# IMPACT OF MATERNAL SMOKING ON THE DEVELOPMENT OF GUT MICROBIOME IN INFANTS

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ABSTRACT

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It has recently been suggested that the development of human microbiome may start in the prenatal period. This hypothesis is based on several studies reporting diverse microbiome in the first-pass meconium, the first stool after birth, formed before birth *in utero*. Earlier, maternal factors, such as maternal overweight, antibiotics and biodiversity of the livening environment during pregnancy have been reported to associate with the microbiome in the first pass meconium. Maternal smoking is a known risk factor for the subsequent health of children. Gut microbiome composition has earlier been associated with several diseases in childhood. In this study, we set out to investigate whether maternal smoking during pregnancy has an impact on the gut microbiome composition in the first pass meconium, developed in the fetal period.

To test this hypothesis, we first made a literature review. PubMed search in November 2019, with the keywords maternal smoking [title] AND microbio* [title], found a single study of the subject. Based on the literature review, in adults, an association between smoking and the changes in gut microbiome have been reported, such as a decrease of abundance of Firmicutes phylum and increase in Bacteroidetes phylum in gut microbiome.

We then investigated 131 first-pass meconium samples with earlier bacterial 16S gene next generation sequencing-based microbiome analysis and maternal smoking status available.

In our study, a cohort of newborn infants, altogether 5 (3.8%) of 131 mothers reported smoking during pregnancy. The relative abundances of Bacteroidetes and Proteobacteria phyla were higher and the relative abundance of Firmicutes phylum was lower in those infants exposed to maternal smoking during pregnancy. In addition, the infants exposed to maternal smoking had less diverse gut microbiome and the number of the operational taxonomic units (OTUs) was lower.

Based on the literature review and our preliminary study with meconium samples, we suggest that the association of maternal smoking and microbiome in the first stool should be further evaluated in a larger cohort since maternal smoking may have clinically significant impact on early gut microbiome of newborn infants.

Keywords: gut, infant, maternal smoking, microbiome

**CONTENTS**

# 1. BACKGROUND

In recent years, the human microbiome has emerged as an essential factor for human health status and morbidity (D'Argenio 2018). Disturbance of the gut microbiome has been associated with various diseases, such as obesity, metabolic disorder, asthma and atherosclerosis. (Lynch et al. 2016, Selber-Hnatiw et al. 2017, Hall et al. 2017). Recently, the interest in pediatric microbiome research has focused on infants and the maternal influence on the fetal microbiome. To date, intestinal dysbiosis in the infants has been associated with development of allergic diseases, asthma, bowel disease and obesity (Saavedra et al. 2012). In previous decades it has been assumed that the fetus is sterile. Several studies have questioned this idea since diverse bacterial DNA has been found in amniotic fluid and meconium samples. (Jiménez et al. 2008, Koleva et al. 2015, Collado et al. 2016). These findings have suggested that human microbiome may start to develop in the prenatal period *in utero*.

Several previous studies have reported the impact of maternal smoking on the morbidity of infants. Furthermore, the associations between infant's early microbiome and infant health have been widely reported. Yet, the impact of maternal smoking on the development of the newborns gut microbiome has rarely been reported.

In this study, we set out to investigate whether maternal smoking during pregnancy has an impact on gut microbiome in the first pass meconium, developed in the fetal period. To test this hypothesis, we first made a literature review. We then investigated first-pass meconium samples with earlier bacterial 16S gene next generation sequencing-based microbiome analysis and maternal smoking status available.

# 2. LITERATURE REVIEW

## 2.1. Gut microbiome

Traditionally, clinical medicine has focused on microbial pathogens. Yet, the human body contains numerous commensals, i.e. normal flora. Most of the human microbiome resides in the gut. Culture independent methods have enabled the modern microbiome research. Most studies have used next generation sequencing of bacterial ribosomal 16S gene as a

marker gene to characterize gut microbiome composition. Research methods based on detecting nucleic acids have demonstrated that mature intestinal microbiome consists even 1200-16 000 different bacterial species. (Bäckhed et al. 2005) The intestinal microbiome of healthy individuals is dominated by Bacteroidetes and Firmicutes phyla, with representatives from additional phyla including Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria. Gut microbiome composition has earlier been associated with several chronic diseases such as cardiovascular diseases, metabolic disorders, asthma, and obesity. Dietary factors, mode of delivery, host immune response, antibiotics and other drugs, infections and environmental factors influence the function and composition of the human gut microbiome. (Huttenhower et al. 2012)

## 2.2. Maternal impact on the fetal microbiome

In previous decades, it was assumed that the fetus is sterile. To date, numerous studies have demonstrated that amniotic fluid and meconium samples contain bacterial DNA. (Jiménez et al. 2008, Koleva et al. 2015, Collado et al. 2016) Jimenez et al. fed pregnant mice with labeled milk supplemented with Enterococcus faecium. The same strain was found in the amniotic fluid and in the meconium of the mice receiving the labeled milk, whereas with the control mice the strain was not detected. Based on these mouse studies they assumed that the bacteria would be able to reach the fetus with potential outflow mechanism. So, contrary to what was previously thought, the human microbiome may commence already *in utero*. This has increased the interest on maternal impact to the development of the fetal microbiome. It has been shown that factors, for example antibiotics during pregnancy and the biodiversity of the prenatal living environment, alters the main phyla of the fetus' gut microbiome (Tapiainen et al. 2014, Tapiainen et al. 2018). In addition, one major maternal factor is the mode of delivery. In 72% of vaginally delivered infants, the intestinal microbiome resembled their mothers' intestinal microbiome but with infants delivered by C-section, gut matched the species found in the stool of their own mother only in 41% of the infants (Bäckhed et al. 2015).

After birth, initial gut microbiome composition starts maturation process, which is modified by breasfeeding, diet, and living environment. In preliminary studies before next-generation sequencings era, Tapiainen et al. made a research in 2006 based on fatty

acid composition of fecal samples in newborn infants and their mothers. The study demonstrated the dynamic process of early gut colonization process. In five of the children, the gut flora changed rapidly within 10 to 20 hours after birth. In three children the change occurred more slowly, at about 50-60 hours, and in two children the composition of the intestinal microbiome varied irregularly. The composition of the intestinal flora began to resemble that of the mothers but also to that of nursing staff flora during the follow-up. (Tapiainen et al. 2006) Since the microbiome development of an infant is a dynamic process and prone to changes right after birth, it sets challenges on studying the maternal impact on the fetal gut microbiome.

## 2.3. Impact of smoking on the gut microbiome: Literature review

To date, the evidence concerning effects of tobacco smoking effects on human gut microbiome is mostly available from adults. Smoking appears to alter the gut microbiome composition, and the relative abundance of main bacterial phyla. The studies show an increase on Proteobacteria and Bacteroidetes phyla and a decrease in Actinobacteria and Firmicutes phyla as a response to tobacco exposure in adults. Also, smoking appears to reduce the diversity of the intestinal microbiome. (Savin et al. 2018, Lee et al. 2018, Huang&Shi 2019) These results are endorsed by research done to ex-smokers. In one study, smoking cessation resulted as a decrease in Proteobacteria and Bacteroidetes phyla and an increase in Actinobacteria and Firmicutes phyla. In addition, the microbial diversity increased after smoking cessation. (Biedermann et al. 2013).

In three cohort studies with newborns and infants, some associations with maternal smoking and later infant gut microbiome have been observed (Table 1). In one study, the impact of maternal smoking on Firmicutes in infant microbiome was suggested to explain the effects on later overweight in children. There was only one small study of 20 newborn infants with meconium samples available.

Table 1. The three studies included in the study by McLean et al. in 2019.

| Author, study type, title | Participants | Data | Smoking outcome measures | Method | Results |
|---|---|---|---|---|---|
| **Tun et al. 2017 Prospective cohort study *** | 959 infants | 3-4 months, fecal stool sample | No exposure; exposure during pregnancy; exposure only postnatally; and exposure during pregnancy and postnatally | 16S rRNA Illumina MiSeq | ↑Firmicutes richness (Ruminococeaceae and Lachnospiraceae) at 3 months in infants exposed to tobacco smoke postnatally or both pre- and postnatally or both pre- and postnatally. Mediation analysis: Firmicutes richness potentially mediated postnatal smoking exposure and risk of childhood overweight at age 1 and 3 years |
| **Levin et al. 2016 Prospective cohort study** | 298 infants | 1 months and 6 months, fecal stool sample | Maternal questionnaire: if any person living in the home smoked Mother smoked: having one cigarette per day | 16S rRNA Illumina MiSeq | Neonates currently exposed to ETS: Ruminococcus and Akkermansia↑, infants of mothers who smoked either during pregnancy or currently: ↑Bacteroides and Staphylococcus<br>↑Bacterial richness, diversity and evenness at 1 mon |
| **Gosables et al. 2013 Prospective cohort study** | 20 infants | Meconium | Maternal questionnaire: Smoked during early pregnancy (Y/N) or smoked entire pregnancy (Y/N) | 16S rRNA 454 Roche | ↑Enterobacteriaceae, less diversity associated with smoking during entire pregnancy |

* The original article was not available. The data in this table is based on the information from the McLean et al. study

# 3. STUDY ABOUT MATERNAL SMOKING AND FIRST-PASS MECONIUM MICROBIOME

## 3.1. Hypothesis, aims and objectives

Smoking has been shown to associate with different gut microbiome in adults, with effects on the relative abundance of the main phyla. Previously, maternal smoking during pregnancy has been widely reported to have negative impact on the subsequent health of the child. We hypothesized, that the maternal smoking may affect on the development of infants' microbiome already in the prenatal and perinatal period. We set out to investigate the impact of maternal smoking during pregnancy on the gut microbiome in the first-pass meconium developed before birth in a cohort of 131 newborn infants with available gut microbiome data of the first-pass meconium and maternal smoking status during pregnancy.

## 3.2. Patients and methods

The study cohort included 212 newborn infants enrolled in Central Finland Central Hospital in Jyväskylä, Finland, between the 3rd of February in 2014 and the 13th of March in 2014. The ethical committee approved the study plan. All families gave their written informed consent before the study (Tapiainen et al. 2018). Earlier, other maternal factors affecting possible fetal microbiome have been reported using the same cohort (Tapiainen et al. 2018). In brief, all mothers delivering during that period were invited to participate. Parents completed medical history by questionnaire including consumption of probiotics and antimicrobials during pregnancy. The first meconium was collected by the midwife from the newborn's diaper and put into two sample tubes. Meconium samples were cooled immediately and kept at a refrigerator. Later, the samples were frozen and transferred to University of Oulu, where the meconium samples were analyzed using 16S rRNA gene sequencing.

The precise stool sample collection and DNA analyzing techniques have been described earlier (Tapiainen et al. 2018). Maternal smoking status was verified at one year of age with a separate follow up questionnaire. To perform a quantitative statistical analysis of the microbiome data, we created sum variables for relative abundances of phyla and genera

in SPSS software using the data received from the bioinformatics analysis. We used the Mann-Whitney U-test for the univariate analyses of relative abundances. In addition, we compared the numbers of operational taxonomic units (OTUs) and the Chao, Simpson and Shannon diversity indices between the smoker and non-smoker groups. The statistical analyses were performed with SPSS 22 software.

## 4. RESULTS

### 4.1. Study population

We had maternal smoking status and gut microbiome data of the first stool available from 131 (62%) of 212 infants. Reported maternal smoking was rare during pregnancy (N=5; 3.8%).

### 4.2. Impact of maternal smoking on the microbial diversity in infants

The observed number of the OTUs was lower in the first stool after birth in infants who had been exposed to maternal smoking during pregnancy. In infants born to mothers who smoked during pregnancy the mean value of OTUs was 134 (SD 52), median 145 and for non-smoking mothers the mean value of OTUs was 200 (SD 116), median 171.

For comparing the diversity of gut microbiome between the study groups, we used Shannon, Simpson and Chao1 diversity indices (Figure 1). Mean Shannon index for mothers who smoked during pregnancy was 4.73 (SD 1.28) and for mothers who did not smoke 5.63 (SD 1.51). Mean Simpson index for mothers who smoked was 0.87 (SD 0.11) and mothers who did not smoke 0.92 (SD 0.09). Mean Chao1 index for mothers who smoked was 202 (SD 81) and for non-smokers 319 (SD 216). As a conclusion, the gut microbiome of the infants who mothers did not smoke during pregnancy appeared to be more diverse. However, due to small number of smokers, we were not able to analyze statistical significance of the differences.

Table 2. The microbial diversity indices and the relative abundances of the main phyla between the study groups.

| | **Smoked during pregnancy** | **Did not smoke** |
|---|---|---|
| **Observed otus** | | |
| **- mean (SD)** | 134.40 (51.79) | 199.92 (116.31) |
| **Chao1** | | |
| **- mean (SD)** | 201.64 (80.45) | 318.94 (215.65) |
| **Simpson** | | |
| **- mean (SD)** | 0.87 (0.11) | 0.92 (0.09) |
| **Shannon** | | |
| **- mean (SD)** | 4.73 (1.28) | 5.63 (1.51) |
| **Actinobacteria** | | |
| **- mean (SD)** | 2.0% (4.3%) | 0.7% (1.6%) |
| **Bacteroidetes** | | |
| **- mean (SD)** | 18.3% (23.3%) | 17.0% (22.5%) |
| **Firmicutes** | | |
| **- mean (SD)** | 41.8% (36.6%) | 45.7% (30.8%) |
| **Proteobacteria** | | |
| **- mean (SD)** | 36.5% (27.2%) | 32.8% (34.7%) |
| **Bacteroides SUM** | | |
| **- mean (SD)** | 3.3% (7.4%) | 14.3% (21%) |
| **Staphylococcus SUM** | | |
| **- mean (SD)** | 36.0% (37.9%) | 14.4% (24.4%) |

### 4.3. Impact of maternal smoking on the gut microbiome in infants at the phylum level

We found that the mean relative abundance of Bacteroidetes phylum was 18% (SD 23%) in infants born to mothers who reported smoking during pregnancy. In infants not exposed to maternal smoking, the mean relative abundance of Bacteroidetes phylum was 17% (SD 23%). However, the box plot analysis (Figure 2) shows higher levels of Bacteroidetes phylum in infants exposed to maternal smoking, with the smoking group median 12% and non-smoking group median 1.2%. Firmicutes phylum appeared to be more similar between the studied groups with smokers mean relative abundance of 42% (SD 37%) and non-smokers mean relative abundance of 46% (SD 31%). The median value for smokers was 40% and 48% for non-smokers, showing slightly decrease on Firmicutes phylum for infants exposed to maternal smoking. The relative abundance of the third main phylum, Proteobacteria was 37% (SD 27%) in infants exposed to maternal smoking and 33% (SD 35%) for infants not exposed to maternal smoking. The median value for infants exposed to maternal smoking was 36% and in infants not exposed to maternal smoking the median was 17%, suggesting increase on the relative abundance of Proteobacteria phylum. The relative abundance of Actinobacteria phylum was 2.0% (SD 4.3%) in infants exposed to maternal smoking and 0.7% (SD 1.6%) in infants not exposed to maternal smoking. In both study groups the median value for Actinobacteria phylum was 0.08%.

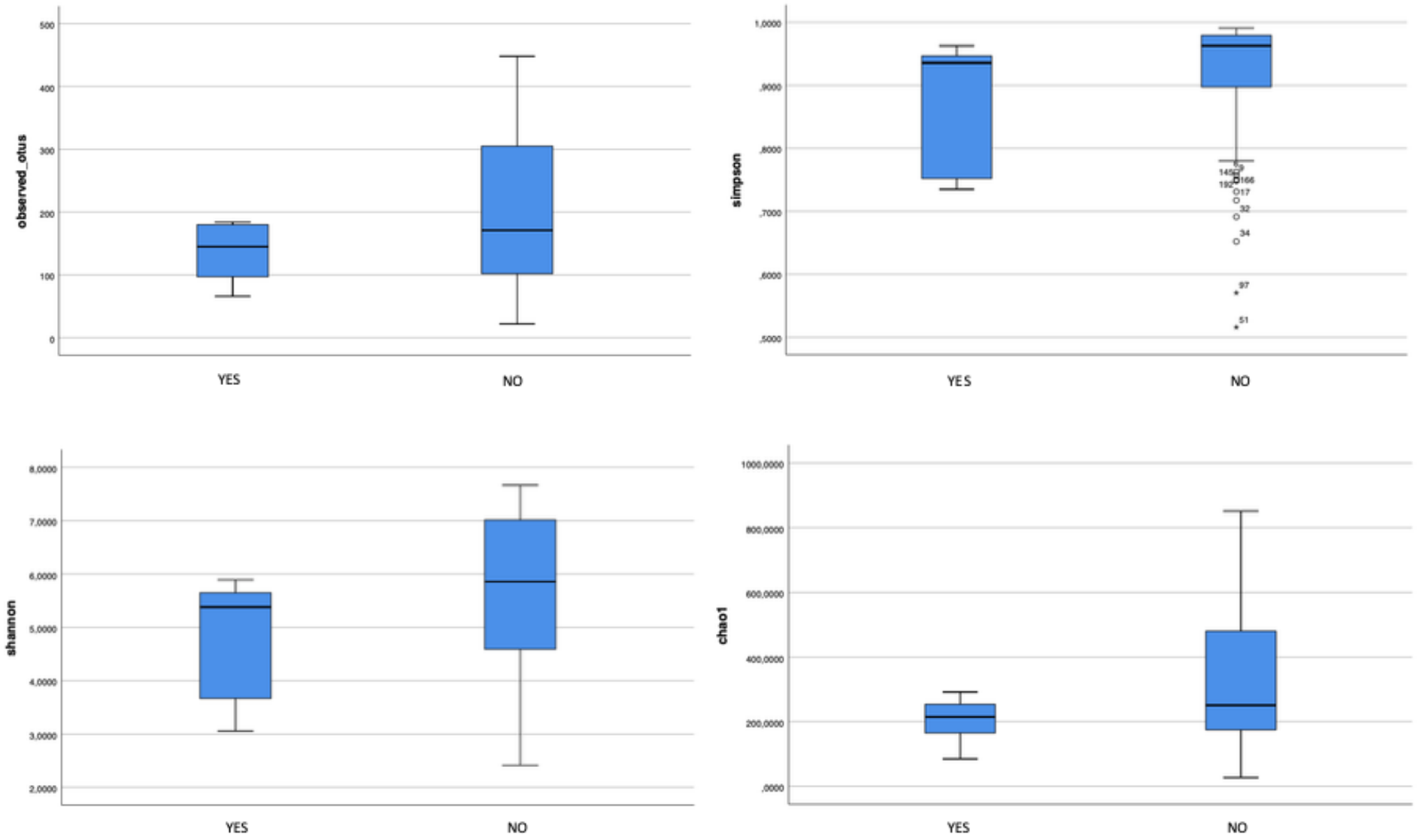


Figure 1. Diversity indices between mothers who smoked (YES) and mothers who did not smoke (NO).

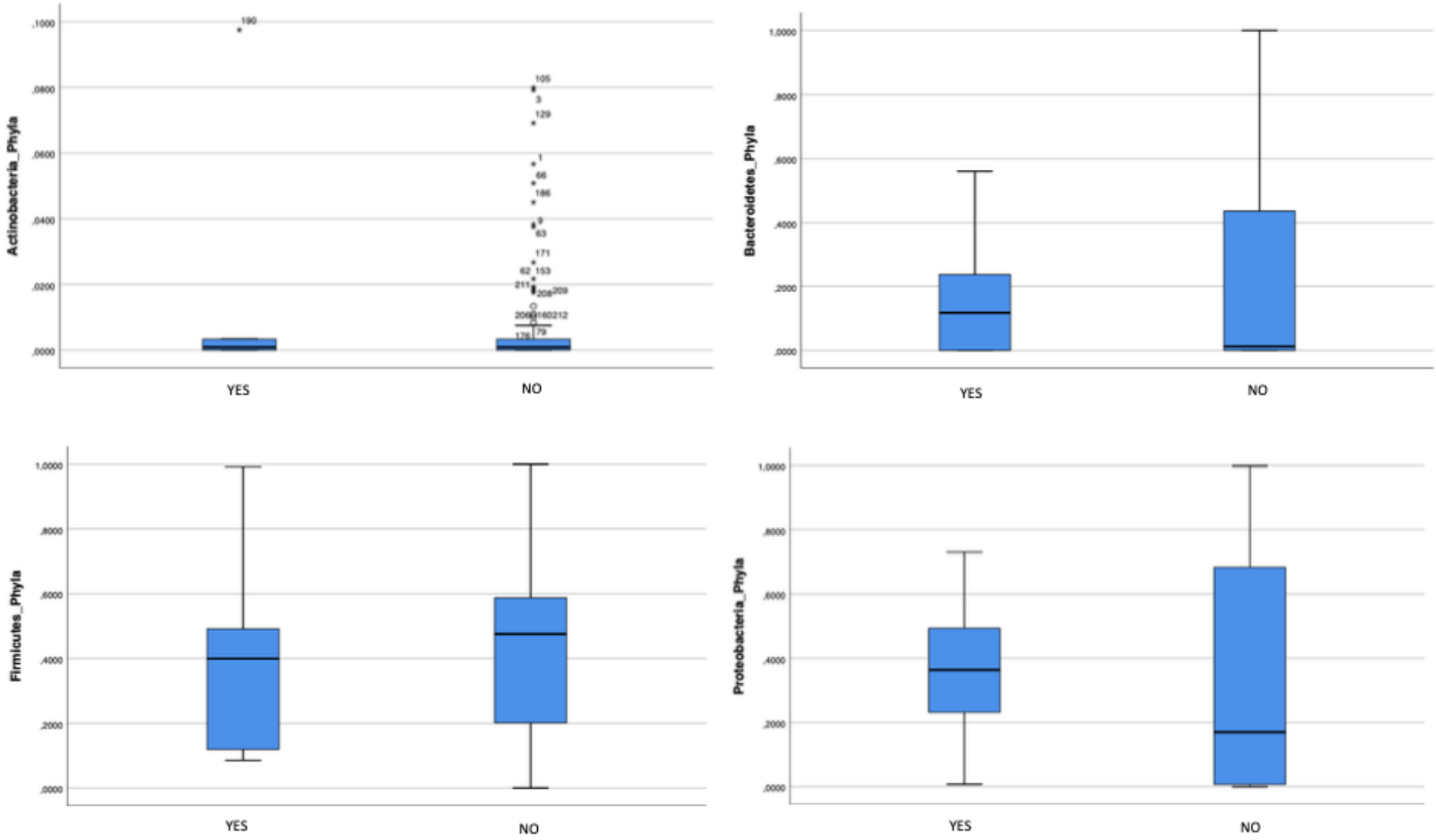


Figure 2. The relative abundances of some of the main phyla between the studied groups. Mothers who smoked (YES) and mothers who did not smoke (NO)

## 5. DISCUSSION

In this preliminary study, maternal smoking during pregnancy was associated with changes in the infants' gut microbiome. In infants whose mothers smoked during pregnancy the relative abundance of Bacteroidetes and Proteobacteria phyla appeared to be increased. The microbiome of infants exposed to maternal smoking during pregnancy appeared to have less diverse intestinal microbiome and lower levels of OTUs.

In a small study by Gosables et al. (2013) analyzed meconium samples from 20 healthy newborns. In their study 10% of the mothers had smoked during entire pregnancy. Gosables et al. found that infants born to mothers who smoked during entire pregnancy had less diverse meconium microbiome and Enterobacteriaceae colonization rates were higher. In our study, infants born to mothers who smoked during pregnancy had also less diverse microbiome. Enterobacteriaceae (part of the Proteobacteria phylum) was not analyzed separately, but the relative abundance of Proteobacteria was decreased in our study. Levin et al. (2016) also analyzed direct maternal smoking and its relation to changes in the infant gut microbiome with stool samples at the age of 1 and 6 months. In their study, 10% of the mothers smoked during pregnancy with the total sample size of 298. They found that infants exposed to maternal smoking had higher abundance of Bacteroides and Staphylococcus at 6 months. In our study infants exposed to maternal smoking had higher abundance of Staphylococcus but lower abundance of Bacteroides. In the study by Tun et al., environmental exposure to tobacco smoke in infants with stool samples in the age of 3-4 months was studies showing Firmicutes richness at 3 months in infants exposed to tobacco smoke postnatally or both pre- and postnatally. (Tun et al. 2018) In our study, the median values suggested decrease on the relative abundance in Firmicutes phylum. The results from these three cohort studies are not directly comparable to our own results, as in two of the articles, the stool samples were taken months after birth, when perinatal factors had had time to modify the microbiome of the infant. In addition, the tobacco smoke was mostly environmental exposure rather than direct maternal smoking. In Gosables' meconium data, the starting points were closest to ours, but the limitation of their study was the small sample size.

Our study results appear to be in line with earlier research in adults. In adults, smoking affects the intestinal microbiome by increasing the relative abundance of Proteobacteria

and Bacteroidetes phyla and decreasing the relative abundance of Actinobacteria and Firmicutes phyla (Savin et al. 2018, Lee et al. 2018, Huang&Shi 2019). Our study suggests that infants of mothers who smoked during pregnancy had decreased relative abundances of Bacteroidetes and Proteobacteria phyla and increased relative abundance of Firmicutes phylum.

### 5.1. Strengths and limitations

The strength of our study is the use of the first stool of life in studying the effects of maternal smoking on the fetal microbiome. By analyzing the first feces of the newborn, we minimize the environmental factors that modify the fetal microbiome immediately after birth. The limitation of the present study is the small sample size. Only 5 of the mothers smoked during pregnancy in our material, and due to small sample size, we were not able to analyze statistical significance of the differences. The amount of maternal smoking such as the number of tobaccos smoked per day and the duration of the maternal smoking (early/late/entire pregnancy) was not asked part of the questionnaire, therefore respond to dose can not be determined. In addition, the environmental exposure to tobacco smoke was not measured.

### 5.2. Future directions

The limited evidence on this subject and the interesting results from our preliminary study requires further large-scale studies to research more precisely the impact of maternal smoking to infants' gut microbiome. We suggest meconium-based studies with smoking data systematically collected with the amount of maternal smoking and expose to environmental smoking.

At the moment, there is an ongoing study conducted by Tapiainen study group with the material of 500 children with smoking status for both of the parents systematically collected. Results from that study will be available in the near future. If the larger scale study shows more direct relation between maternal smoking and changes in infants gut microbiome, it rises more interesting questions about this topic.

In conclusion, smoking is a risk factor for many diseases, and maternal smoking increases morbidity and obesity of the infant in later childhood. With the results from our literature review and our preliminary study, we hypothesize that maternal smoking may impact on the development of the early microbiome and may interferes the immunological maturation. We suggest that larger population-based studies explore the impact of maternal smoking on the infant gut microbiome in the future.

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