and 3) taxa that were similar in abundance in non-T1D and T1D women in trimester 1 but decreased or increased to be differentially abundant in trimester 3. In the first category, in which taxa were differentially abundant between non-T1D and T1D women across all trimesters, the order Enterobacteriales, the family Enterobacteriaceae and six species including Escherichia coli were increased in T1D compared to non-T1D women across all trimesters. Enrichment in Enterobacteriaceae, in particular E. coli, is associated with intestinal inflammation [26, 27]. Moreover, an increase in these facultative anaerobic bacteria may displace obligate anaerobic bacteria that produce SCFAs, further accentuating inflammation. In keeping with this, T1D women had a decrease in the abundance of the family Prevotellaceae, which produces succinate and the SCFAs propionate and acetate known to be associated with improved glucose metabolism [18, 28, 29]. In T1D women, E. coli contributed to an increased abundance of the major curlin subunit (K04334) involved in biofilm formation and of a tyrosine

aminotransferase (EC. 2.6.1.107) involved in the biosynthesis of mannopeptimycin, a peptide antibiotic active on gram-positive bacteria. Bacterial biofilms confer increased tolerance to antibiotics and host immune responses [30] and may provide E. coli with a protective advantage over other more sensitive bacteria that compete for the same resources in the gut. Moreover, mannopeptimycin may provide E. coli with a competitive advantage to displace gram-positive butyrate-producing bacteria [17] such as B. adolescentis and R. bromii, which we observed to be decreased in T1D.

In the second category, taxa decreased in T1D women only or in contrast to non-T1D women demonstrated no increase as pregnancy progressed. We observed a decrease in Roseburia intestinalis (and the order Clostridiales) and Akkermansia muciniphila (and the order Verrucomicrobiales) from trimester 1 to trimester 3. Deficiency of these taxa is associated with gut inflammation and impaired gut barrier function. R. intestinalis produces butyrate [31, 32]. The abundance of A. muciniphila correlates with richness of the gut microbiome and protection from type 1 diabetes in the NOD mouse model [33] and inversely with markers of dysmetabolism [34]. Early life treatment with vancomycin propagated A. muciniphila and reduced diabetes incidence in the NOD mouse [33]. The benefits of A. muciniphila relate to its ability to promote host production of anti-inflammatory lipids and mucus, and glucagon-like peptides which regulate glucose homeostasis [35]. Furthermore, by also degrading mucins, A. muciniphila produces oligosaccharides, and acetate and propionate, which together then stimulate mucus production and enhance epithelial integrity [36]. Beta-N-acetylhexosaminidase (K01207), which degrades mucin [37], was associated mostly with A. muciniphila, and decreased in women with T1D from trimester 1 to 3. Women with T1D also had a decrease in the abundance of pathways involved in the synthesis of the BCAAs, leucine, isoleucine and valine, contributed mainly by F. prausnitzii, A. muciniphila, B. adolescentis and some unidentified bacteria. BCAAs have a wide range of beneficial anabolic functions including promotion of glucose utilization, protein synthesis, intestinal epithelial integrity and mucin production, milk protein production and immune function [38]. Their deficiency could therefore be detrimental to both mother and fetus in the T1D pregnancy and ENDIA will provides the opportunity to investigate this.

In the third category (taxa that became differentially abundant by trimester 3) we observed that the family Bacteroidaceae and five species, four of which belonged to the genus Bacteroides (B. caccae, B. uniformis, B. salyersiae and B. faecis) and Parabacteroides distasonis, increased in T1D and decreased in non-T1D women. Bacteroidaceae and its genus Bacteroidess [11, 12], and Parabacteroides distasonis [39], were reported to be significantly more abundant in children with islet autoimmunity compared to healthy controls. The CMP-3-deoxy-D-manno-octulosonate biosynthesis I complete pathway and enzymes UDP-N-acetylglucosamine 2-epimerase (K01791) and P-N-acetyl-D-mannosamino uronate:lipid I N-acetyl-D- mannosamino uronosyltransferase (K02852) involved in LPS biosynthesis, contributed mostly by bacteria from the genus Bacteroides (including B. caccae) and by Parabacteroides distasonis, increased in T1D and decreased in non-T1D women throughout pregnancy, becoming differentially abundant by trimester 3. Immunostimulatory LPS could contribute to the pro-inflammatory gut microbiome, again potentially increasing risk of complications for mother and fetus in the T1D pregnancy [40]. Within this third category, we also observed that the species Bifidobacterium adolescentis and Ruminococcus bromii, and the order Bifidobacteriales, decreased in T1D by the third trimester. In addition,

the phylum Actinobacteria (spp. B. adolescentis) and the order Clostridiales (spp. Ruminococcus bromii), decreased only in women with T1D. Bifidobacterium adolescentis degrades resistant starches into lactate, acetate and malto-oligosaccharides, which then act as substrates to 'cross-feed' butyrate-producing bacteria [41]. Ruminococcus bromii is a 'keystone' degrader of resistant starches to butyrate [42]. Our functional analysis revealed that the enzyme cyclomaltodextrinase (K01208), involved in the degradation of starch into maltodextrin, contributed mainly by Bifidobacterium adolescentis, decreased in T1D women as pregnancy progressed and was significantly less abundant compared to non-T1D women by trimester 3. Enzymes involved in the conversion of pyruvate to acetyl-CoA (pyruvate ferredoxin oxidoreductase [K00171] and phosphate acetyltransferase [K13788]) and acetate (acylphosphatase [K01512]) decreased during pregnancy only in T1D. For the synthesis of butyrate, acetyl-CoA is converted into butyryl-CoA ultimately by butyryl-CoA dehydrogenase (K00248) [42] and trans-2-enoyl-CoA reductase (K00209) [43], both of which also decreased in T1D women by trimester 3. Thus, different bacterial species with diverse biochemical pathways implicated in the production of acetate and butyrate were decreased in T1D compared to non-T1D women, especially towards the end of pregnancy.

We also detected a decrease in T1D women over pregnancy of gut microbiome functions associated with the metabolism of the B-group vitamins B1 (thiamine), B5 (pantothenate), B6 (pyridoxine), B7 (biotin) and B9 (folate). Mammals cannot synthesize B-group vitamins and must acquire them from the diet or gut microorganisms [44]. All B-group vitamins contribute to regulation of immunity-inflammation and their deficiency may be associated with inflammatory disorders [45]. In addition, within the tricarboxylic acid (TCA) or Krebs cycle, which fuels ATP production via acetyl-CoA, B1 is a cofactor for pyruvate dehydrogenase to catalyse formation of acetyl CoA, B5 is a precursor of coenzyme A (CoA) and B7 is a cofactor for acetyl-CoA [44, 45]. Succinate, a precursor of propionate and butyrate, is produced by the TCA cycle [46]. Thus, the lower abundance of B1, B5 and B7 could also contribute to the relative deficiency of SCFAs in T1D women. In addition to modulating immune responses, B9 (folate) is essential for the growth and development of the fetus; the association of folate deficiency in early pregnancy with neural tube defects is well-established [47]. However, pregnancy in women with T1D carries an increased risk for a range of fetal malformations [48] and is also associated with an increased risk of pre-eclampsia in which folate deficiency has been implicated [49, 50]. B6 deficiency has been associated with inflammatory markers in population-based studies [51] and is reported to be common in T1D [52, 53]. Of interest therefore, we found that the key enzymes in B6 synthesis, pyridoxine kinase (K00868) and pyridoxal 5'-phosphate synthase subunits pdxT and pdxS (K08681 and K06215), were decreased in T1D women across pregnancy. The majority of both non-T1D and T1D women reported taking multi B-group vitamins from early pregnancy (Table 1) and there was no difference in the circulating concentrations of vitamins B6 or B9 between non-T1D and T1D women (Table 1). However, the relative deficiency of these vitamin-synthesizing bacteria in T1D women might not only compound other changes in the gut microbiome but underscores the importance of dietary supplementation in this group of women.

Our findings show that the composition of the gut microbiome not only changes across pregnancy but in a distinct way in women with T1D. By the third trimester, T1D women exhibit a more pro-inflammatory and catabolic gut microbiome profile, reflected by a deficiency of SCFA-producing bacteria, as well a

decrease in B-group vitamin-synthesizing bacteria. These changes could impair epithelial barrier function and contribute to systemic inflammation, a risk marker for pre-eclampsia, which is more common in T1D [54], as well as accentuating the insulin resistance of later pregnancy. Furthermore, a pro-inflammatory gut microbiome in the mother may impact the infant postnatally. In an elegant study in mice, Aguero et al [55] found that transient exposure to an auxotrophic E. coli mutant in the intestine of germ-free mothers in pregnancy accentuated innate immune development in the intestine of their germ-free offspring. This effect was mediated by the transfer, in part via maternal antibodies, of a range of E. coli products across the placenta and in the mother's serum and milk. Thus, with a single gut bacterium, the mother conditioned the immune system of her offspring, before their exposure to the external environment. This was proposed to be an adaptive mechanism to cope with the known higher risk of infection in neonates [55]. The question arises therefore could T1D mothers who display an increased abundance of E. coli and other LPS-producing bacteria induce stronger conditioning of innate immunity in their offspring? Moreover, if T1D is triggered by an infectious agent [56] this mechanism could account for the lower transmission of T1D from maternal compared to paternal probands [57].

Conclusions

The gut microbiome changes across normal pregnancy. However, in women with T1D these changes are distinct from other pregnancies. They include an increase in pro-inflammatory bacteria, a decrease in anti-inflammatory bacteria and a decrease in bacteria that synthesize essential vitamins and metabolites. This gut microbiome dysbiosis could contribute to the known increased risks for mother and fetus in the T1D pregnancy and activate innate immunity in the fetus to modify its immune reactivity postnatally. In T1D women in pregnancy the relative deficiency of gut bacteria that synthesize a range of anti-inflammatory compounds and B-group vitamins has implications for management of the T1D pregnancy. It remains to be determined if intervention by safe means to promote a less proinflammatory gut microbiome, e.g. with dietary fiber and other pre-biotics, could decrease the higher risk of maternal and fetal complications in the T1D pregnancy.

Methods

Participants, study design and sample collection

This study involved 145 pregnancies in women participating in the ENDIA pregnancy-birth cohort study [22], 51 in healthy women with no history of gestational diabetes and 94 in women with established T1D on daily insulin treatment. The main criterion for participation in ENDIA was an unborn child with a first-degree relative with T1D. Table 1 provides summary statistics for participant pregnancies, on 139 women with 145 pregnancies (six sibling pairs) and 147 babies (two sets of twins). Therefore, six women were included twice in the study population (each with two different pregnancies). The unit of observation is the *pregnancy*, and therefore observations from the same mother but different pregnancies have been included as separate observations, as many characteristics might change between pregnancies. Trimesters were categorised according to gestational age: T1 0-99 days; T2 100-196; T3 197-274.

Women provided written informed consent and were enrolled into the study between 2013-2016 at one of eight clinical sites. Up to three study visits occurred during pregnancy, ideally one in each trimester. The study was approved by a Human Research Ethics Committee (HREC) at each clinical site, with the Women's and Children's Health Network HREC in Adelaide acting as the lead HREC under the Australian National Mutual Acceptance Scheme (reference number HREC/16/WCHN/066). ENDIA is registered on the Australia New Zealand Clinical Trials Registry (ACTRN1261300794707).

Maternal and paternal demographics, medical history, past-pregnancy history, pre-pregnancy weight, assisted conception status, and plurality of pregnancy were recorded at the first opportunity. Standardized questionnaires were offered at each pregnancy visit to record pregnancy complications, antibiotic and supplement usage, maternal and household smoking, household composition and pet ownership. Maternal diet during pregnancy was measured at the third trimester visit using a validated 74 item food frequency questionnaire (Dietary Questionnaire for Epidemiological Studies version 2) [58]. Women were advised to take multi B-group vitamin supplements from as early as possible in pregnancy. Serum 1,5-AG, a measure of glucose control in pregnancy [24], was measured by GlycoMark (Nippon Kayaku Co. Ltd., New York, NY, US) in a single batch. Serum vitamin D3 was measured with a Liaison Analyser by the DiaSorin method (DiaSorin, Turin, Italy). Plasma vitamin B6 was measured by the Chromsystems HPLC-based assay (Chromsystems Instruments & Chemicals, Gräfelfing, Germany). Serum vitamin B9 (folate) was measured by the chemiluminescent microparticle folate binding protein-based ARCHITECT assay (Abbott Laboratories Abbott Park, IL USA).

HLA DR typing was performed on DNA in saliva collected with OG-500 Oragene DNA tubes (DNA Genotek, Ontario, Canada) by TaqMan-based PCR-typing and imputation from three single nucleotide polymorphisms (rs3104413, rs2187668 and rs9275495), as described previously [59].

For continuous responses, where appropriate the summary tables present the mean and standard deviation derived from fitting a linear mixed model. The model fit for each continuous response adjusts for the fact that the observations from mothers with more than one pregnancy are not fully independent but may be correlated. For some response variables, the assumption of normally distributed residuals was not met. In these analyses, the response variable was transformed using a square root or log transformation, as appropriate. For transformed responses, the back transformed means and approximated standard deviations are presented. A Wald's test is used to determine whether the groups are significantly different.

For categorical responses, summary tables show numbers and percentages. The percentage was calculated using the total number of pregnancies or samples as the denominator. To determine whether the distribution of observations between groups for categorical data were similar or not, a generalized linear mixed model was fitted, with a random effect for mother. Such models adjust for correlated mother observations. To determine whether groups were significantly different, the change in deviance of the final model (i.e. a likelihood ratio test), which includes and excludes the treatment term, was examined. A pre-set p-value of 0.05 was used as a cut-off for determining statistical significance for all models. Data

analysis was performed in R (R Core Team, 2018; [60]), with the R packages lme4 v1.1.21 [61], car v3.0.3 [62], predictmeans v1.0.1 [63] and nnet v7.3.12 [64].

Fecal samples were collected in accordance with our validated collection-processing-storage method [65]. Briefly, samples were captured in a toilet using the Easy Sampler device (Co-Vertec Ltd, Waterlooville, UK) then transferred into a sterile 70 mL collection jar. Participants were instructed to store the sample in the refrigerator prior to transport to the laboratory in an insulated bag within 24 h. Samples were divided into aliquots with a sterile spatula in a Biosafety Level 2 cabinet, then stored at -80°C. A total of 354 fecal samples were collected from the 145 pregnancies with either two or three samples collected longitudinally in each pregnancy (Figure S1).

DNA extraction, whole metagenome shotgun sequencing and 16S rRNA gene amplicon sequencing

DNA was extracted from stool samples at the Walter and Eliza Hall Institute of Medical Research (WEHI) with the MoBio PowerSoil kit (MoBio Laboratories, Carlsbad, CA) as per manufacturer's instructions. WMS libraries were generated and sequenced with the 2x150 bp paired-end chemistry on an Illumina NovaSeq 6000 (Illumina, San Diego, California, USA) sequencer at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia. www.ramaciotti.unsw.edu.au). The hypervariable region 4 (V4) of the 16S rRNA gene was amplified using the universal bacterial/archaeal primers 515F/806R. Detailed methods used for 16S amplification and sequencing are as previously described [65].

Sequence processing and generation of taxonomic and functional profiles

Whole metagenome sequencing data were quality controlled, and reads aligning to the human genome were removed using KneadData (v0.6.1) [23]. For the functional analysis, filtered reads classified using Kraken2 with the standard database [66], were further processed using HUMAnN2 (v0.11.1) [67] with the UniRef90 database to generate functional annotations (i.e. genes and metabolic pathways) and define the metabolic potential of the microbial communities. A functional profile (i.e. function-per-sample counts matrix) for metaCyc [68] complete metabolic pathways was obtained. In addition, two functional profiles were generated by grouping genes into KO [69] and MetaCyc-reactions functional categories using the humann2_regroup_table command. As part of the HUMAnN2 pipeline, MetaPhLan2 (v 2.7.5) [70] was used on the reads filtered with KneadData, to detect and quantify individual species with a library of clade-specific markers (ChocoPhlAn database) and generate whole-metagenome-based profiles at strain, species, genus, order and phylum taxonomic levels.

The 16S rRNA gene amplicon sequence data from six sequencing runs were separately processed using QIIME2 (version 2017.12) [71]. Briefly, Q2-dada2 denoise-paired [72] was used to construct features (amplicon sequence variants; ASV). Denoised feature matrices, i.e. features and their counts per sample, and representative sequences obtained from the six different sequencing runs were merged and a taxonomic profile was obtained. A naive Bayes classifier pre-trained with the Silva 16S rRNA reference sequence database [73] (v119; clustered at 99% similarity) was used to taxonomically classify representative features and a phylogenetic tree was built.

Comparative diversity and statistical analyses

Taxonomic and functional profiles were imported into the phyloseq [74] package in R [60]. The features from the 16S rRNA-based-taxonomic profile were agglomerated into OTUs based on a phylogenetic tree (i.e. cophenetic distance of 0.03, used as proxy for the difference between species) with the phyloseq function tip_glom. Due to the difference in the mean library size between sequencing runs, samples were normalized, i.e. subsampled without replacement, to the size of the smallest sample (4,892 sequences) using the phyloseq function rarefy_even_depth.

Alpha diversity (diversity within microbial communities) was obtained from the number of observed taxa (richness) using the function estimate_richness from the R package phyloseq. For testing differences in alpha diversity between groups of interest, GEEs [75] were applied using the R function geeglm from package geepack v1.2-1 ([76]; parameter family set to default “Gaussian”) to account for possible correlation of multiple measurements within a woman over time. The default empirical (robust or ‘sandwich’) estimator was used to ensure that estimates are robust to misspecification of the correlation structure. The model used for the regression included T1D status and time (i.e. two models were tested considering time as a continuous [gestational days] or categorical [trimesters] variable) as well as their interaction term (T1D x days or trimester; to test if differences in alpha diversity between T1D and non-T1D women change across days or trimesters) and was adjusted for sample processing batches (which includes sequencing run), conception age, BMI, parity and HLA type. Mean-centred values were used for gestational days, conception age and BMI to ensure that the model coefficients are meaningful.

Beta diversity (diversity between microbial communities) was determined with phyloseq (function distance, method=“bray”). This function calculates Bray-Curtis coefficients, which measure the distance between communities based on the taxa/functions that they contain and their abundances. Differences in beta diversity were assessed by PERMANOVA using Bray-Curtis dissimilarities with the Adonis function from the vegan [77] R package. For tests that included multiple samples across trimesters from the same participant (i.e. longitudinal analysis) a modified version of Adonis, which performs a RMA-PERMANOVA test [27] was employed. This statistical model included T1D status and time with their interaction adjusted as in the alpha diversity model. In addition, interactions between time and other factors were also tested as described in the results section. When an interaction was either significant (i.e. FDR<0.05) or borderline significant (i.e. FDR greater than 0.05 and less than 0.1) statistical analysis was performed within trimester (i.e. when testing for differences between non-T1D and T1D women) or by separating data from non-T1D women and T1D women (i.e. when testing for differences in time).

Differential abundance of taxa and functions was analysed with the R package limma [78]. Library sizes were normalized using the trimmed mean of log expression ratios (TMM) method [79]. Counts were transformed to log2-counts per million (CPM) with associated precision weights using voom [80]. In the models, a consensus correlation was estimated with the limma duplicateCorrelation function in which “women IDs” are blocks and the consensus correlation is incorporated to account for multiple measurements while estimating statistics using linear models with the lmFit function and empirical

Bayes moderated t statistics. For the taxonomic analysis, a correction was applied in limma to account for high sparsity of data (i.e. the large number of zeroes) that leads to underestimation of the “genewise” variances [81]. Differential abundance analyses of gene categories and metabolic pathways from HUMAnN2 was performed similarly to that of taxa but without the sparsity correction in limma. Since we have samples for all possible combinations of T1D status and trimester, this is a factorial design. Therefore, in order to build our model, factors T1D status and trimester were combined into a single factor with six levels and the comparisons of interests were defined as contrasts. P-values were adjusted with the Benjamini and Hochberg method to control the FDR. FDR $\leq$ 0.05 were considered significant. FDR greater than 0.05 but less than 0.1 were considered borderline. Taxa or functions significantly different with an abundance logFC greater than 0.5 or less than -0.5 and present in at least 50% of the samples in either of the groups being compared were regarded as biologically significant.

Abbreviations

AG
anhydroglucitrol
ASV
amplicon sequence variants
BMI
body mass index
BCAA
branched chain amino acid
CoA
coenzyme A
CPM
counts per million
ENDIA
environmental determinants of islet autoimmunity
FDR
false discovery rate
GEE
generalized estimating equations
HLA
human leukocyte antigen
HPLC
high performance liquid chromatography
HREC
human research ethics committee
KO
Kegg orthology

LCBD
local contribution to beta diversity
LogFC
log2 fold-change
LPS
lipopolysaccharide
NOD
non-obese diabetic
OTU
operational taxonomic unit
PCoA
principal components analysis
RMA-PERMANOVA
repeated measure-aware permutation analysis of variance
SCFA
short chain fatty acid
SD
standard deviation
T
trimester
TCA
tricarboxylic acid
T1D
type 1 diabetes
TMM
trimmed mean of log expression ratios
WEHI
Walter and Eliza Hall Institute of Medical Research
WMS
whole metagenomic sequencing

Declarations

The authors declare they have no conflicts of interest.

Ethics approval and consent to participate

The ENDIA study is approved by Women's and Children's Health Network Human Research Ethics Committee (HREC) under the National Mutual Acceptance Scheme for conduct in South Australia, Victoria, New South Wales, and Queensland (HREC/16/WCHN/66). Conduct in Western Australia is approved by the Child and Adolescent Health Service HREC (RGS0000002402). The investigators and

study staff conduct the study in accordance with International Conference on Harmonisation Good Clinical Practice guidelines, the Declaration of Helsinki, the Australian National Statement on Ethical Conduct in Human Research and the Australian Code of the Responsible Conduct of Research. All participants provided written informed consent and were free to withdraw from the study at any time.

Consent for publication

Not applicable

Availability of data and materials

The demultiplexed raw datasets supporting the conclusions of this study can be accessed in the NCBI SRA https://www.ncbi.nlm.nih.gov/sra (project number PRJNA604850). All the commands used to run QIIME2, the python commands used to run HUMAnN2 and the R code used to perform statistical analyses are available at GitHub (https://github.com/PapenfussLab/RothSchulze_pregnancy-gut-microbiome-T1D) as R markdown coding and knitr html files along with the necessary R objects which contain taxonomic and functional profiles with metadata.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

AJR-S performed bioinformatic analyses under the supervision of ATP and LCH, and wrote the manuscript with LCH;

MASP contributed to study conception and design, compiled metadata and edited the manuscript;

KMN performed DNA extraction and sequencing;

HO prepared maternal summary tables and advised on statistical analysis;

RLT analysed dietary and physical activity data;

PJV contributed to study design, recruitment and sample collection;

MEC contributed to study design, participant recruitment and sample collection;

WDR contributed microbiology expertise and edited the manuscript;

EAD contributed to participant recruitment and sample collection;

MH contributed to participant recruitment and sample collection;

GS contributed to participant recruitment and sample collection;

PCG contributed to participant recruitment and sample collection;

JMW contributed to participant recruitment and sample collection;

AH contributed to participant recruitment and sample collection;

GM analysed HLA types

NGB contributed to bioinformatic analysis;

GKS contributed to bioinformatic analysis;

ATP contributed to bioinformatic analysis;

JJC contributed to study design, participant recruitment and sample collection, and edited the manuscript;

LCH designed and supervised the study, analysed data and wrote the manuscript with AJR-S.

All authors reviewed the manuscript.

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Tables

Table 1. Summary of characteristics of the non-T1D and T1D pregnancies.

	Whole metagenomic sequencing			16S rRNA sequencing		
Characteristics	Control	T1D	P-value **	Control	T1D	P-value **
Overall number of samples	48	48		119	235	
Trimester 1	11 (22.9)	12 (25.0)		18 (15.1)	38 (16.2)	
Trimester 2	20 (41.7)	20 (41.7)		51 (42.9)	107 (45.5)	
Trimester 3	17 (35.4)	16 (33.3)		50 (42.0)	90 (38.3)	
All three trimesters (%pregnancies)	11 (68.8)	12 (75.0)		16 (31.4)	30 (31.9)	
All three trimesters (%samples)	11 (22.9)	12 (25.0)		16 (13.4)	30 (25.2)	
Gestational age at stool sample (days): Mean (SD)						
Trimester 1	75.4 (16.5)	75.3 (16.5)		75.4 (15.0)	78.3 (15.0)	
Trimester 2	148.9 (25.4)	148.0 (25.4)		156.5 (29.4)	150.3 (29.4)	
Trimester 3	242.2 (16.7)	231.8 (16.7)		243.9 (17.3)	233.1 (17.3)	<0.001
MATERNAL CLINICAL AND PREGNANCY CHARACTERISTICS						
Overall number of pregnancies	16	16		51	94	
Age at conception: Mean (SD)						
Maternal	31.5 (3.4)	32.0 (3.3)		33.0 (4.7)	31.6 (4.7)	
Paternal	33.9 (5.3)	32.5 (4.9)		34.8 (5.4)	32.6 (5.0)	0.014
Paternal: Missing	1 (6.3)	1 (6.3)		1 (2.0)	2 (2.1)	
Assisted Conception: n (%)	1 (6.3)	1 (6.3)		4 (7.8)	12 (12.8)	
Twin Pregnancy: n (%)	0 (0.0)	0 (0.0)		1 (2.0)	1 (1.1)	
Nulliparous: n (%)	6 (37.5)	7 (43.8)		15 (29.4)	52 (55.3)	
Preeclampsia: n (%)	0 (0.0)	1 (6.3)		1 (2.0)	15 (16.0)	
Group B Streptococcus: n (%)	4 (25.0)	1 (6.3)		8 (15.7)	5 (5.3)	
GU tract infections: n (%)	2 (12.5)	3 (18.8)		2 (3.9)	7 (7.4)	
Maternal Pre-Pregnancy BMI: Mean (SD)	24.3 (5.0)	26.2 (4.8)		25.2 (5.6)	26.5 (5.6)	
Maternal underweight (<18.5): n (%)	0 (0.0)	0 (0.0)		0 (0.0)	2 (2.1)	
Maternal normal weight (18.5-24.9): n (%)	11 (68.8)	7 (43.8)		30 (58.8)	38 (40.4)	
Maternal overweight weight (25-29.9): n (%)	2 (12.5)	5 (31.3)		9 (17.6)	30 (31.9)	
Maternal obese (>30): n (%)	3 (18.8)	4 (25.0)		12 (23.5)	24 (25.5)	
Paternal Pre-Pregnancy BMI: Mean (SD)	28.4 (4.8)	29.0 (4.8)		28.6 (3.9)	27.0 (3.9)	
Paternal underweight (<18.5): n (%)	0 (0.0)	0 (0.0)		0 (0.0)	1 (1.1)	
Paternal normal weight (18.5-24.9): n (%)	2 (12.5)	2 (12.5)		4 (7.8)	16 (17.0)	
Paternal overweight weight (25-29.9): n (%)	4 (25.0)	4 (25.0)		14 (27.5)	23 (24.5)	
Paternal obese (>30): n (%)	3 (18.8)	5 (31.3)		7 (13.7)	15 (16.0)	
Paternal: Missing: n (%)	7 (43.8)	5 (31.3)		26 (51.0)	39 (41.5)	
Gestational Weight gain (kg): Mean (SD)	12.9 (4.3)	11.5 (4.2)		12.7 (5.4)	12.3 (5.3)	
Gestational Weight gain (kg): Missing: n (%)	0 (0.0)	1 (6.3)		6 (11.8)	8 (8.5)	
DEMOGRAPHICS						
Born in Australia: n (%)						
Yes	14 (87.5)	9 (56.3)		41 (80.4)	67 (71.3)	
Unknown	0 (0.0)	1 (6.3)		0 (0.0)	1 (1.1)	
Education beyond High School: n (%)						
Yes	12 (75.0)	13 (81.3)		42 (82.4)	74 (78.7)	
Unknown	0 (0.0)	0 (0.0)		0 (0.0)	1 (1.1)	
Lives in a metro area: n (%)	14 (87.5)	16 (100.0)		48 (94.1)	87 (92.6)	
SEIFA IRSD						
Quintile 1	1 (6.3)	0 (0.0)		3 (5.9)	6 (6.4)	
Quintile 2	2 (12.5)	2 (12.5)		4 (7.8)	11 (11.7)	
Quintile 3	4 (25.0)	1 (6.3)		15 (29.4)	19 (20.2)	
Quintile 4	2 (12.5)	5 (31.3)		11 (21.6)	22 (23.4)	
Quintile 5	7 (43.8)	8 (50.0)		18 (35.3)	36 (38.3)	
Maternal smoking: n (%)	0 (0.0)	0 (0.0)		3 (5.9)	1 (1.1)	
Household smoking: n (%)	1 (6.3)	1 (6.3)		6 (11.8)	17 (18.1)	
Adults in house: n (%)						
One	0 (0.0)	2 (12.5)		0 (0.0)	2 (2.1)	
Two	16 (100.0)	14 (87.5)		48 (94.1)	84 (89.4)	

More than 2	0 (0.0)	0 (0.0)		3 (5.9)	8 (8.5)	
Children in house: n (%)						
None	6 (37.5)	7 (43.8)		15 (29.4)	52 (55.3)	
One	4 (25.0)	4 (25.0)		17 (33.3)	28 (29.8)	
Two	3 (18.8)	5 (31.3)		11 (21.6)	13 (13.8)	
More than 2	3 (18.8)	0 (0.0)		8 (15.7)	1 (1.1)	<0.001
Furred pets ownership: n (%)	10 (62.5)	8 (50.0)		32 (62.7)	53 (56.4)	
DIET AND PHYSICAL ACTIVITY						
Diet: Mean (SD)						
Energy/day (kJ)	6917.1 (2551.8)	5711.8 (2318.8)		6852.9 (2245.8)	6536.0 (2160.6)	
Fat (g)	74.1 (29.7)	62.3 (26.5)		71.5 (27.2)	72.9 (27.0)	
Protein (g)	81.8 (31.1)	70.3 (28.1)		79.1 (27.7)	80.4 (27.6)	
Carbohydrate (g)	169.3 (63.2)	137.2 (63.2)		168.9 (58.6)	144.3 (53.4)	0.016
Fibre (g)	19.3 (6.8)	17.1 (6.8)		18.6 (5.9)	18.4 (5.8)	
Diet: Missing: n (%)	0 (0.0)	0 (0.0)		4 (7.8)	10 (10.6)	
Consumed Alcohol: n (%)						
Yes	3 (18.8)	3 (18.8)		13 (25.5)	21 (22.3)	
Unknown	0 (0.0)	0 (0.0)		4 (7.8)	10 (10.6)	
Metabolic equivalent of task (MET) (h/wk): mean (SD)	273.4 (108.5)	272.6 (106.9)		253.1 (110.2)	241.8 (103.7)	
BIOLOGICAL DATA						
HbA1c (%) in samples						
Trimester 1: Median (IQR)		6.8 (1.6)			6.8 (1.4)	
Trimester 2: Median (IQR)		6.0 (1.2)			6.4 (1.1)	
Trimester 3: Median (IQR)						
Trimester 1: Missing		1 (8.3)			7 (18.4)	
Trimester 2: Missing		2 (10.0)			31 (29.0)	
Trimester 3: Missing		16 (100.0)			90 (100.0)	
1,5-anhydroglucitol (AG) (ug/mL) in samples						
Trimester 1: Median (IQR)	14.1 (13.1)	3.4 (1.5)		14.1 (11.6)	3.3 (3.0)	
Trimester 2: Median (IQR)	11.5 (4.9)	2.5 (2.6)		10.3 (6.3)	2.0 (2.5)	
Trimester 3: Median (IQR)	9.3 (4.5)	1.4 (1.6)		9.0 (6.1)	2.8 (2.2)	
Trimester 1: Mean (SD)	14.1 (5.9)	3.4 (2.9)	<0.001	14.0 (5.8)	3.6 (2.9)	<0.001
Trimester 2: Mean (SD)	11.2 (5.2)	2.3 (2.3)	<0.001	10.3 (4.3)	2.3 (2.1)	<0.001
Trimester 3: Mean (SD)	9.0 (4.5)	1.7 (1.9)	<0.001	8.9 (4.1)	2.9 (2.2)	<0.001
Trimester 1: Missing	0 (0.0)	0 (0.0)		1 (5.6)	0 (0.0)	
Trimester 2: Missing	0 (0.0)	1 (5.0)		3 (5.9)	10 (9.3)	
Trimester 3: Missing	0 (0.0)	6 (37.5)		4 (8.0)	14 (15.6)	
Vitamin D (nmol/L) in samples						
Trimester 1: Mean (SD)	85.3 (26.8)	78.4 (26.8)		78.8 (23.8)	77.5 (23.6)	
Trimester 2: Mean (SD)	96.7 (22.2)	85.6 (20.9)		91.7 (31.6)	86.3 (32.0)	
Trimester 3: Mean (SD)	88.1 (34.3)	97.1 (32.2)		91 (33.5)	94 (31.8)	
Trimester 1: Missing	0.0 (0.0)	1 (8.3)		1 (5.6)	2 (5.3)	
Trimester 2: Missing	0.0 (0.0)	0 (0.0)		0 (0.0)	5 (4.7)	
Trimester 3: Missing	1 (5.9)	1 (6.3)		2 (4.0)	9 (10.0)	
Vitamin B6 (nmol/L): mean (SD)						
Trimester 3	76 (75.8)	70 (102.4)		NM	NM	
Vitamin B9 (folate) (nmol/L): mean (SD)						
Trimester 3	37 (9.6)	36 (9.4)		38 (8.1)	38 (8.8)	
Maternal HLA type: n (%)						
DR34	2 (12.5)	7 (43.8)		28 (54.9)	54 (57.4)	<0.001
DR3orDR4	8 (50.0)	7 (43.8)		5 (9.8)	31 (33.0)	0.004
DRXX	6 (37.5)	2 (12.5)		18 (35.3)	9 (9.6)	
KNOWN SUPPLEMENTS IN PREGNANCY						
Antibiotics: n (%)	5 (31.3)	6 (37.5)		12 (23.5)	25 (26.6)	
Anticoagulants, antithrombotic: n (%)	3 (18.8)	4 (25)		4 (7.8)	25 (26.6)	

Antihypertensive agents: n (%)	0 (0)	1 (6.3)		1 (2.0)	17 (18.1)	
KNOWN OTHER SUPPLEMENTS IN PRE-PREGNANCY_PREGNANCY						
Biotin: n (%)	7 (43.8)_14 (87.5)	5 (31.3)_14 (87.5)		15 (29.4)_44 (86.3)	18 (19.1)_81 (86.2)	
Calcium: n (%)	8 (50)_14 (87.5)	5 (31.3)_15 (93.8)		16 (31.4)_46 (90.2)	20 (21.3)_86 (91.5)	
Iron	9 (56.3)_16 (100)	5 (31.3)_15 (93.8)		17 (33.3)_48 (94.1)	18 (19.1)_91 (96.8)	
Magnesium: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_47 (92.2)	18 (19.1)_83 (88.3)	
Selenium: n (%)	7 (43.8)_14 (87.5)	5 (31.3)_13 (81.8)		15 (29.4)_45 (88.2)	18 (19.1)_81 (86.2)	
Vitamin B1: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_48 (94.1)	18 (19.1)_84 (89.4)	
Vitamin B2: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_47 (92.2)	18 (19.1)_84 (89.4)	
Vitamin B3: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_47 (92.2)	18 (19.1)_84 (89.4)	
Vitamin B5: n (%)	5 (31.3)_9 (56.3)	3 (18.8)_7 (43.8)		11 (21.6)_28 (54.9)	14 (14.9)_52 (55.3)	
Vitamin B6: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_48 (94.1)	18 (19.1)_84 (89.4)	
Vitamin B9 (Folate) : n (%)	8 (50)_16(100)	7 (43.8)_16 (100)		18 (35.3)_48 (94.1)	26 (27.7)_91 (96.8)	
Vitamin B12: n (%)	9 (56.3)_15 (93.8)	5 (31.3)_14 (87.5)		17 (33.3)_47 (92.2)	18 (19.1)_81 (86.2)	
Vitamin D: n (%)	9 (56.3)_16 (100)	5 (31.3)_15 (93.8)		17 (33.3)_49 (96.1)	20 (21.3)_88 (93.6)	
Vitamin E: n (%)	8 (50)_13 (81.3)	5 (31.3)_13 (81.8)		16 (31.4)_43 (84.3)	16 (17.0)_75 (79.8)	
OTHER						
Vaccine: n (%)						
Yes (Flu only)	1 (6.3)	3 (18.8)		2 (3.9)	4 (4.3)	
Yes (Pertussis only)	2 (12.5)	2 (12.5)		4 (7.8)	11 (11.7)	
Yes (Flu and Pertussis)	6 (37.5)	6 (37.5)		12 (23.5)	19 (20.2)	
Mode of Delivery: n (%)						
Vaginal	14 (87.5)	5 (31.3)		37 (72.5)	25 (26.6)	
Caesarean (with labour)	0 (0.0)	1 (6.3)		1 (2.0)	14 (14.9)	
Caesarean (without labour)	2 (12.5)	10 (62.5)	0.003	13 (25.5)	55 (58.5)	<0.001
Log transformation used for Age at conception: Paternal						
Square root transformation used for all diet variables and log transformation was used for MET.						
Square root transformations were used for 1,5-AG (ug/mL) in all trimesters and for Vitamin D in Trimesters 1 and 2.						
Hb1A-c, 1,5-AG (ug/mL) and Vitamin D are based on samples rather than pregnancies						
** Blank cells indicate P value non-significant. P values for HLA are determined against DRXX as baseline.						
NM = not measured						

Table 2. Pathways and enzymes differentially abundant in T1D women.

KO/MetaCyc ID	Function	Taxonomic contribution
CATEGORY 1 (increased in T1D relative to non-T1D women across all trimesters)		
PWY-1269	LPS biosynthesis	*Alistipes shahii, Bacteroides finegoldii, Bacteroides ovatus, Bacteroides thetaiotaomicron, Escherichia coli, Odoribacter splanchnicus* and unclassified bacteria
K04334	Biofilm formation	*Escherichia coli*
K00815	Antibiotic biosynthesis	*Escherichia coli*
CATEGORY 2 (decreased from trimester 1 to trimester 3 in T1D only)		
K01207	Mucin degradation	*Akkermansia muciniphila, Roseburia hominis, Escherichia coli, Bifidobacterium longum* and*Bifidobacterium adolescentis*
K01512	SCFA biosynthesis	*Eubacterium rectale, Roseburia hominis, Escherichia coli, Haemophilus parainfluenzae* and unclassified bacteria
K00248	SCFA biosynthesis	*Eubacterium rectale, Odoribacter splanchnicus, Roseburia hominis, Comamonas testosteroni* and unclassified bacteria
CATEGORY 3 (decreased in T1D relative to non-T1D as pregnancy progressed)		
BCAA-SYN-PWY	BCAA biosynthesis	*Faecalibacterium prausnitzii, Treponema succinifaciens, Akkermansia muciniphila, Odoribacter splanchnicus* and unclassified bacteria
K00171	SCFA biosynthesis	*Methanobrevibacter smithii, Anaerostipes hadrus, Adlercreutzia equolifaciens, Lachnospiraceae*bacterium 5 1 63FAA and unclassified bacteria
K13788	SCFA biosynthesis	*Bifidobacterium adolescentis, Bifidobacterium bifidum, Haemophilus parainfluenzae, Bifidobacterium animalis* and *Enterobacter cloacae*
K00209	SCFA biosynthesis	*Lachnospiraceae* bacterium 5 1 63FAA, *Anaerostipes hadrus, Treponema succinifaciens*and unclassified bacteria
K00941	Vitamin B1 biosynthesis	*Parabacteroides distasonis, Faecalibacterium prausnitzii, Treponema succinifaciens, Eubacterium rectale* and unclassified bacteria
K04487	Vitamin B1 biosynthesis	*Faecalibacterium prausnitzii, Eubacterium rectale, Eubacterium eligens, Treponema succinifaciens* and unclassified bacteria
K00878	Vitamin B1 biosynthesis	*Faecalibacterium prausnitzii, Eubacterium rectale, Treponema succinifaciens, Akkermansia muciniphila* and *Bifidobacterium adolescentis*
K00997	Vitamin B5 biosynthesis	*Faecalibacterium prausnitzii, Bifidobacterium adolescentis, Treponema succinifaciens, Akkermansia muciniphila* and *Bifidobacterium longum*
K00868	Vitamin B6 biosynthesis	*Eubacterium rectale, Parabacteroides distasonis, Bacteroides uniformis, Eubacterium eligens* and unclassified bacteria
K06215	Vitamin B6 biosynthesis	*Eubacterium rectale, Treponema succinifaciens, Bifidobacterium adolescentis, Bifidobacterium longum* and unclassified bacteria
K08681	Vitamin B6 biosynthesis	*Eubacterium rectale, Odoribacter splanchnicus, Lachnospiraceae* bacterium 5 1 63FAA, *Treponema succinifaciens* and unclassified bacteria
K16784	Vitamin B7 biosynthesis	*Bifidobacterium adolescentis, Bifidobacterium longum, Treponema succinifaciens, Bifidobacterium bifidum* and *Bifidobacterium animalis*
K13940	Vitamin B9 biosynthesis	*Bifidobacterium adolescentis, Anaerostipes hadrus, Lachnospiraceae* bacterium 5 1 63FAA, *Bifidobacterium bifidum* and unclassified bacteria
K01208	Starch degradation	*Bifidobacterium adolescentis, Bifidobacterium dentium* and *Gardnerella vaginalis*

Figures

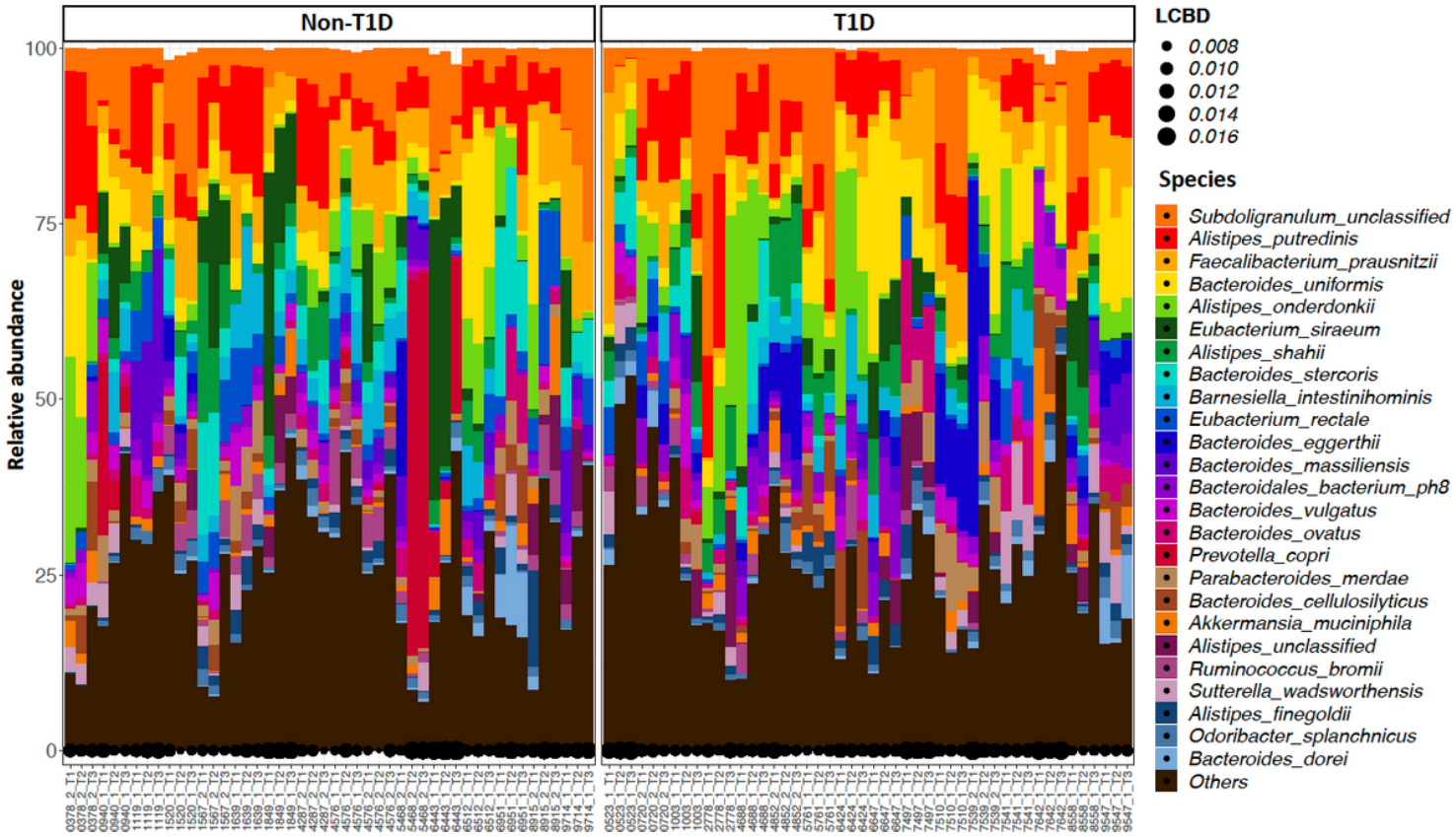

Figure 1

Taxonomic composition of the 25 most abundant species as measured by WMS in fecal samples collected in trimesters 1, 2 and 3 of pregnancy from 32 women (16 each without and with T1D). X-axis depicts the non-informative study ID in the format womanID_pregnancy.number_trimester. LCBD: local contribution to beta diversity (a measure of the uniqueness of communities).

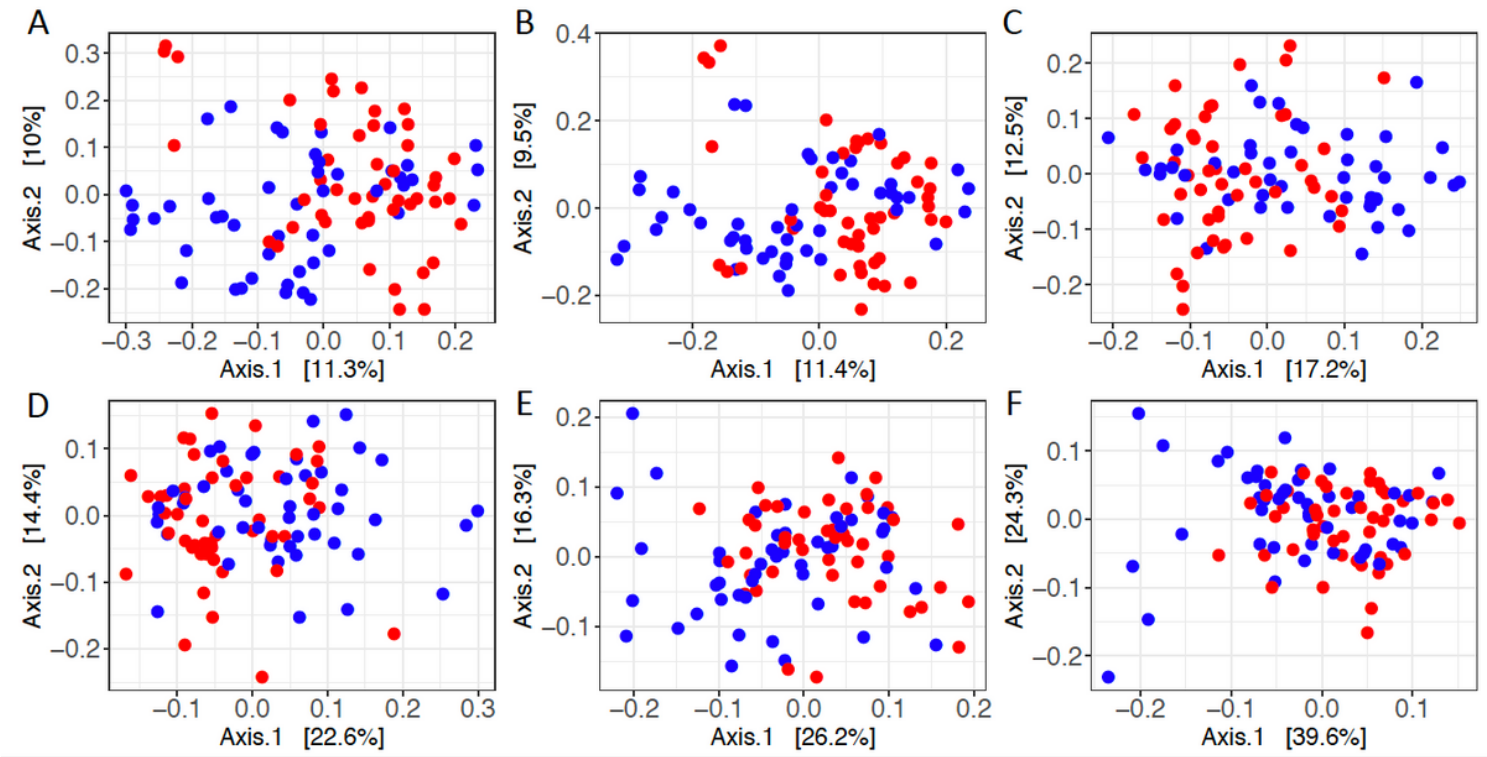

Figure 2

Beta diversity analysis by T1D status. PCoA ordination plots based on Bray-Curtis distances between samples at the A) Strain, B) Species, C) Genus, D) Family, E) Order and F) Phylum taxonomic levels across all trimesters. Blue, non-T1D; Red, T1D.

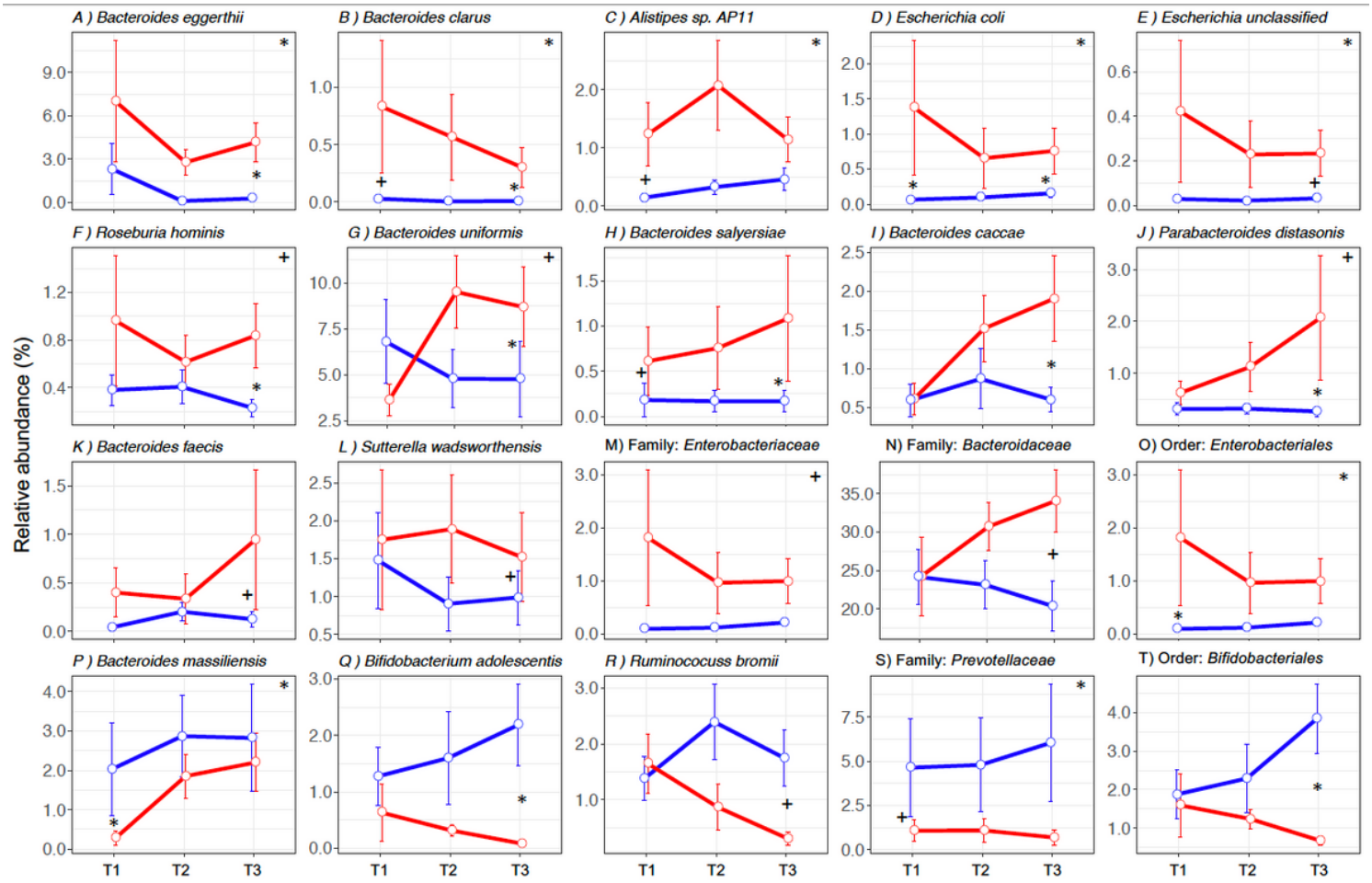

Figure 3

Differentially abundant species (A-L and P-R), families (M, N and S) and orders (O and T) (mean ± SEM) detected by WMS between non-T1D (blue) and T1D (red) women within a specific trimester or across trimesters. * Denotes a significant difference between non-T1D and T1D; + denotes borderline (FDR > 0.05 and< 0.1) significance. * or + in the top right corner denotes a difference across the trimesters; * or + between points denotes a difference between groups in that trimester.

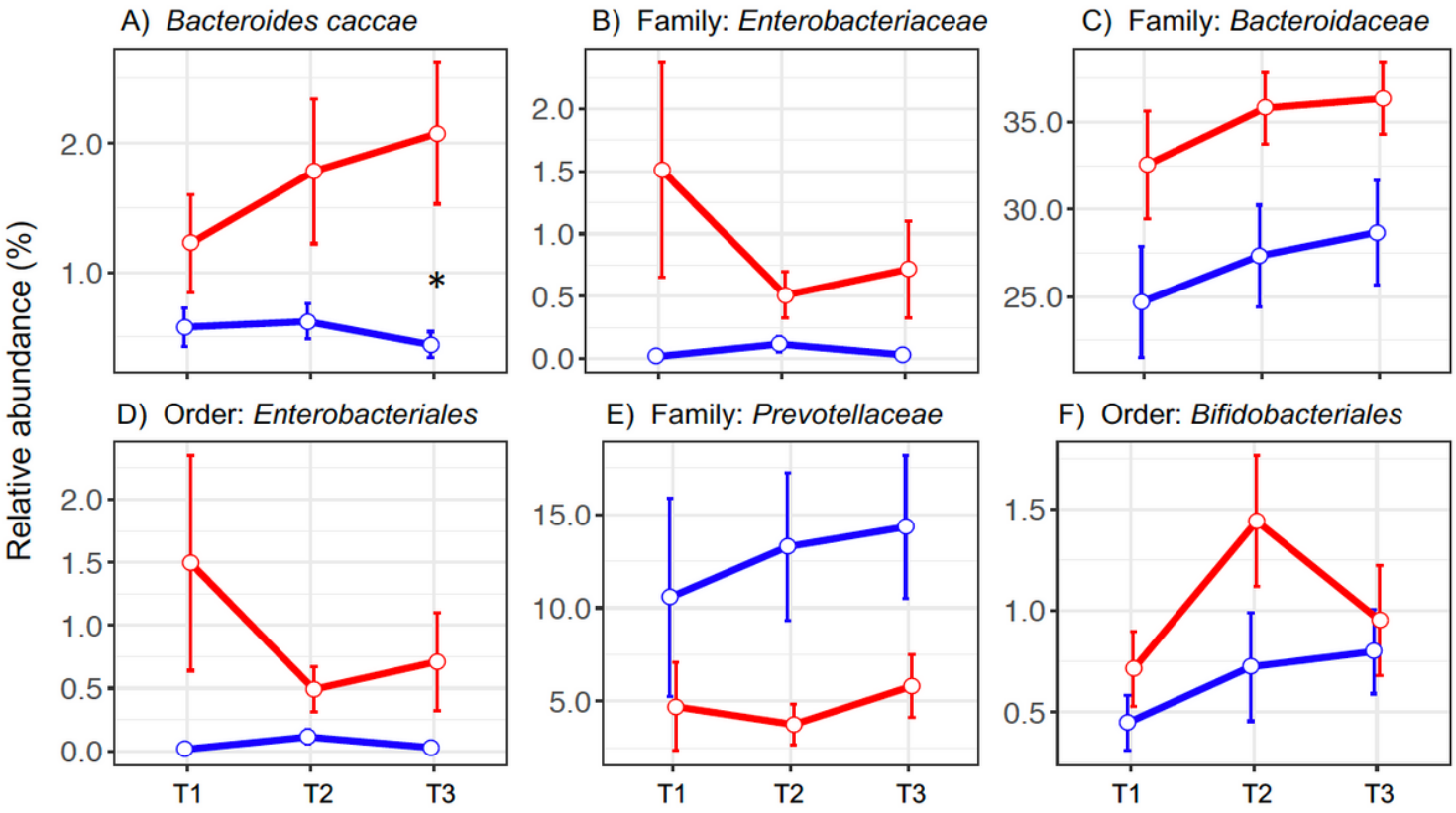

Figure 4

Abundance of taxa detected by WMS between non-T1D (blue) and T1D (red) women across trimesters (mean ± SEM). *denotes a significant difference in the trimester.

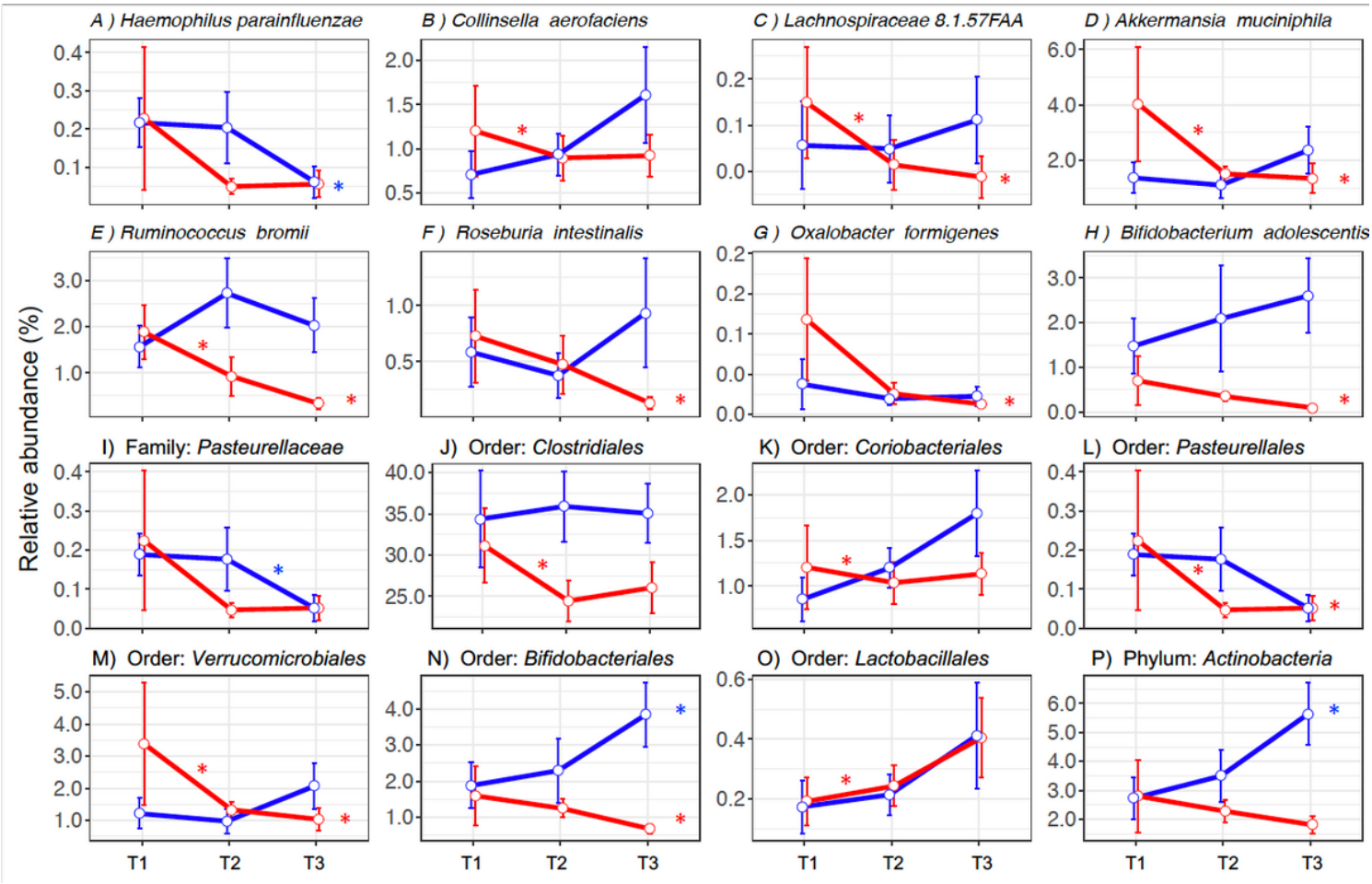

Figure 5

Differentially abundant taxa detected by WMS (mean ± SEM) between trimesters in non-T1D (blue) and T1D (red) women. The * between trimesters denotes a significant difference between those trimesters while the * after the trend line denotes significant difference between trimesters 1 and 3. The * color denotes if differences between trimesters are within non-T1D (blue) or T1D (red) women.

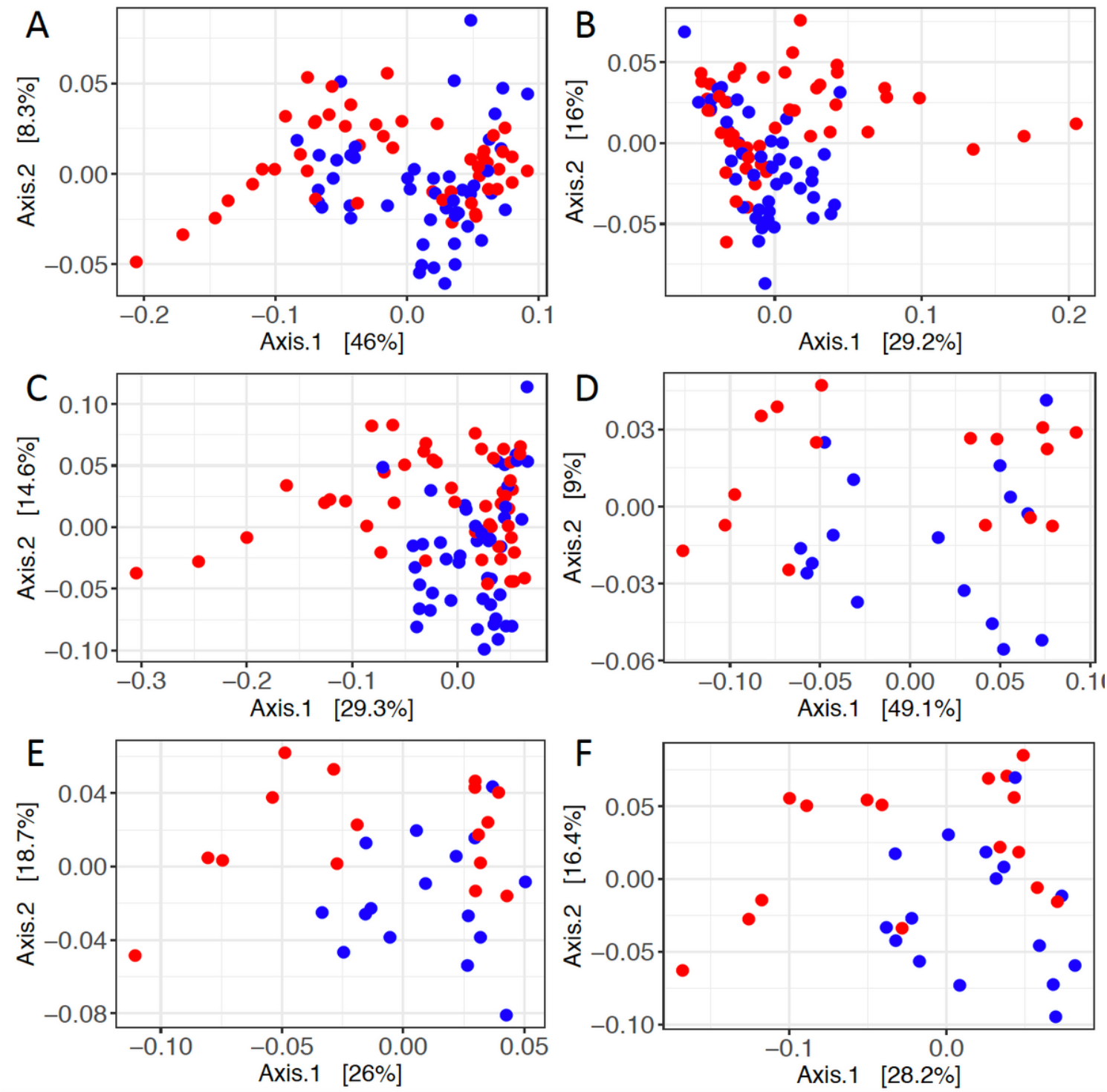

Figure 6

Beta diversity analysis by T1D status. PCoA ordination plots based on Bray-Curtis distances between samples for A) pathways, B) MetaCyc reactions and C) KOs across all trimesters; D) pathways, E) MetaCyc reactions and E) KOs in trimester 3. Non-T1D, blue; T1D, red.

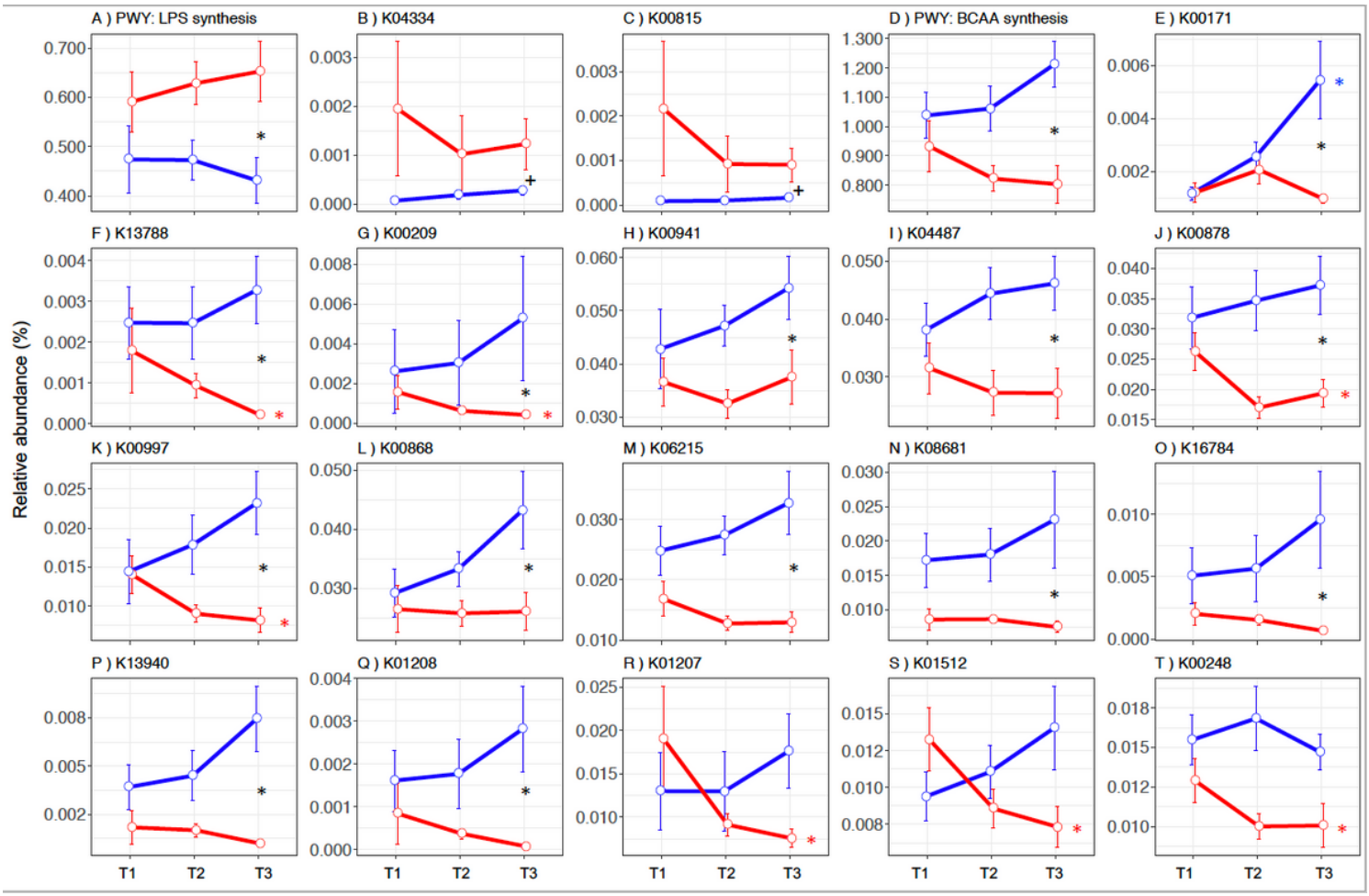

Figure 7

Pathways (A and D) and enzymes (B, C and E-T) (mean ± SEM) differentially abundant between non-T1D (blue) and T1D (red) women in trimester 3 (A-Q) or between trimesters (E-G, J-K and R-T). (A to C) represent Category 1 functions that were consistently enriched in T1D relative to non-T1D women across all trimesters. (D to Q) represent Category 2 functions that became less abundant in T1D relative to non-T1D women as pregnancy progressed. (R to T) represent Category 3 functions that were decreased from trimester 1 to 3 in T1D women. * Denotes a significant difference in the trimester; + denotes borderline (FDR > 0.05 and < 0.1) significance. A black * or + in trimester 3 denotes differences between T1D and non-T1D in that trimester; a colored * denotes significant differences between trimester 1 and 3 within non-T1D (blue) or T1D (red) women.

Supplementary Files

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

- Supplementaryfigures.pptx
- Excelfile.xlsx