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Dynamics of gut microbiota emergence during fetal development in mice model*

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Formation of prenatal gut microbiota is extremely important in shaping balance between health and disease. Until now, the course of pregnancy was believed to be a sterile environment. However, recent studies have suggested that microbial populations are present in the fetus also prior to birth. Present study for the first time looked at the origin of fetal gut microbiota in mice model, and indicated its exact route of transmission. Our data was able to report for the first time signature of microbial presence during fetal development, by identifying commensal microbiota as early as few days after implantation, including both bacterial and fungi species. Interestingly, we detected few species specifically related to gestation period. This strongly indicates that the first colonization occurs prenatally with species specificity manner. In conclusion, the study provides a great basis for tests on possible modulation properties of detected key-players microbes. The promising results could become a potential for future biotherapies.

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The animals and human body is a host to a large and complex beneficial (commensal) microbial communities consisted of hundreds of microbial species including bacteria and fungi, that inhabit areas of nasal, oral, skin, gastrointestinal, and urogenital areas [Human Microbiome Project Consortium 2012, Turnbaugh 2007]. However, the greater parts of these microbes reside mainly in the gastrointestinal tract [Lozupone *et al*. 2012, Ursell *et al.* 2012]. Such microbial communities play an important homeostatic role by metabolizing indigestible polysaccharides, producing essential nutrients, regulating fat storage, protecting from pathogens, and healthy immune development where can have both immune-stimulatory and immune-regulatory effects [Ackerman 2012]. Interestingly, differences in microbiota composition have been correlated with diverse infections and inflammatory diseases, showing that targeting or modulating the microbiota may be a novel therapeutic strategy that could nicely complement established treatments for inflammatory conditions.

The gut microbiota is established for its long-term prevalence. Different individuals might be a host to different microbial species, but any one individual is inhabited by the same core set of species for a long period of time [Dethlefsen and Relman 2011]. Therefore, maintaining the persistence of commensal gut microbiota is thought to be important for maintaining health by diseases prevention. Especially, that microbiota is capable of triggering the effect on health at body parts remote far from the gastrointestinal tract, by modulating responses in the immune system and/or by modulating the metabolites that are carried to distal organs in the bloodstream [May 1972, Coyte *et al.* 2015]. Nonetheless, our understanding of what enables the microbiota formation, what maintains their prevalence, and what can break this stability is still very obscure. Improving our knowledge on these aspects is particularly important for disease predisposition and prevention prognosis.

The past years have attracted remarkable attention of gastrointestinal tract (GIT) and its accompanying microbial communities, and how their diversity influences the biology of many disorders and diseases [Sekirov *et al.* 2010, Shanahan 2013, Yeung *et al*. 2018]. The research has been focusing on diseases ranging from allergies [Fujimura and Lynch 2015], inflammatory bowel disease, colorectal cancer [Yamamoto and Matsumoto 2016], obesity [Musso *et al.* 2010], altherosclerosis [Huminiecki *et al.* 2017, Huminiecki and Horbańczuk 2018, Yeung *et al*. 2019, 2020, 2021abc, Wang *et al.* 2020], and neurological conditions eg. autism, schizophrenia, depression and migraine [Dinan and Cryan 2013, Gonzalez *et al.* 2016, Moos *et al.* 2016, Tewari *et al.* 2017ab, 2018, Singh *et al.* 2020]. Furthermore, an increasing interest has been put on finding a link between disruption in normal balance between gut microbiota and their host and the predisposition in the development of autoimmune diseases [Vieira *et al.* 2014]. Moreover, it has been recently indicated that the formation of microbiota initiates with the beginning of life, during gestation and develops promptly after birth. Therefore, the placenta and growing fetus are not a sterile environment as it was believed until now [Hu *et al*. 2013, Wassenaar and Panigrahi 2014].

Many factors including environment, mother's genetic set-up, hormonal changes, immune system alterations, have a substantial impact on how the composition of the microbiota community shapes during gestation and how they contribute to the health of the individuals in the postnatal life. Additionally, the antibiotic used during pregnancy, breastfeeding and the type of delivery are well established factors that affect the early life microbial composition [Dominguez-Bello *et al.* 2010], but whether embryonic implantation itself modulate the fetus and infant microbiota has not been investigated yet. Furthermore, it is insufficiently understood whether the maternal microbiota can predict the set-up of fetus composition and therefore its predisposition to diseases in postnatal life.

Little is known about the time the first microbial composition begins its formation and what factors, including implantation, maternal microbiota set-up and possible microbiome dysbiosis play a crucial role in that process and how does it affect later health and development of diseases, which is particularly important for healthy life prognosis. In particular, what microbial components of primary gut microbiota shape the immune system responses to basic infections in the early life development, contributing to predispositions to certain diseases in the later life. To this end, would be beneficial to find commensal microbial resources that can modulate the microbiome composition and maintain their healthy composition or shift into the natural when is misbalanced.

In order to track the role of gestation related microbiota, it is important to consider the time when and how the host-microbial association begins and which factors play a role in their development, as in relation to mother and fetus microbiome separately. This allows to address the questions more specifically on when and under what conditions the growing fetus first encounter microbial colonization and whether this process is influenced by maternal microbiota.

Herein, we investigated the dynamics of gut microbiota development during mice fetal development. By employing shotgun metagenomics approach, we investigated and identified the specific core bacterial and fungi species associated with fetal development from the moment as early as few days after implantation. Our results provided a comprehensive view on what microbial species are associated with healthy mice pregnancy. These prevalent gut species could be further used as important key individuals in the re-balancing maternal gut microbiota, which might directly shape the fetal microbiota composition, contributing to disease predisposition in the postnatal life.

Material and methods

Samples /mice model

The project used 45 female and male mice belonging to C57BL6 strain derived from local laboratory of the Institute of Genetics and Animal Biotechnology PAS in Jastrzebiec. C57BL/6J mice were maintained under inbreeding in the animal facility of the Institute. Mice were housed in 265 x 207 x 140 mm transparent polycarbonate cages in groups of 3-5 mice per cage, and maintained under 12h light-dark cycle, 20-

22°C and 40-60% humidity into a HEPA-filtered room, with food (Labofeed H, Kcynia Poland) and water ad libitum. All procedures used in the experiments were reviewed and approved by the II Local Ethics Committee for Experiments on Animals in Warsaw (Approval no.: 62/2015; 25.06.2015). C57BL6 inbred mice was reproduced in the local laboratory. This strain was chosen due to its most widely used "genetic background", to have its full genome sequenced. Additionally, it is easy breeding, and robust model for studying diseases, immunological responses and genes expression. As an inbred mice are as genetically identical as possible, therefore no genetic variation was introduced that could have influenced results outcome. The mice used in the study were 7-9 weeks old, since at this age range are considered to have reached sexual maturity.

Initially, five virgin female mice were screened for the diversity of the present gut microbiota by collecting gut homogenate samples and performing shotgun metagenomics analysis.

Natural fertilization

Five experimental groups of five female mice were set-up, representing following time points: developing fetus on 10^{th} day of pregnancy, developing fetus on 14^{th} day of pregnancy, developing fetus on 18^{th} day of pregnancy, neonate immediately after delivery and neonate at 10 days after delivery. Initially in each group, female mice were kept with males in separate cages, however with the close contact (to stimulate hormonal production and increase fertility rate/fertility success). After 48 hours males and females were put together into one cage in order to unable insemination (three male on five female mice). After 24 hours females were checked for the presence of vaginal plugs and ones carrying them were put into new, sterile cages allocated in number of five and treated as one experimental group. Such an experimental group was kept in sterile cage under conditions optimal for mice growth and development.

At 10^{th}, 14^{th}, 18^{th} day of pregnancy mice were euthanized via cervical dislocation, embryos removed and gut organs collected and processed under aseptic conditions (Tab. 1). All intestines of the fetuses and neonates of the same litter were weighed, pooled and immediately homogenized in PBS and treated as one sample. All samples were assessed in metagenomic studies to capture differences in microbiota composition, taking into account bacteria and fungi species (Fig. 1).

Isolation of gut (intestine) samples

Whole intestine collection was collected from removed embryos after killing pregnant female mice by cervical dislocation, followed by homogenization in PBS and used for prompt DNA isolation for NGS analysis. Intestines isolated from embryos belonging to one animal were mixed and used as one sample for further analysis.

Isolation of microbial DNA

Microbial DNA was extracted from homogenized gut samples using commercially available kit (Quiagen, The QIAamp *cador* Pathogen Mini Kit). Briefly, fetal samples

Table 1. The gut samples of maternal and fetal mice in five replicates used in the shotgun metagenomics study

Time point	Maternal	Fetal/newborn
10th day of pregnancy	5 replicates	5 replicates
14th day of pregnancy	5 replicates	5 replicates
18th day of pregnancy	5 replicates	5 replicates
Delivery	5 replicates	5 replicates
10th day after delivery	5 replicates	5 replicates

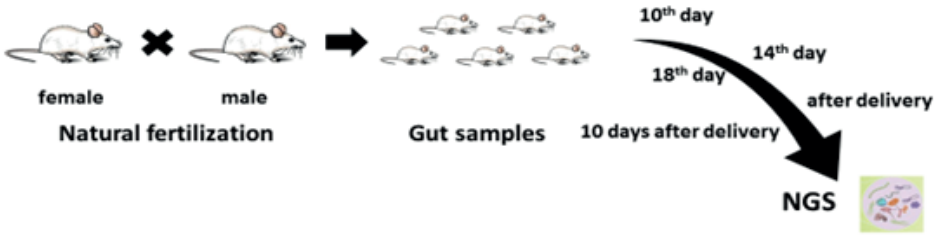

Fig. 1. Experimental approach of the *in vivo* fetal gut microbiota experiment. Figure represents steps including: natural fertilization, intestine isolation from fetus and neonate at five different time points, and NGS analysis.

from the same mice that were to be sequenced were pooled, and 25 mg of this pool was used isolate genomic DNA. 25 mg of the maternal intestine was used, accordingly. Samples were suspended in 200 µl freshly prepared PBS and homogenised using MagnaLyser at 2000 rpm for 2 minutes. The rest of the sample of pooled endpoints was stored at -20°C in case DNA isolation needed to be repeated. DNA concentration of both fetal and maternal DNA was determined using the Nanodrop (Thermo Scientific) and quality was determined by gel electrophoresis. Equimolar amounts of genomic DNA were mixed to yield final DNA samples containing >3 µg DNA. These samples were analysed by ServiceXS B.V. using Illumina HiSeq (paired-end, 100 read length).

Gut microbiota profiling

Shotgun metagenomics approach allowed comprehensively sample all genes of all microbes present in the intestine tissue.

Libraries were prepared with Nextera DNA Flex Library Prep Kit (Illumina, 20018704, 20018707 and 20024144). Five biological replicates were prepared for each sample. Libraries were sequenced on Illumina MiSeq platform for 300 bp paired-end reads. Obtained genomic data was analyzed on BaseSpace and OneCodex applications for k-mer alignments and taxonomic classification of microbial populations.

Statystical analyses

All data was representing the mean of samples from indvidual groups of independent experiments. Results were presented as mean and standard deviation

(mean±SD). One-way *ANOVA* was employed for testing the effect of time after implantation on the fetal microbiota composition. A p value less than 0.05 was considered being statistically significant.

Results and discussion

We performed series of experiments using inbred C57BL6 mice. We first followed natural fertilization. Then, total fetal and maternal gut samples representing time points: 10th, 14h and 18th day of gestation and the moment of delivery, accordingly were collected and immediately homogenized under aseptic conditions. Part of the homogenate was used for inoculation SD (Sabouraud Dextrose) and LB (Lysogeny broth) media to obtain growth of microbial species. Another part of the homogenate was used for direct total microbiome isolation, using specifically designed kits for bacterial and fungi species. DNA samples were later subjected to shotgun metagenomics sequencing on MiSeq Illumina device. In parallel, multiple controls of possible microbial contamination were run, to confirm sterile dissection techniques, and therefore to eliminate any faulty results. Growth of bacterial and fungi cultures of: cages where mice were kept, fume cabinet and surgical sets, which were used for gut samples' isolation, were propagated. All cultures were negative after 48-hours interval. A positive control of non-sterile lab areas, and mixture of several bacterial and fungi strains were included, in order to maintain normal growth conditions. Furthermore, DNA template free controls of isolation and sequencing kits were performed, using protocols run for core experimental samples. Obtained set of data is based on mixed/metagenomics samples, where each ~3-7% reads were classified using both: BaseSpace and One Codex databases.

Identification of bacteria and fungi in maternal and fetal mice species

Our study reported for the first time signature of microbial presence during fetal development, by revealing identification of commensal microbiota as early as few days after implantation (10th day of pregnancy), including bacterial and fungi species (Fig. 2AB, and Fig. 3). Among most important healthy gut bacterial genuses, we identified following: *Bacteroides, Butyrivibrio, Bifidobacterium, Lactobacillus, Roseburia, Ruminococcus, Eubacterium, Parabacteroides, Enterococcus, Desulfovibrio, Odoribacter, Escherichia, Streptococcus, Oscillibacter, Prevotella, Alistipes*. Furthermore, we detected six fundamental fungi species naturally colonizing healthy gut: *Fusarium, Saccharomyces, Penicillium, Pichia, Cladosporium, Candida*. The link between abundance of species present in fetal gut with the time of gestation was reported in Figure 1A. The diversity of fetal gut community was significantly changing across developmental stages (one-way *ANOVA*: p=0.0434) In contrast, there were no significant differences reported between different stages of gestation and microbiota composition in maternal gut (one-way *ANOVA*: p=0.295). Additionally, we found higher diversity of bacterial and fungi species after delivery.

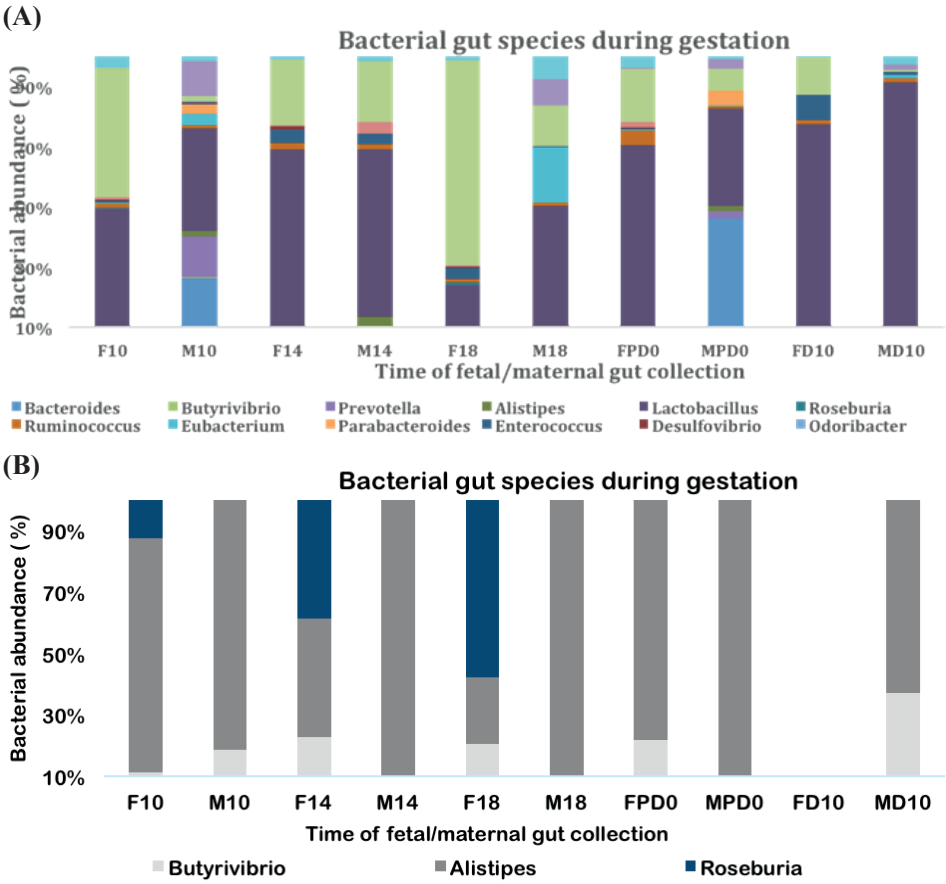

Fig. 2. Bacterial abundance in fetal and maternal gut samples at multiple time points. F stands for fetal, whereas M for maternal samples. Fig. A represents all bacterial species reported during gestation, whereas Fig. B only selected three that proved gestation specificity.

Identification of strains associated with pregnancy

We finally hypothetised that the presence of certain strains is strongly associated with maintenance of the pregnancy. Our data support such an assumption as we reported very interesting result in fetuses species, where *Roseburia* bacterial genus increases while the pregnancy progresses and vanishes upon delivery (one-way *ANOVA*: p=0.041). In contrast, maternal gut did not show any sign of such bacteria presence. Similarly, two other bacterial species: *Butyrivibrio* and *Alistipes* present during gestation were lost by newborn after delivery, whereas maintain at the same time by mothers' gut (Fig. 2B). This particular result strongly suggests species specificity manner during gestation, where certain bacteria might be required for healthy fetal development and perhaps maintaining the course of pregnancy. Furthermore, in terms

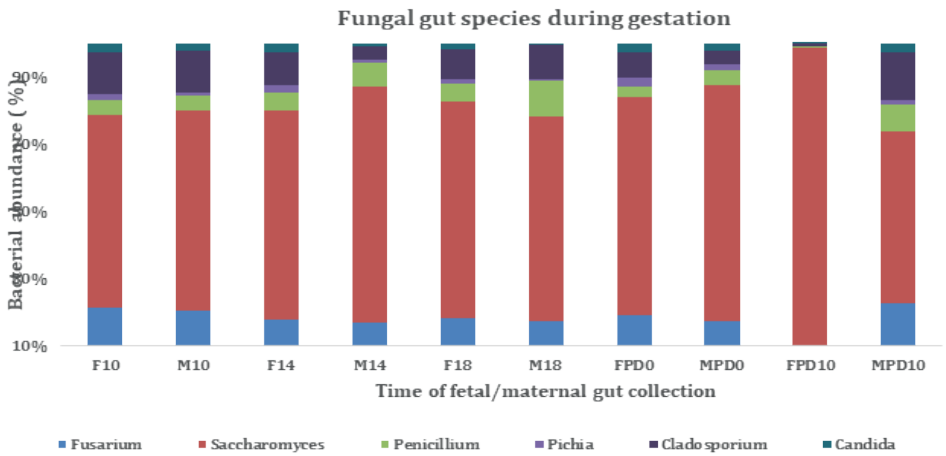

Fig. 3. Fungal abundance in fetal and maternal gut samples at multiple time points. F stands for fetal, whereas M for maternal samples.

of fungi species we reported *Fusarium* fungi genus disappearing after delivery in the favor of *Saccharomyces spp.* overtaking the majority of population. Based on this data set, we can strongly conclude that the first colonization indeed initiates prenatally, with few species specifically related to gestation. However, to rule out our experimental data set further studies are required to unravel their origin, exact time of formation and explain detected lost/maintained interactions.

Only a decade ago the course of pregnancy as well as the newborn were presumed to be so called "sterile" (free of microbial communities) - Tissier [1900]. Till today, some of the researchers still hold to that phenomenon. However, there is an increasing evidence gathered around microbial colonization beginning its root during gestation [Aagaard *et al.* 2014], and microbial species appearing in the amniotic fluid [Wang *et al.* 2013], umbilical cord blood [Jiménez *et al.* 2005], fetal membranes [Satokari *et al.* 2009], and placenta [Stout *et al.* 2013]. Placenta is among least studied organs of living organisms in terms of the microbial communities present within it. In a previous study, where nearly 30 biopsies, isolated from the placenta after the C-section delivery has demonstrated presence of the widely met gut *Lactobacillus* and *Bifidobacterium spp*. bacteria. In the investigation there have been neither damages in the membranes, nor indications of maternal infection, therefore clearly pointing to the placenta specific microbiota [Satokari *et al.* 2009]. Yet, another study has demonstrated that bacteria are commonly present in the placenta during the normal pregnancy [Stout *et al.* 2013]. Species colonizing placenta resemble oral commensal microbes, belonging to non-pathogenic *Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes* and *Fusobacteria* species. Such results have been presented for both mice [Fardini *et al.* 2010] and human [Fardini *et al.* 2010]. Additionally, other researchers have shown that the bacterial compositions from the preterm placentas are richer and more diverse when

compared with placentas from full-term gestations [Aagaard *et al.* 2014]. Until now, it has been believed that the amniotic sac is occupied by microbes derived from the vaginal barrier. However, researchers have suggested that uterus is inhabited by microbes that have been incorporated into the basal plate during the process of placenta implantation [Stout *et al.* 2013]. Furthermore, it has been suggested that uterus contains its own microbiome that can promote fetal colonization, as the placenta is developing from inner lining from the uterus [Mitchell *et al.* 2015, Verstraelen *et al.* 2016]. Others have claimed, that placenta microbiome starts the colonization of the fetus as part of the regular developmental process [Aagaard *et al.* 2014, Jiménez *et al.* 2008, Collado *et al.* 2016, Fardini *et al.* 2010]. Hence, focusing and tracking implantation allows unraveling explicit time and conditions of the launch of fetal gut microbiota and changes in their composition in pre and early postnatal.

To this end, in this work, we presented for the first time the evidence that microbial development initiates during fetal development and that the course of pregnancy is not a sterile environment as it was believed until now. Tracking implantation allowed us to unravel explicit time and conditions of the launch of fetal gut microbiota and changes in their composition in pre and early postnatal. We found that the first colonisation of fetal gut initiates as shortly as few days after implantation (10th day of pregnancy), including both: bacterial and fungi species (Fig. 2 A and B, and Fig. 3). Moreover, we reported that such prenatal colonization is driven by few species specifically related to gestation.

Metagenomics and related methods have increased our understanding of the human and animal microbiome. Although, components of widely known probiotics used for re-balancing gut microbiota belongs mainly to *Lactobacillus* genus, it has also become a core specimen in various of studies based on untargeted gut microbiomes in humans and animals. Recent findings on *Lactobacillus* species in human and animal microbiome research, together with the increased knowledge on probiotic and other ingested lactobacilli, have resulted in new perspectives on the importance of this genus to health [Heeney *et al.* 2018]. To this end, the notable variation in intestinal abundance of this genus between healthy and disease-carrier, or health-compromised, individuals indicates that *Lactobacillus*, or at least certain species or genotypes of *Lactobacillus*, might be useful gut biomarkers for many conditions. In our data the abundance of *Lactobacillus* in mice fetal was stable during pregnancy. However, we found strong increase of this genus in newborn mice in 10^{th} day after delivery. Less profound effect was reported in maternal gut.

Research on healthy gut microbiota development focusing on other microbial domains than bacteria has limited number of studies to date. Majority of studies focusing on gut microbiota ignore the Fungi due to their low abundance, since they make up ~0.1% of the total microorganisms in the gastrointestinal tract of human and animals. However, still dysbiosis of the gut fungal community might have serious consequences resulting in many intestinal diseases [Ott *et al.* 2008]. Recent study with the use of gene marker sequencing conducted on purified stool specimens showed that

the most prevalent fungal genus is *Saccharomyces* (in 89% of the samples) [Hoffmann *et al.* 2013]. Interestingly, it has been also indicated that newborn are characterised by much higher richness of fungi compared to adults. This assumption is demonstrated in our results, since *Saccharomyces* comensal fungus reaches higher abundance in 10 days old neonate mice compared to their adult mothers (Fig. 3).

In summary, our study examined for the first time the course of emergence of microbiota during fetal development in mice model. We identified the composition and the diversity of the gut microbial communities in mice during pregnancy and shortly after delivery in order to establish the point of gut colonisation emergence during gestation. Specifically, dynamic of changes in the gut microbiota of maternal and accompanying fetal species were monitored during different time points, and the gut microbiota communities of maternal and fetal species were compared. The results presented herein provide a new knowledge on microbiota origin and the dynamics of gut microbiota changes with the development of pregnancy, and open the window into further studies on mechanism and steps involved in gut development, as well as possible application of detected as important key player's microbial species in the re-balancing maternal gut microbiota, which directly shapes the fetal microbiota composition, contributing to disease predisposition in the postnatal life. In conclusion, the study provide a great basis for tests on possible modulation properties of detected key-players microbes. The promising results could become a potential for future biotherapies.

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