**Vaginal microbiota in pregnant women with Type 1 Diabetes and its mother-to-neonate transmission**

**Type 1 diabetes predictors identified based on the assessment of microbiota in maternal-neonatal dyads**

*Other suggestions?*

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**Abstract**

Vaginal microbiota in pregnant women with type 1 diabetes (T1D) and its mother-to-neonate transmission has not been quantitatively and functionally examined. We characterized microbiota in total of 527 vaginal, rectum, and ear-skin swabs and stool samples derived from 92 maternal - neonatal dyads. The impact of the delivery mode and antibiotics administrated to the mothers on microbiota composition in neonates was found together with microbial diversity specific for neonates delivered by women with T1D. In women with T1D, overrepresentative bacterial genera were identified and the mother-to-neonate transmission of these differentially expressed bacteria was observed. Forty-one shared bacterial genera were revealed in the maternal-neonatal dyads and their functional potential was characterized pointing to numerous common metabolic pathways and only a few specific for women with T1D and their neonates. The probiotics and/or synbiotics intake declared by women was compared with the presence of major probiotic genera in the maternal and neonatal samples. These findings define the structural and functional features of the maternal and neonatal microbiota in T1D and provide a basis for further targeted study of the T1D-specific bacteria and their function.

**INTRODUCTION**

Type 1 diabetes mellitus (T1D) is one of the most common chronic diseases in childhood and adolescence (1/4 of cases are diagnosed in adults) [ref], caused by insulin deficiency following destruction of the insulin-producing pancreatic beta cells. The incidence of T1D varies based upon geography, age, gender, family history, and ethnicity [Borchers et al., 2010]. The incidence of childhood T1D was reported to be rising worldwide, with an average annual increase of approx. 2% [ref].

T1D is a heterogeneous disorder with a multifactorial pathogenesis involving both genetic and environmental factors, including episodes of viral infections, particularly respiratory or enterovirus infections, immunizations, diet, higher socioeconomic status, obesity, vitamin D deficiency, perinatal factors such as maternal age, history of preeclampsia, and neonatal jaundice [ref]. Low birth weight decreases the risk of developing T1D, while high birth weight for gestational age and lower gestational age at birth may increase the risk for T1D [ref].

Currently, human microbiomes are extensively investigated as an undervalued factor influencing both disease and health, including the role of female reproductive track microbiota [Kaminska and Gajecka Benef Microbes. 2017; Aagaard, Kjersti M. *EBioMedicine* 2020]. There is an urgent need to explain the role of disturbed microbiomes in etiology of various diseases, including T1D. Previously, it has been proposed that an aberrant gut microbiota, a permeable intestinal mucosal barrier, and an altered mucosal immune response collectively contribute to the development of T1D (Vaarala et al., 2008). Also, the involvement of microbiome in the pathogenesis of anti-islet cell autoimmunity has been suggested [Endesfelder et al, 2014]. We hypothesize that some vaginal bacteria that operate collectively in the vaginal microbiome are related to T1D. Since the vertical transmission of the microbiome from mother to fetus has been proved [Liu C-J et al. 2016], we assumed that comparing various microbiota characteristics from pregnant women with T1D and unaffected controls and their offspring we will identify bacteria specific for the mothers with T1D and will evaluate mother-to-newborn transition of these bacteria. As the data regarding vaginal microbiota of pregnant women and its composition seem to vary depending on the population and implemented methodology [Dobbler et al, 2019; Tajesvi et al., 2019], we designed a study of pregnant women (and their offspring) residing in the same region of the country, of the same ethnic origin and giving birth in the same obstetrical hospital, and all experimental samples were subjected to the same sequencing experiment. To minimize the effect of insulin administration on microbiota composition [Su et al, 2018] in pregnant women with T1D, patients with satisfactory glycaemic control in second and third trimesters were investigated.

Therefore, T1D-specific microbiota profiles in swab samples derived from maternal 1) vaginal introitus, 2) vaginal canal in the middle, 3) cervix, and 4) rectum as well as neonatal 1) stool samples, and 2) ear-skin swabs were investigated. First, to point to specific microbial predictors in T1D, both representative and recurring taxa in the analyzed microbiomes were assessed. Next, mother-to-neonate microbiota transition, taking into account the mode of delivery and the effects of antibiotics given to the mothers was examined. Finally, the effects of chosen elements of maternal diet, probiotics supplementation and breastfeeding on microbiota profiles were evaluated.

**RESULTS**

**Clinical characteristics of maternal-neonatal dyads**

50 pregnant women with T1D and their neonates and 42 unaffected control pregnant women and their neonates were ascertained.

Detailed clinical characteristics of the women are presented in Table\_1. There were differences in the gestation (weeks) between women with T1D (38.1 ± 0.7) and unaffected women (39.2 ± 1.0) (P<0.0001) (Figure S1A). There was no statistically significant difference in terms of delivery mode comparing subgroups of Caesarian and vaginal (including the vaginal and vacuum-assisted vaginal) deliveries in the women with T1D [33 (66%) vs. 17 (34%)] and unaffected women [20 (47.5%) vs. 22 (52.5%)]. At the delivery time women with T1D were younger (30 ± 4) comparing to the unaffected women (32 ± 4) (Table 1). BMI before pregnancy, weight gain during pregnancy and BMI before delivery were comparable in the studied groups of women (Table 1). Detailed dietary aspects recorded for the studied women are described elsewhere [Gutaj et al. 2020], while the selected nutritional aspects (percentages of energy from fat, saturated fatty acids, carbohydrates and proteins) are presented in the Table 1. The percentage of energy derived from total protein in the diet of women with T1D was higher compared to the diet of unaffected women (P= 0.029). The percentage of energy derived from carbohydrates in the diet of women with T1D was lower compared to the diet of unaffected women (P= 0.0171) (Table 1).

Glycaemic control was satisfactory in second and third trimesters in the women with T1D, in accordance with the recommendations of the Polish Diabetes Association, in which one of the criteria for good diabetes control during pregnancy is a glycated hemoglobin level ≤ 6.1% (43 mmol/mol) (Figure S1B). There was no substantial difference in the results of diabetes control when comparing the data of the second and third trimesters of pregnancy (P= 0.682).

Clinical characteristics of the neonates are presented in Table 2. No preterm neonates were involved in the study. 64% of newborns born to the women with T1D and 93% of newborns of the unaffected women were classified as appropriate for gestational age (AGA), 28% and 7%, respectively, were considered large for gestational age (LGA) (P= 0.0102), while newborn small for gestational age (SGA) were born only to the women with T1D (8%) (Table 2; Figure S2A).

In newborns born to women with T1D, 2-5 measurements of blood glucose levels were performed over time (Figure S2B). Generally, no hypoglycemia was observed in the newborns (Figure S2C). No correlation was found between the glycated hemoglobin level in women with T1D measured before delivery and the level of glucose in newborns born to these women (Figure S3).

14% and 95% of the newborns were breastfed, respectively, by mothers with T1D and unaffected women. Mixed feeding, including mother's milk and formula milk, was administered to 82% of newborns of mothers with T1D (Table 2).

**Study materials**

Material sample sets derived from 50 pregnant women with T1D and 42 unaffected control pregnant women and their neonates comprised swab samples derived from maternal 1) vaginal introitus, 2) vaginal canal in the middle, 3) cervix, and 4) rectum as well as neonatal 1) stool samples, and 2) ear-skin swabs. A total of 530 of these material samples were collected from women and their neonates.

**16S rRNA sequencing and data analysis**

A total of 530 DNA samples were sequenced. Sequencing row data of 527 samples was analyzed in accordance with the bioinformatics protocols established for the purpose of this study.

**Diversity of bacteria in the three assessed vaginal sampling sites and rectum swabs, and differentially expressed bacteria associated with T1D**

No substantial differences in the number of species and between species abundances compared for the vaginal samples derived from the three sampling sites in the women with T1D and the unaffected women were found. No substantial differences in the number of species and species abundances between the rectum swabs were found for the women with T1D and the unaffected women. Alpha diversity estimates are presented in Figure 1A.

No substantial differences in microbial composition at the three vaginal sampling sites and in the rectum swabs between the women with T1D and the unaffected women were found. Beta Diversity PCoA plots are presented in Figure 1B. However, the delivery week (37-41) affected the microbial composition (P=0.0024) in the assessed samples. In addition, the status of the T1D disease influenced the results (P=0.002) (Figure S4A, B).

The differences in phylum relative abundance in the 3 assessed vaginal sampling sites were found. Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes dominated among the 12 examined phyla. The low variability of bacteria near the cervix and the ascendent trend of phylum Firmicutes, increasing from introitus through the center of the vagina to the cervix were found in both T1D and control women (Figure 1C). Proteobacteria dominated the introitus of the vagina of both women with T1D and unaffected women compared to other sampling sites, while relative abundance of Actinobacteria and Bacteroidetes were comparable in the assessed vaginal sampling sites (Figure 1C).

Differentially expressed bacteria associated with T1Dwere revealed in the three assessed vaginal sampling sites with the majority of these bacteria recognized in the cervix (Table 3), namely *Enhydrobacter, Parabacteroides, Collinsella, Intestinibacter, Sneathia, Fusicatenibacter, Terrisporobacter, Bacteroides, Jonquetella, Atopobium, Bifidobacterium, Anaerococcus, Gemella, Enterococcus, Escherichia/Shigella, Staphylococcus,* and *Fusobacterium* genera. Among these 17 genera, 11 were overrepresented and 6 were underrepresented in the cervix of the women with T1D (Table 3, Figure 2). In the vaginal introitus and vaginal canal in the middle numbers of differentially expressed bacteria associated with T1D were similar. Two genera, *Staphylococcus* and *Sneathia* were found as overrepresented in the samples taken from rectum of the women with T1D.

Comparing the four types of material samples taken from women, *Snetahia, Gemella,* and *Staphylococcus as well as Intestinibacter, Atopobium, Terrisporobacter,* and *Enhydrobacter* were identified as differentially expressed genera associated with T1D in more than one sampling site, respectively in 3 and 2 material types derived from the women with T1D (Table 3).

**Diversity of bacteria in the samples from the neonates and differentially expressed bacteria associated with T1D**

Differences in the number of species and between species abundances compared for the microbiota from the ear-skin swabs and stool samples were found in the neonatal materials collected up to 72h after the birth. However, there were no significant differences in ear swabs and stool samples in neonates born to women with T1D comparing to those born to unaffected women. Alpha diversity estimates are presented in Figure 1A.

No substantial differences in phylum relative abundance in the materials derived from neonates were found. Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes dominated among the 12 examined phyla. The relative abundance of Bacteroidetes in stool samples of neonates delivered by unaffected women was similar to the level found for samples derived from the maternal rectum swabs (Figure 1C).

Differentially expressed bacteria associated with T1Din the mothers of neonates were assessed again and identified as overrepresented or underrepresented in the neonatal ear-skin swabs and/or stool samples (Table 3). No overrepresented bacteria associated with T1D in the ear-skin swabs were identified. *Rothia, Micrococcus, Escherichia/Shigella*, and *Kocuria* were underrepresented in the ear-skin swabs derived from the neonates of the women with T1D, comparing to the ear-skin swabs derived from the neonates of the control women. *Fusicatenibacter, Fusobacterium, Megasphaera, Anaeroglobus, Pseudomonas, Romboutsia, Peptoniphilus, Dialister, Actinomyces*, and *Lachnoclostridium* were overrepresented in the stool samples derived from the neonates of the women with T1D, while *Collinsella, Terrisporobacter, Sutterella*, and *Bacteroides* were underrepresented in these samples (Figure 2).

**Transmission of T1D specific bacteria from woman to neonate (Mother-to-neonate T1D specific bacteria transition)**

Analyzing the microbiota composition of maternal-neonatal dyads, 41 genera were identified as common to women and their neonates, presented as numbers of the dyads in the Figure 3.

Seven of the differentially expressed genera associated with T1D found in the vaginal and rectum samples were also revealed to be specific for the ear-skin swabs or stool samples in the offspring of the women with T1D. *Fusicatenibacter, Fusobacterium, Megasphaera, Collinsella, Terrisporobacter,* and *Bacteroides* as well as *Escherichia/Shigella* were indicated as differentially expressed genera in the neonatal stool samples and the ear-skin swabs of the neonates born to women with T1D, respectively.

**Differences in microbiota composition between neonates born by Cesarean section and vaginally delivered**

The influence of the delivery mode on neonatal microbiota composition was found in the neonates regardless of the mother’ disease status (P=0.0008; Figure 4, Table 4A).

Microbiota composition could not be distinguished among Cesarean and vaginally born offspring of the T1D mothers (P>0.05; Table 4A).

**Impact of antibiotic treatment on bacterial diversity**

There were no statistically significant differences in numbers of antibiotic prophylaxis between the studied groups (P>0.05; Table 1).

Cesarean section prophylaxis, *Streptococcus agalactiae* colonization and premature rupture of membranes prophylaxis were the causes of antibiotic treatment in both women with T1D and unaffected controls (Table 1). Cefuroxime and cefazolin were the most frequently administrated antibiotics in the women with T1D (51.5% and 31.5.%, respectively) and unaffected women (30.5% and 43.5%, respectively) (Table 1).

The influence of the antibiotic treatment in the mother on microbiota composition in the neonates was found regardless of the mother’ disease status (P=0.0013; Figure 4, Table\_Beta Diversity Bray Curtis adonis permanova of VST transformed counts versus Antibiotics stratified by sample type).

In addition to antibiotic therapy, the maternal T1D disease status influenced the composition of microbiota in samples obtained from neonates (P=0.0064; Table 4B).

**Functional potential of microbiota (based on the PICRUST analysis)**

1. The PICRUST analysis of maternal and neonatal samples analysed together (in contrast to the data of maternal - neonatal dyads presented later) pointed to 18 predicted classes of pathways involved in microbiota metabolism. Further evaluating these results, 6 pathways were predicted as enriched in the samples derived from the unaffected women and their neonates, namely pathways of lactose and galactose degradation (class: Carbohydrate Degradation), 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I (class: Folate Biosynthesis), acetyl-CoA fermentation to butanoate II (class: Generation of Precursor Metabolites and Energy), superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks (class: Carbohydrate Biosynthesis), aromatic biogenic amine degradation (bacteria) (class: Amine and Polyamine Degradation), and S-methyl-5-thio-&alpha;-D-ribose 1-phosphate degradation (class: Nucleoside and Nucleotide Degradation) (Figure 5).

Based on microbiota data obtained from three vaginal sampling sites, it was noticed that 165 pathways are significantly differentiated when comparing samples from women with T1D with samples from unaffected women (Table S2\_**Sheet1**). Pathways belonging to Amino Acid Biosynthesis node were overrepresented in T1D. Predicted lactate production as an indirect product of selected Carbohydrate Degradation pathways (superpathway of glucose and xylose degradation, fucose degradation, L-rhamnose degradation I) was significantly enriched in samples of women with T1D, but as a product of selected Fermentation pathways (pyruvate fermentation to acetate and lactate II, homolactic fermentation) was enriched in samples derived from unaffected women.

In the rectum swabs 10 pathways were predicted including 2 pathways from the carbohydrate biosynthesis class (Figure S5, Table S2\_**Sheet2**). In women with T1D decreased folate biosynthesis (N10-formyl-tetrahydrofolate biosynthesis) was noticed. The SCFA production in the rectum swabs of women with T1D was found lowered (acetyl-CoA fermentation to butanoate II, purine nucleobases degradation I (anaerobic)). The Generation of Precursor Metabolites and Energy (from superpathway of glycolysis and Entner-Doudoroff) was also decreased.

The analysis of combined neonatal ear-skin swabs and stool samples made it possible to predict the 6-hydroxymethyl-dihydropterin diphosphate biosynthesis I being a part of folate biosynthesis, and lactose and galactose degradation I as the mostly differentiated pathways, both enriched in neonates of unaffected women (Table S2\_**Sheet3)**.

Regarding neonatal stool samples and maternal disease status, it was possible to indicate 4 functional pathways of microbiota significantly reduced in neonates born to T1D women: catechol degradation I (meta-cleavage pathway), reductive TCA cycle I, superpathway of heme biosynthesis from glycine, and superpathway of L-methionine biosynthesis (by sulfhydrylation)(Table S2\_**Sheet4)**. In ear-skin swabs derived from neonates of the women with T1D enriched L-lysine biosynthesis I, L-lysine biosynthesis III, and L-lysine biosynthesis VI pathways were found (Table S2\_**Sheet5)**.

Regarding the mode of delivery, regardless of the maternal disease status and the sample type, the most differentiated pathway was the degradation of glycerol to butanol (P= 0.0011), which was found to be enriched in offspring after caesarean section (Table S2\_**Sheet6)**.

2. The results of the analysis of shared pathways of samples from maternal - neonatal dyads, indicate that the major covariate driving the clustering is the maternal disease status rather than the mode of delivery (Figure 6A). Among those pathways the reactions including branched amino acids degradation (L-leucine degradation I), cob(II)yrinate a,c-diamide biosynthesis I (early cobalt insertion) (vit. B12 biosynhesis), adenosylcobalamin biosynthesis from cobyrinate a,c-diamide I (vit. B12 biosynthesis), and acetyl-CoA fermentation to butanoate II (indirect SCFA production) were predicted to be underrepresented in the women with T1D and their neonates. In contrast, biotin biosynthesis pathways (biotin biosynthesis I, 8-amino-7-oxononanoate biosynthesis I), the glyoxylate cycle, superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation were more commonly shared in the women with T1D and their neonates comparing to the unaffected women and their neonates.

3. Further characteristics of the predicted shared pathways in the maternal - neonatal dyads are presented in the Figure 6B-D. The 191 pathways from unaffected mother-neonatal dyads and 270 pathways from mother with T1D-neonatal dyads were shared regardless the delivery mode and neonatal sample type (Figure 6B, C). The 79 out of 87 pathways predicted in the vaginally and C-section delivered neonates’ ear-skin swabs and vaginally delivered neonates’ stool samples in the unaffected mother-neonatal dyads were also predicted in both C-section and vaginally delivered born neonates’ samples in the mother with T1D-neonatal dyads. The analysis, independently of neonatal sample type (Figure 6D) showed 283 metagenomic pathways, which were predicted as shared in the all studied dyads. Three each were predicted as exclusively shared within the unaffected mother-neonatal dyads and the mother with T1D-neonatal dyads (Table 5, Figure 6D).

**Probiotics and/or synbiotics intake and the presence of the genera *Lactobacillus, Bifidobacterium*, and *Streptococcus* in the assessed maternal and neonatal samples**

Relative abundances of the genera *Lactobacillus, Bifidobacterium*, and *Streptococcus* were estimated in the particular maternal and neonatal samples (Figure S6). There were statistically significant differences in the relative abundance of the genera of *Lactobacillus* (P=0.029) and *Bifidobacterium* (P=0.0054) in rectal swabs as well as *Streptococcus* (P=0.043) in the vaginal introitus samples between women with T1D and unaffected women which declared consumption of the probiotic food products during pregnancy.

Since pregnant women took different types of supplements, and the supplements contained a different number of bacterial strains, it was impossible to accurately determine the amounts of probiotics taken. The intake of probiotics during pregnancy in the form of probiotics and/or synbiotics (the qualitative data) is summarized in Table S1. The assessment did not take into account the actual amounts of taken probiotic and/or synbiotic supplements, and probiotics and prebiotics from various types of probiotic food products consumed by the surveyed women.

**The percentage of energy derived from protein in diet of women with T1D and microbiota composition**

The influence of the percentage of energy derived from protein on maternal and neonatal microbiota composition was found, regardless of the T1D disease status (P=0.002). Also, combined, the disease and energy derived from proteins, influenced the microbiota composition (P=0.0027) (Figure S7).

**Discussion**

**Diversity of bacteria in the three assessed vaginal sampling sites.** In contrast to previously reported microbiota homogeneity in three vaginal sampling sites in unaffected pregnant women [Huang YE, 2015], in this study variability in the relative abundance of the leading bacterium phyla in the assessed sample sites was observed. Generally, the low variability of bacteria near the cervix and the ascendent trend of phylum Firmicutes, increasing from introitus through the center of the vagina to the cervix were revealed in both women with T1D and unaffected. Previously, vaginal species in 34 Chinese women during different pregnancy stages were characterized at the cervix, posterior fornix, and vaginal canal, using the Illumina sequencing of 16S rRNA tag sequences [Huang YE, 2015]. Little heterogeneity across community structures within each individual was reported, as determined by LEfSe, indicating high vaginal microbiome homogeneity at the examined vaginal sites [Huang YE 2015].

**Diversity of bacteria in the** **neonatal samples.** In contrast to previous data indicating the homogeneous microbiota distribution across the newborn body obtained based on samples of skin, oral and rectal swabs and nasopharyngeal aspirate from newborns within 24 hours from birth [Dominguez-Bello et al, 2010], in this study, differences in the number of species and between species abundances compared for the microbiota from the ear-skin swabs and stool samples were found in the materials collected up to 72h after the birth. However, there were no significant differences in ear swabs and stool samples in neonates born to women with T1D and those born to unaffected women.

To evaluatethe impact of **the delivery mode** and **antibiotics** administrated to the women on microbiota composition in the neonates the maternal samples were assessed along with neonatal ear-skin swabs and stool samples (no meconium) collected up to 72h after the birth. As previously the lack of negative impact of mothers’ gestational diabetes mellitus (GDM) disease on infants’ gut microbiome through first years of life was reported [Koren et al, 2012] and later, on the contrary, significant differences in meconium’s microbiome composition of full-term and C-sectioned newborns of mothers diagnosed with GDM were reported [Su et al, 2018], here the influence of the maternal T1D disease status on the microbiota diversity in neonates was also investigated.

There is a growing evidence suggesting that Cesarean delivery per se is not associated with any appreciable differences in the neonatal microbiota [Liu C-J et al. 2016, Hu et al., 2013; Wang et al., 2018]. Here, in line with opposite reports [Backhed et al., 2015, Yassour M, et al., 2016], we confirmed the influence of **the delivery mode** on the microbiota composition of the assessed neonatal sample types. However, no T1D disease effect on microbiota (compositional) heterogeneity was observed, indicating that the delivery mode differentiated the microbiota more strongly than the disease itself. On the other hand, here the delivery week (37-41) significantly influenced the microbial composition of the analysed samples. Women with T1D gave birth earlier, at 37-39 weeks, compared to unaffected women at 38-41 weeks of gestation, and in this aspect the (compositional) heterogeneity was related to the disease status.

Previously,no differences were found in microbiota composition of neonates within 24 hours after the **antibiotics** (cephalosporins) administration as the C-section prophylaxis [Dominguez-Bello et al, 2010]. In prospective studies the impact of intrapartum antibiotics on infant gut microbiota composition and maturation were reported as significant and characterized by the depleted abundance of *Bacteroides* after penicillin and *Bifidobacterium* after cephalosporin administration, as well as enrichment in *Veillonella dispar* associated with multi-drag intervention [Coker et al, 2020]. However, analyzing the 1-year postpartum samples, differences in alpha-diversity scores and UniFrac distances weren’t significant [Coker et al, 2020]. Here, the Cesarean section prophylaxis, *Streptococcus agalactiae* colonization and premature rupture of membranes prophylaxis were the causes of antibiotic treatment in both women with T1D and unaffected controls. Cefuroxime and cefazolin were the most frequently administrated antibiotics in both the women with T1D and unaffected. Although no statistically significant differences were found between the compared groups of women in the numbers of applied antibiotic prophylaxis, the antibiotic therapy itself influenced the composition of the neonate microflora, regardless of the maternal T1D. Subsequent division of neonatal samples into those from neonates delivered by women with T1D and those from unaffected women additionally confirmed the influence of antibiotic therapy on the composition of the neonate microflora. Thus, when comparing the effects of the disease of T1D itself, delivery mode and antibiotic prophylaxis in the women, on the diversity of microbiota of neonates, the latter seems to be of greater importance in the investigated groups of mothers and their offspring.

Although the disease of T1D itself generally has not impacted the microbiota composition (beta-diversity) of the maternal samples we were able to point to the **differentially expressed microbiota associated with T1D**. The identified differentially expressed genera associated with T1Din majority originated from the maternal cervix, however some of the genera were found in more than one vaginal sampling sites. In addition, two genera, *Staphylococcus* and *Sneathia* were found as overrepresented in the samples taken from rectum of the women with T1D. Comparing the four types of material samples taken from women, *Snetahia, Gemella,* and *Staphylococcus* as well as *Intestinibacter, Atopobium, Terrisporobacter,* and *Enhydrobacter* were identified as differentially expressed genera associated with T1D in more than one sampling site, respectively in 3 and 2 material types derived from the women with T1D. Previously, in the TEDDY study, a nested case–control analysis revealed subtle associations between microbial taxonomy and the development of islet autoimmunity or T1D [Stewart et al. TEDDY study, 2018]. In the early childhood, five bacterial genera were associated with T1D onset, with *Parabacteroides* the most significant (P < 0.001). Eleven bacterial genera were lower in T1D cases, including four unclassified *Ruminococcaceae*, *Lactococcus* (P = 0.020), *Streptococcus* (P = 0.032), and *Akkermansia* (P = 0.045) [Stewart et al. TEDDY study, 2018]. In our study, *Parabacteroides* was also found as overrepresented (in the cervix, P<0.0001) while the *Streptococcus* underrepresented (in the vaginal canal in the middle; P<0.0001) in samples derived from women with T1D. Although the differential expression of the listed genera remains to be further elucidated, the presence of the indicated bacteria, including the known pathogens, might not be incidental. For example, *Sneathia*, in our study overrepresented in the rectum swabs, vaginal canal in the middle and cervix of women with T1D, has been currently characterized as an emerging pathogen (*Sneathia amnii*) that could affect pregnancy outcome and cause urethritis and other infections [Gentile et al. 2020].

In addition, seven of the differentially expressed genera associated with T1D in this study were also recognized as differentially expressed in the neonates born to the women with T1D. While *Fusicatenibacter* and *Megasphaera* were found as overrepresented in both maternal and neonatal samples, the genera *Bacteroides, Escherichia/Shigella* and *Terrisporobacter*, overrepresented in the cervix of the women with T1D (P<0001) were found as underrepresented (P<0.0001) in the stool samples of the neonates delivered by these women. However, since no medical information about the offspring of women with T1D is available, we cannot infer these genera of bacteria as indicators of T1D in the offspring. Still, these and other microbiota shared by the maternal-neonatal dyads were further assessed to characterize in details **the mother-neonate microbiota transfer.**

Vertical transmission of the microbiome from mother to fetus [Aagaard et al., 2014; Liu C-J et al. 2016] as well as direct transfer from mother to infant of some genera, including *Bifidobacterium* spp. and *Lactobacillus* spp. existing in breast milk or *Staphylococcus* spp. colonizing the areolar skin [Martín, R. et al. 2009; Hunt, K. M. et al. 2011; Pannaraj, P. S. et al. 2017; Soeorg, H. et al. 2017; Stewart et al. TEDDY study, 2018] have been previously investigated. Temporal alpha diversity and community dynamics were comparable between cases and controls in the TEDDY study [Stewart et al. TEDDY study, 2018], which was in contrast to other studies on the infant gut microbiome and T1D [Giongo, A. et al. 2011; Kostic, A. D. et al. 2015] and might reflect the increased number of subjects and samples in the TEDDY cohort [Stewart et al. TEDDY study, 2018]. Here, characterizing the shared microbiota of the three vaginal sampling sites and neonatal samples in the maternal-neonatal dyads, out of X, 41 genera were identified as common to women and their neonates. Previously, it was found, based on studies of 9 mother-neonate pairs that vaginally delivered neonates received their mother’s vaginal microbiota (*Lactobacillus, Prevotella,* or *Sneathia spp*.), and C-section delivered neonates acquired microbiota characteristic for human skin surface (*Staphylococcus, Corynebacterium,* and *Propionibacterium spp*), but from nonmaternal source [Dominguez-Bello et al, 2010]. Also, Korpela et al., using publicly available and own metagenomic data, by strain source tracking (SNVs), found only selective maternal transmission of gut microbiome, exclusively limited to *Actinobacteria* and *Bacteroidia* classes in vaginally born infants [Korpela et al., 2018]. In our study, *Streptococcus, Staphylococcus, Lactobacillus, Escherichia/Shigella, Enterococcus*, and *Corynebacterium* were the most frequently recognized in neonates delivered by c-section by women with T1D, what indicates vertical transmission of these genera from mother to fetus rather than horizontal mother-neonate microbiota transfer. In line to a study performed in a Swedish cohort [Backhed et al., 2015], in our investigation *Bacteroides*, *Bifidobacterium* and *Escherichia/Shigella* were found as frequently shared by mothers and neonates, regardless of the delivery mode in the neonates born to the women with T1D but decreased in the neonates born to unaffected women via C-section. Overall, little microbiota variation was found, discussing these 41 genera in vaginally versus cesarean delivered neonates, regardless the disease of T1D in women. This suggests no impact of the disease of T1D and the delivery mode on the mother-neonate microbiota transfer of these 41 identified genera. As bacterial genera were identified in maternal-neonatal dyads, the next step was to look at the potential T1D-altered microbiota metabolic pathways in these dyads.

The effect of the gut microflora on the host as a complex interaction, mainly based on the products of **bacterial metabolism** that affect the host's metabolism [Fan and Pedersen, 2021], intestinal barrier integrity [Lavelle and Sokol, 2020], and immunity has been reported [Vatanen et al, 2016]. However, since multiple taxa have overlapping metabolic function, subtle variations in which microbiota occupy a niche may not contribute to physiologically meaningful differences in the host [Aagaard, *EBioMedicine*, 2020]. Still, the influence of the microbiota of the female reproductive track can be considered in relation to the influence of the products of bacterial metabolism on the course of pregnancy, especially in pregnancy with T1D, in which numerous complications arise [ref.]

Previously, in the study on gut microbiome in infants from Finland and Estonia carried T1D-predisposing HLA alleles, extensive variation in overall taxonomic composition between and within individuals over time was found, but at the same time there was significantly less variation in the metabolic composition of the microbiome, and almost no variation in its metabolic pathway coding potential [Kostic et al. 2015]. Therefore, we assumed that **the metabolic pathways predictions** should be informative if performed in maternal-neonatal dyads and should allow for the identification of many common metabolic pathways and only a few, if any, **pathways specific for women with T1D** and their newborns. Using a PICRUST, 191 and 270 pathways, respectively, in unaffected mother-neonatal and mother with T1D-neonatal dyads were found to be common, irrespective of the mode of delivery and the type of neonatal sample. The 79 out of 87 pathways predicted in the vaginally and C-section delivered neonates’ ear-skin swabs and vaginally delivered neonates’ stool samples in the unaffected mother-neonatal dyads were also predicted in both C-section and vaginally delivered neonates’ samples in the mother with T1D-neonatal dyads. In the analysis carried out independently of neonates’ sample type**,** 283 predicted metagenomic pathways were found as shared in the all studied dyads. Interestingly, three biosynthesis pathways (class of Cofactor, Carrier, and Vitamin Biosynthesis, and class of Carbohydrate Biosynthesis) were predicted as exclusively shared within unaffected mother-neonatal dyads, while three pathways (class of Aromatic Compound Degradation, class of Carboxylase degradation, and class of Amine and Polyamine Biosynthesis) were predicted as exclusively shared within mother with T1D-neonatal dyads. Previously, specific metabolic pathways (involved in the utilization of D-galactose, D-xylose, L-arabinose, D-glucose, and D-mannose; and the biosynthesis of a number of amino acids) shifting from biosynthesis to utilization/degradation were predicted in the T1D infants [Kostic et al. 2015].

In the TEDDY study [Stewart et al. TEDDY study, 2018], **breastfeeding** was the only covariate that was significantly associated with metabolic potential. In another study, the delivery mode and feeding mode were found as the main covariates shaping the dissimilarity (Bray-Curtis distances) between newborns’ stool microbiota at day 3 [Sakwinska et al., 2017]. The C-section delivery and mixed feeding resulted in decreased of *Bifidobacterium* species relative abundances, and absence of *Bacteroides*, in newborns’ stool microbiota at day 3 [Sakwinska et al., 2017]. We observed similar results for the stool samples of C-section delivered neonates of unaffected women but definitely different for the stool samples of C-section delivered neonates of women with T1D, analyzing the 41 bacterial genera in aspect of delivery mode in the maternal-neonatal dyads. However, as our study showed significant differences in the numbers of breastfed and mixed breastmilk/formula fed neonates, the influence of the way the neonates were fed on microbial diversity was not further investigated.

Detailed information of **dietary aspects in pregnant women with T1D** examined in this study were discussed in details elsewhere [Gutaj et al., 2020]. We found no significant differences in energy intake between studied groups, which was reflected in similar weight gain during pregnancy, regardless of T1D disease state. However, women with T1D consumed less carbohydrates and more proteins, and the percentage of energy derived from carbohydrates was decreased in their diet while the percentage of energy derived from proteins was increased. These results seem to be related to the differences found in microbiota composition in the assessed groups of pregnant women. Previously, diet was assumed to have a central role determining the T1D-associated dysbiosis evolution. The decrease of protein consumption was reported to correlate with *Bacteroides* increase in T1D patients [Meija-Leon et al., 2018]. In our study, relative abundance of phylum *Bacteroidetes* in the maternal rectum swabs was comparable to that of the neonatal stool samples, regardless of the T1D disease status in the mother. However, *Bacteroides*, as we already mentioned, was found to be one of dominating shared genera in the maternal-neonatal dyads, more frequently observed in the women with T1D and their offspring.

**Summarizing**…

***Other aspects to consider, study limitations***

Since in our study satisfactory glycemic control in the second and third trimesters was achieved in women with T1D, we did not take blood glucose levels to as a variable to consider.

Although various aspects were taken into consideration in the maternal and neonatal microbiota characterization, others were not possible to be evaluated at the same time, including the hospital environment effect, and actual maternal probiotic/synbiotic/prebiotic intake and vitamin supplementation.

**Materials and methods**

*Patient ascertainment and study materials*

Pregnant women with T1D and unaffected control pregnant women, and their neonates were ascertained in the Gynecologic and Obstetrical University Hospital at the Poznan University of Medical Sciences, Poznan, Poland. Inclusion criteria for both groups were age 18 to 45 years and single, term pregnancy (37+0–41+0). Multiple pregnancies, genitourinary infections diagnosed in the last four weeks, use of oral or vaginal antibiotics/antifungal medicines/ probiotics in pregnancy in the previous four weeks, and vaginal irrigation/ sexual intercourse in last 72 hours were the exclusion criteria. The time of recruitment was the same in both study groups.

Clinical information concerning the mothers, pregnancy and offspring was collected during the clinic stay by questionnaire and included the mode of birth (vaginal vs. Caesarean section delivery), the newborn’s Apgar score, pregnancy complications, information about maternal T1D, gestational age, and maternal medication use during pregnancy.

Material sample sets derived from pregnant women with T1D and unaffected control pregnant women comprised swab samples derived from maternal 1) vaginal introitus, 2) vaginal canal in the middle, 3) cervix, and 4) rectum, collected before delivery. Stool samples (no meconium samples, later feces) and ear skin swabs were collected from newborns up to 72 h after birth.

Receipt of breast milk, either exclusive or partial was recorded in the newborns.

The study was approved by the Institutional Review Boards at the Poznan University of Medical Sciences, Poznan, Poland. The mothers provided written informed consent before enrolment. The study was performed in compliance with all relevant ethical regulations.

***16S rRNA gene sequencing***

16S rRNA gene sequencing method was adapted from the methods developed by Weisburg (Weisburg et al., 1991). Bacterial DNA was extracted using the ZymoBiomics DNA Miniprep Kit following the manufacturer’s instructions. The V3-V4 region of the 16S rRNA gene was amplified by PCR and sequenced on the MiSeq platform (Illumina) using the 2×250 bp paired-end read protocol. To be continued (Gosia Rydzanicz)

***16S rRNA sequencing data analysis***

- alpha diversity (richness and Shannon diversity) was calculated at the OTU-level, alpha diversity and taxonomic abundance were modelled in R (http://www.R-project.org) using the ggplot package,

- as some covariates might influence the microbiome before the start date, the fallowing factors were taken into consideration: antibiotic administration, delivery mode, … . The effect size and significance of each covariate were determined using …. Ordination was performed using NMDS based on Bray–Curtis dissimilarity. To be continued (MG, MJ)

***Nutritional assessment***

Mothers with T1D participated in a dietary education program led by diabetes educators during pregnancy. The control group women did not receive any nutritional counseling either in early pregnancy or during term hospitalization.

A nutritional assessment was performed in the ascertained mothers as previously described [Gutaj et al. Pol Arch Intern Med. 2020]. Briefly, it was carried out face‑to‑face, using 24‑hour dietary recalls for 7 consecutive days (day‑by‑day) before delivery. The Dietetyk 2011 computer software (JuMaR, Poznan, Poland) based on a Polish database comprising tables of the nutritional value of food [Kunachowicz M et al. 2005] was used to perform a qualitative and quantitative analysis of daily food intake. Moreover, information concerning dietary habits was collected based on the authors’ survey and the assessment of the validated Food Frequency Questionnaire (FFQ‑D10) [Gutaj et al. Pol Arch Intern Med. 2020]. Women were asked to indicate the frequency of consumption of foodstuffs within the last 12 months with a special focus on the period of their pregnancy. The results were converted to a daily frequency indicating how many portions of a selected product were consumed within one day [Gutaj et al. Pol Arch Intern Med. 2020].

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**Competing interests**

The authors declare no competing interests.

**Author contributions**

MG, EWO, PG, and RP designed the study. PG, EWO, and TS participated in patient recruitment and diagnosis. PG, EWO, TS, DK, and GK participated in sample collection and patient surveys. PG, KJ, DK, TS, MJ and MG participated in generation of the metadata. KJ extracted the microbial DNA and generated the 16S rRNA libraries. MR, and KJ generated and processed the raw sequencing data. MG, MJ, KJ and KMA performed the data analysis. MG, MJ, KJ, EWO, and KMA performed the data interpretation. MJ performed the figure generation. MG and KJ wrote the paper. GK and JP performed the nutritional assessment. All authors contributed to critical revisions and approved the final manuscript.

**Figure titles and legends**

**Figure 1.** Characterization of the vaginal microbiome using targeted 16S rRNA gene amplicon sequencing. **A.** The Alpha diversity (Shannon index) of maternal vaginal introitus, vaginal canal in the middle, cervix and rectum swabs, as well as neonatal ear-skin swabs and stool samples. The Wilcoxon test showed no significant differences in species abundance in terms of maternal disease status. **B.** Beta Diversity PCoA plots showing the microbial composition of T1D and control samples in all tested sample types. **C**. The mean relative abundance of 12 most prevalent phyla (Verrucomicrobia, Tenericutes, Synergistetes, Proteobacteria, Fusobacteria, Firmicutes, Euryarcheota, Epsilonbacteraeota, Deinococcus-Thermus, Bacteroidetes, Actinobacteria and Acidobacteria) across all studied sample types. The Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes dominated among the 12 examined phyla. The differences in phylum relative abundance in the 3 assessed vaginal sampling sites were found. The low variability of bacteria near the cervix and the ascendent trend of phylum Firmicutes, increasing from introitus through the center of the vagina to the cervix were found in both T1D and control women. No substantial differences in phylum relative abundance in the materials derived from neonates were found.

**Figure 2**. Differentially expressed microbiota (genera) associated with T1D in the assessed sample types.

**Figure 3.** Bacterial genera shared in the maternal - neonatal dyads. The identified 41 genera, common to women and their neonates, presented as numbers of the maternal-neonatal dyads and presented regarding maternal T1D disease status, delivery mode and neonatal sampling site.

**Figure** **4**. Mosaic plot of the dataset, the maternal T1D disease status, delivery mode and antibiotic prophylaxis.

**Figure 5**. The PICRUSt analysis of combined maternal and neonatal samples. The most significantly underrepresented predicted pathways (6 out of 18 recognised) in T1D women and their neonates.

**Figure 6**. Prediction of pathways common to the samples of the maternal-neonatal dyads. **A**. The top most common predicted to share pathways, presented as percentage of dyads with presence of particular pathway in maternal vaginal sites and neonatal samples. The results indicate that the major covariate driving the clustering is the maternal disease status rather than the mode of delivery. **B-C.** Metagenomic pathways found in at least 50% of dyads of unaffected women (**B**) and women with T1D (**C**), predicted using PICRUSt. Each prediction of a specific metagenomic pathway common to one of the maternal sample types and one of the neonatal sample types was counted in the maternal - neonatal dyads (the ear-skin swab from Csection born neonates, ear-skin swab from Vaginally delivered neonates, stool samples from Csection born neonates, and stool samples from Vaginally delivered samples were assessed separately). The 191 pathways from unaffected mother-neonatal dyads and 270 pathways from mother with T1D-neonatal dyads were shared regardless the delivery mode and neonatal sample type. **D**. Independently of neonatal sample type, 283 metagenomic pathways shared in the all studied dyads were predicted. Three biosynthesis pathways were predicted as exclusively shared within unaffected mother-neonatal dyads, while three pathways were predicted as exclusively shared within mother with T1D-neonatal dyads.

**Table titles and legends**

**Table 1**. Clinical characteristics of women with T1D and unaffected women. The detailed clinical characteristics of the pregnant women, including age, ethnicity, delivery mode, BMI, pregnancy weight gain, diabetic complications, antibiotic prophylaxis, selected dietary aspects and other characteristics, together with statistical data presented as P-values.

**Table 2**. Clinical characteristics of neonates delivered by women with T1D and unaffected. The detailed clinical characteristics of the neonates, including sex, birth weight, feeding methods and other characteristics, together with statistical data presented as P-values.

**Table 3**. Differentially expressed microbiota (genera) associated with T1D identified in the assessed sample types (**A-F**). The log2FoldChange values show the direction of change in pregnant women with T1D (under- or overrepresentation). All presented genera showed significant (padj value) variation in relative abundance.

**Table 4**. Beta Diversity Bray Curtis adonis permanova of VST transformed counts versus Delivery mode (**A**) and Antibiotics administration in the women (**B**), stratified by sample type.

**Table 5.** Metagenomic pathways predicted as exclusively shared within unaffected mother-neonatal dyads (**A**), and within mother with T1D-neonatal dyads (**B**). These pathways were predicted regardless of the type of neonatal sample type, out of 283 predicted pathways common to all dyads.

**Supplemental Information titles and legends**

**Supplemental Figure** **1.** Additional clinical findings in women with T1D. (**A)** Disease and week of delivery. The box plot presents the differences in the gestation (weeks) between women with T1D and unaffected women (P<0.0001). Each dot represents one woman. **(B)** Glycaemic control during first, second and third (before delivery) trimesters in women with T1DThe HbA1C levels measured during first, second and third (before delivery) trimesters in women with T1D. Each dot represents one woman with T1D. The glycaemic control was found to be satisfactory in second and third trimesters (in accordance with the recommendations of the Polish Diabetes Association, HbA1C ≤ 6.1% (43 mmol/mol)). The Tukey multiple comparisons of means show that no substantial difference in the results of diabetes control when comparing the data of the second and third trimesters of pregnancy (P= 0.682) was found.

**Supplemental Figure** **2.** Additional clinical findings in newborns delivered by the women with T1D. **(A)** Newborn weight distribution. Newborns born to mothers with T1D had higher body weight (in grams), which is indicated by the Gaussian curve shift to the right (P=0.0947). (**B)** The measurements of plasma glucose level [mg/dl] in two to five time points (the number of measurements depended on the condition of the newborn), performed in newborns born to women with T1D. (**C)** Glucose level measurements over time in particular newborns of women with T1D, presented in 50 individual graphs corresponding to 50 newborns of women with T1D. [If a figure 2.C. is selected for this publication, it must be changed to 50 neonates reported in this ms]

**Supplemental Figure** **3.** The correlation between the glycated hemoglobin level in women with T1D measured before delivery, and the level of glucose in newborns born to these women. No correlations using Pearson's product-moment test were found regarding HbA1C levels of mothers with T1D, in first, second and third (delivery) trimesters, and first measurement of glucose level in newborns (t = -0.62595. df = 45. p-value = 0.5345).

**Supplemental Figure** **4.** The Beta Diversity PCoA plots presenting the T1D and control samples’ microbial composition across all studied sample types regarding delivery week. (**A**) The number of samples analyzed, taking into account the given week of labor. (**B**) The influence of delivery week was found to affect the microbial composition (P=0.0024) in the assessed samples (Beta Diversity Bray Curtis adonis permanova of VST transformed counts versus week of delivery stratified by sample type). The T1D disease status also influenced the microbial composition (P=0.002).

**Supplemental Figure** **5.** The predicted differently represented pathways in the rectum swabs of women with T1D. The 10 pathways were recognised including 2 pathways from the carbohydrate biosynthesis class. In women with T1D decreased folate biosynthesis (N10-formyl-tetrahydrofolate biosynthesis) was noticed. The SCFA production in the rectum swabs of women with T1D was found lowered (acetyl-CoA fermentation to butanoate II, purine nucleobases degradation I (anaerobic)). The Generation of Precursor Metabolites and Energy (from superpathway of glycolysis and Entner-Doudoroff) was also decreased.

**Supplemental Figure** **6.** Comparison of *Lactobacillus, Bifidobacterium,* and *Streptococcus* genera relative abundances by disease and probiotic food products consumed during pregnancy. The relative abundances of the selected genera *Lactobacillus, Bifidobacterium*, and *Streptococcus* estimated in the particular maternal and neonatal samples. The differences in the relative abundance of the genera of *Lactobacillus* (P=0.029) and *Bifidobacterium* (P=0.0054) in rectal swabs as well as *Streptococcus* (P=0.043) in the vaginal introitus samples between women with T1D and unaffected women which declared consumption of the probiotic food products during pregnancy were found [the Figure 5 will be modified].

**Supplemental Figure** **7.** The Beta Diversity PCoA plots presenting the T1D and control samples’ microbial composition across all studied sample types regarding the percentage of energy derived from protein. The influence of the percentage of energy derived from protein on maternal and neonatal microbiota composition was found, regardless of the T1D disease status (P=0.002). Also, combined, the disease and energy derived from proteins, influenced the microbiota composition (P=0.0027) (Beta Diversity Bray Curtis adonis permanova of VST transformed counts versus the percentage of energy derived from protein, stratified by sample type).

**Supplemental Table** **1**. The summary of the intake of probiotics during pregnancy in the form of particular probiotics and/or synbiotics (products commonly available). Supplements, including bacterial strains and their percentage content are presented. The supplements consumption as qualitative data (yes/no) is shown. The assessment did not take into account the actual amounts of taken probiotic and/or synbiotic supplements, and probiotics and prebiotics from various types of probiotic food products consumed by the surveyed women.

**Data only tables (xls extended data tables):**

**Supplemental Table** **2**. Differentially represented metagenomic pathways, predicted using a PICRUSt bioinformatics software package. **Sheet1** presents 165 predicted pathways, significantly differentiated by comparing three vaginal sampling sites of women with T1D with samples of unaffected women. **Sheet2** contains 10 pathways predicted as differentially represented in the rectum swabs. **Sheet 3** shows the analysis of combined neonatal ear-skin swabs and stool samples. **Sheet 4** presents pathways predicted to differ depending on maternal disease status regarding neonatal stool samples. **Sheet5** shows pathways of ear-skin swabs differently represented in neonates born to T1D and unaffected mothers. The influence of mode of delivery on pathways prediction is presented in **Sheet6**.

**Supplemental Table 3**. A list of differentially expressed genera associated with T1D (in accordance with the Table\_Differentially expressed microbiota (genera) associated with T1D) and their predicted contribution to selected pathways (PICRUSt). The 33 metagenomic pathways from the Figure\_Top\_pathways\_shared\_maternal-neonatal dyads were chosen based on the literature and possible microbiome-host effect (listed in sheet **pathway\_list** of the SuppTable). The list of predicted pathways in differentially expressed genera regarding sampling site is presented in sheet **taxa\_contribution\_pathways**. Next, based on presence of particular genus in particular sample from sampling site of interest, its abundance, relative abundance, taxon functional abundance and taxon relative function abundance, the median values of taxon relative function abundance for sample sites were calculated. The sheet **taxa\_contribution\_pathway\_value** presents the median values of taxon relative function abundance regarding mother’s T1D disease status. The medians were calculated only from samples in which selected genus were present. Value “0” stands for lack or minimal presence of selected pathways in selected genus.

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