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Method paper

A workflow to study the microbiota profile of piglet's umbilical cord blood: from sampling to data analysis

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ABSTRACT

The possibility of pre-birth microbiota colonisation remains controversial in the scientific community. Due to the placenta's characteristics in pigs, the umbilical cord is the sole way for mother-foetus microbial transmission to occur. Studies on this topic have demonstrated conflicting results; some of these discrepancies might be due to differences during sampling, DNA extraction, bioinformatics and data analysis. The aim of this study is to assess a workflow for characterising the umbilical cord blood microbial profile by adjusting for the contaminating sources of bacterial DNA during the extraction procedure. The results show that among 735 amplicon sequence variants (**ASVs**), 568 ASVs were contaminants, while 165 ASVs were true samples. Using this workflow, we could distinguish the contaminant ASVs introduced during bacterial DNA extraction and amplification. With the results of the present study, however, we cannot confirm the pre-birth bacterial transfer by the umbilical cord blood due to the lack of samples representative of the contaminants in the surrounding sampling environment. Nevertheless, the present study can be used as a reference to address low microbial biomass, particularly with umbilical cord blood.

Implications

Conducting analyses of low microbial mass, such as with umbilical cord blood, can be challenging because of the presence of contaminants in the surrounding sampling environment and in the laboratory. Such challenges can lead to the misinterpretation of results. The present study proposes a workflow – from sampling methods to DNA extraction, bioinformatics and data analysis – that characterises the bacterial profile of umbilical cord blood samples, taking into account the contaminants found throughout the procedure of bacterial DNA extraction and amplification.

Specification table

Subject	Physiology and Functional Biology
Type of data	Boxplots, R code (version 4.0.2)
How data were acquired	Umbilical cord blood samples were collected with a 3 ml disposable sterile syringe (Covetrus BV, Cuijk, Netherlands) and a 21 G × $^{5}/_{8}''$ (0.8 × 16 mm) sterile injection needle (Kruuse, Marslev, Denmark) and transferred into a 4 ml BD Vacutainer K2E (BD Vacutainer Systems, Plymouth, UK); bacterial DNA was extracted using the HostZero Microbial DNA Kit (Zymo Research, Irvine, CA, USA).

(*continued on next page*)

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Data format	Raw data, analysed data and output from RStudio (version 4.0.2)
Parameters for data collection	Data were collected from piglets' umbilical cords at birth; during each farrowing, one piglet per litter of medium visual weight was randomly selected.
Description of data collection	Amplicon sequence variants (ASVs) were generated using DADA2 1.14.0 (Callahan et al., 2016), running on R 4.0.2; for taxonomic assignment, the Silva database, release 138 (Quast et al., 2012, was used as reference. The V3-V4 region of the 16S rRNA gene (~460 bp) was then amplified; amplicons were produced using the universal primers Pro341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014) using the Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Monza, Italy). Sequencing was performed using the Illumina MiSeq platform 300 × 2bp. The libraries were prepared using the standard protocol for MiSeq Reagent Kit V3 and sequenced on the MiSeq platform (Illumina Inc., San Diego, CA, USA).
Data source location	Institution: Agroscope City/Town/Region: Posieux, Fribourg Canton Country: Switzerland Latitude and longitude (and GPS coordinates, if possible) for collected samples/data: 46°46′07.5″N, 7°06′17.9″E
Data accessibility	Repository name: Sequence Read Archive (SRA) Data identification number: PRJNA880850 The DADA2 phyloseq object and R scripts used for analysis are available at: https://doi.org/10.5281/zenodo.

Introduction

The uterus is generally accepted to represent a sterile environment for the foetus (Tissier, 1900). The first bacterial colonisation of the offspring gastrointestinal tract seems to occur mainly when the newborn is passing through the birth canal, via contact with the vaginal microbiota (Mackie et al., 1999). However, Walker et al. (2017) hypothesised that in humans, the establishment of the intestinal microbiota starts before birth via the passage of bacteria through the placental barrier or through the ingestion of amniotic fluids. Several studies using 16S rRNA sequencing agreed on the hypothesis that a vertical transfer from mother to foetus already occurs before birth. One finding that could confirm this hypothesis is the umbilical cord blood microbial profile reported in human and pig studies (Jiménez et al., 2005; Leblois et al., 2017).

Nonetheless, the existence of pre-birth microbiota is still questioned, as reported by Gschwind et al. (2020), who found that bacterial DNA extracted from the in utero environment might be the result of samples exposed to contaminant bacteria during farrowing. The discrepancies between studies might be the result of different methods of sampling and handling as well as the absence of true negative controls, such as blank samples for estimating the bias that may occur in every step of the analysis. Especially when considering low microbial biomass samples, estimating the amount of contaminant bacteria introduced during each step of the analysis (from DNA extraction to the library preparation) is crucial (Glassing et al., 2016). In this sense, tools that can recognise, remove and classify contaminant amplicon sequence variants (**ASVs**), such as the "Decontam" R package developed by Davis et al. (2018), have been successfully applied in different studies (Karstens et al., 2019; Claassen-Weitz et al., 2020). The aim of the present work was to define a detailed workflow – from sampling methods to DNA extraction, bioinformatics and data analysis – to allow for investigating pre-birth microbiota transfer through umbilical cord blood analysis.

Material and methods

Animal housing

The experiment was performed on 13 Swiss Large White sows originating from the Agroscope herd, divided into two farrowing batches separated by three weeks. The first farrowing batch was composed of seven animals, while the second farrowing batch was composed of six animals. The sows were individually housed in pens of 7 m^2 and bedded with straw. Room temperature was maintained at 24 °C, and artificial lights were kept on from 0800 h to 1700 h. Feed was provided three times per day (at 0700, 1200 and 1700 h), and they had free access to water. Farrowing was induced once the gestation period exceeded 115 and 116 days for primiparous and multiparous sows, respectively. Sows received two intramuscular doses (0.5 ml each) of cloprostenol (0.25 g/ml) (Estrumate, MSD Animal Health GmbH, Luzern, Switzerland) at 24-hour intervals. Two people were present during the whole time of farrowing.

Material preparation and sampling procedures

The sampling methods and materials used in the present study followed the procedure described by Leblois et al. (2017). During each farrowing, one piglet per litter of medium visual weight (total 13 piglets) was randomly selected. The sampling procedure required two people. When a piglet was expulsed, one person held the newborn and took care that it would not touch the floor and then clamped the cord. The umbilical cord surface was then disinfected with 70% ethanol by the other person. After disinfection, the second person collected blood while wearing sterile gloves, using a 21 G × $^5/_8$″ (0.8 × 16 mm) sterile injection needle (Kruuse, Marslev, Denmark) and a 3 ml disposable sterile syringe (Covetrus BV, Cuijk, Netherlands). Blood was immediately transferred into a 4 ml BD Vacutainer K2E tube (BD Vacutainer Systems, Plymouth, UK) and mixed thoroughly. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. Sterile gloves were worn during the whole sampling process.

Analytical methods

Bacterial DNA extraction and sequencing

The DNA of the umbilical cord blood samples was extracted in two extraction batches using a HostZero Microbial DNA Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The DNA extraction of the 13 samples was carried out on the same day. Seven samples were extracted in the morning from

0900 to 1200 h and six samples were extracted in the afternoon from 1400 to 1700 h. To determine possible reagent and laboratory contamination, in both the morning and afternoon extraction series, the same DNA procedure as for the umbilical cord blood was used for the nuclease-free water provided with the extraction kit (negative control). The yield and the purity (ratio of absorbance 260/280 and 260/230) of the extracted DNA were measured using a NanoDrop spectrophotometer (Fisher Scientific, Schwerte, Germany) and by a 1% (w/v) agarose gel (1%). The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified, and amplicons were produced using the universal primers Pro341F: 5′-TCGTCGGCAGC GTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC TACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014) using Platinum Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Monza, Italy). The DNA samples were stored at −20 °C until sequencing, which was performed using the Illumina MiSeq platform 300 × 2bp. The libraries were prepared using the standard protocol for MiSeq Reagent Kit V3 and sequenced on the MiSeq platform (Illumina Inc., San Diego, CA, USA).

Bioinformatics analysis

The ASVs were generated using DADA2 1.14.0 (Callahan et al., 2016) running on R 4.0.2; the Silva database, release 138 (Quast et al., 2012), was used as reference for the taxonomic assignment. The Decontam R package was used to identify contaminant ASVs using the prevalence method and a false discovery rate (**FDR**) threshold of 0.5 (Davis et al., 2018). Because the umbilical cord is generally considered a low microbial biomass (Glassing et al., 2016), and according to the observations of Davis et al. (2018), the *isNotContaminant* function was also implemented to select non-contaminant candidate ASVs. This function identifies non-contaminant sequences based on the prevalence of each ASV in the input feature table across true samples and negative controls (rdrr.io/bioc/decontam/man/isNotContaminant.html). Results about taxonomical composition are expressed as mean ± SD. When a specific taxon is present in only one sample, the calculation of the SD was not possible and was defined as not applicable **(NA)**.

Results

A total of 57,347 reads were attributed to 735 ASVs for the 13 umbilical cord blood samples and the two negative control samples. Before the application of the Decontam R package, 17 phyla (mainly Firmicutes 61 ± 4.1% and Proteobacteria 26 ± 4.5%), 99 families (mainly Lactobacillaceae 44 ± 8.7%, Pseudomonadaceae 16 ± 10.6% and Muribaculaceae 5 ± 3.7%), and 196 genera (mainly *Lactobacillus* 44 ± 8.7%, *Pseudomonas* 16 ± 10.6% and *Muribaculaceae_CAG-873* 5 ± 4.5%) were identified in the umbilical blood cord samples (Fig. 1). In the negative control samples, two phyla (mainly Proteobacteria 50 ± NA% and Firmicutes 5 ± NA%), two families (mainly Pseudomonadaceae 50 ± NA% and Lactobacillaceae 5 ± NA%) and two genera (mainly *Pseudomonas* 50 ± NA% and *Lactobacillus* 5 ± NA%) were identified. Using the Decontam R package (Davis et al., 2018), 568 ASVs were identified as contaminants, representing 77% of the previously identified ASVs.

After the application of the Decontam R package, two ASVs were identified as belonging to the Eukaryote kingdom and removed. Only 165 ASVs of the 735 ASVs we detected (22%) were thus identified as characterising the umbilical cord blood samples. The latter resulted in 10 phyla (Firmicutes 63 ± 6.2% and Proteobacteria 27 ± 6.3%, composing the major part), 58 families (Lactobacillaceae 50 ± 10.5%, Pseudomonadaceae 19 ± 13.0% and Muribaculaceae 6 ± 7.8%, composing the major part) and 89 genera (*Lactobacillus* 50 ± 10.5%, *Pseudomonas* 19.4 ± 13.0% and *Muribaculaceae_CAG-873* 6 ± 8.9%, composing the major part); see Fig. 2. Table 1 summarises the major bacterial profiles detected in the umbilical cord blood samples at the phylum, family and genus levels; before and after that, microbial sequences were processed using the Decontam R package.

Authors' points of view

The present study showed how most of the ASVs isolated from samples with low microbial biomass (such as umbilical cord blood) were derived from the negative control samples. Almost 78% of the total ASVs were considered contaminants, while only the remainder may be considered a picture of the microbial composition of the true samples. Similar observations were reported by Lauder et al. (2016), who sequenced the 16S rRNA gene on samples of placenta biopsy and on several negative control samples from the sampling environment, the reagents of two different DNA extraction kits and the laboratory where the bacterial DNA extraction was performed. The authors showed that most of the sequences observed in the placenta samples originated from sample contamination during the sampling and extraction procedures. Using the dataset of Lauder et al. (2016), dataset, Davis et al. (2018) implemented the same bioinformatics analysis method as the one

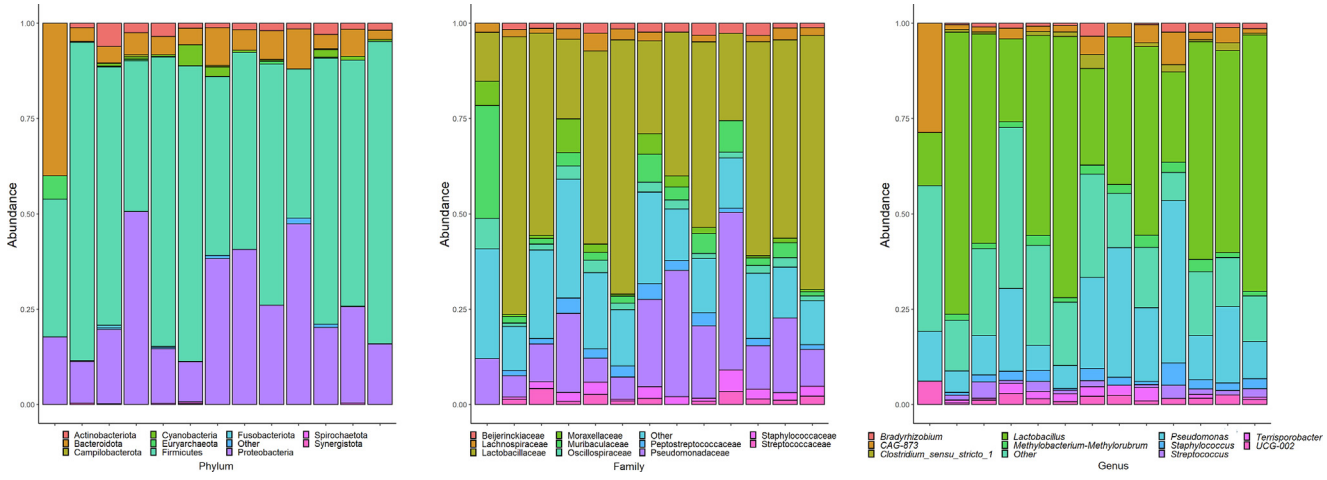

Fig. 1. Boxplot illustrating the major bacterial profile detected in each piglet's umbilical cord blood sample at the phylum, family and genus levels; before that, microbial sequences were processed using the Decontam R package (Davis et al., 2018).

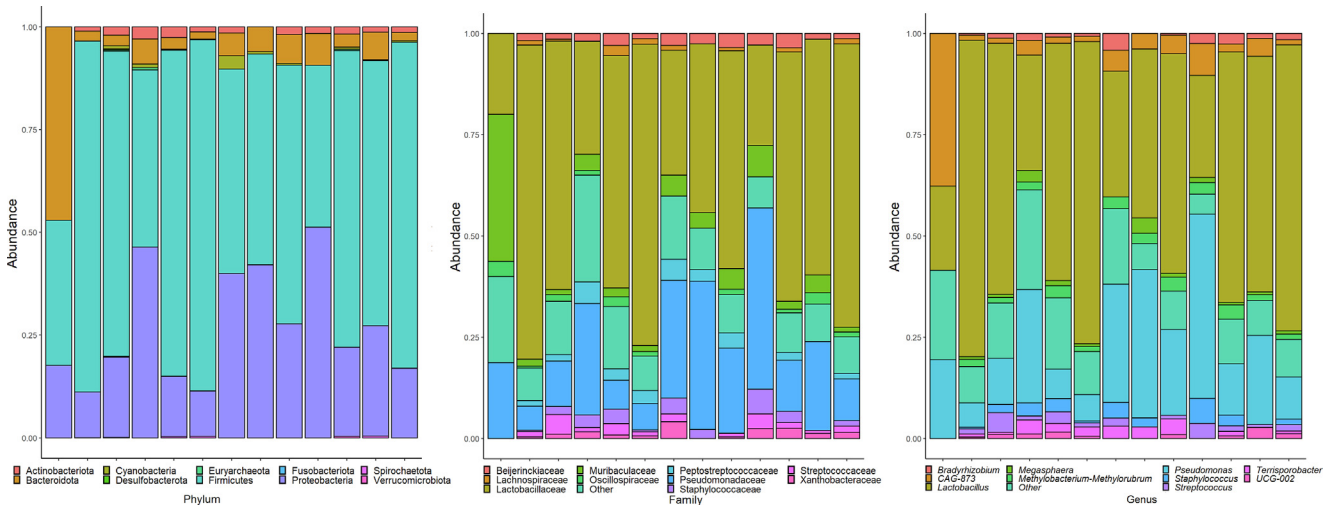

Fig. 2. Boxplot illustrating the major non-contaminant bacterial profile detected in each piglet's umbilical cord blood sample at the phylum, family and genus levels; after that, microbial sequences were processed using the Decontam R package (Davis et al., 2018).

Table 1
Major bacterial profiles detected in piglet's umbilical cord blood samples at the phylum, family and genus levels; before and after that, microbial sequences were processed using the Decontam R package (Davis et al., 2018).

	The Decontam R package			
	Before[1]		After[2]	
	Mean	SD	Mean	SD
Number of				
Phyla	17	–	10	–
Families	99	–	58	–
Genera	196	–	89	–
Relative abundance;%				
Phyla				
Firmicutes	61	4.1	63	6.2
Proteobacteria	26	4.5	27	6.3
Families				
Lactobacillaceae	44	8.7	50	10.5
Pseudomonadaceae	16	10.6	19	13.0
Muribaculaceae	5	3.7	6	7.8
Genera				
Lactobacillus	44	8.7	50	10.5
Pseudomonas	16	10.6	19	13.0
Muribaculaceae_CAG-873	5	4.5	6	8.9

[1] The major bacterial profile detected in umbilical cord blood samples of piglets at the phylum, family and genus levels; before that, microbial sequences were processed using the Decontam R package (Davis et al., 2018).
[2] The major bacterial profile detected in umbilical cord blood samples of piglets at the phylum, family and genus levels; after that, microbial sequences were processed using the Decontam R package (Davis et al., 2018).

described in the present study. Using the function *IsNotContaminant* of the Decontam R package and a FDR threshold of 0.5, the authors observed that more than 93% of the 810 ASVs were identified as contaminants.

The main limitation of the present study is that, in contrast with the experiment of Lauder et al. (2016), no negative control samples from the sampling area or from the farm environment were collected during the sampling procedure. Indeed, our results after the application of the Decontam R package showed the presence of bacteria belonging to the *Pseudomonas* genus. These bacteria are generally considered environmental contaminants, especially in water and soil, and can survive to common disinfectants and adapt to a wide range of environments (Kerr and Snelling, 2009). Similarly, a relatively high abundance of bacteria belonging to the genus *Lactobacillus* were isolated in the present samples. These bacteria are mostly found in the intestinal microflora of humans and animals; they are also common in environments contaminated by human and animal faecal material (Kagkli et al., 2007). The samples in the present study were collected in an experimental farm, and despite careful sampling precautions, faecal contamination cannot be excluded. Part of the 165 ASVs considered to not be contaminants from the extraction process thus might be contaminants from the sampling process. The hypothesis of Leblois et al. (2017) about maternal microbial transfer during gestation through the umbilical cord blood thus cannot be fully supported by the present study. In future studies aiming at investigating the microbial profile of the umbilical cord blood, one may consider sampling the vaginal mucus layer and the environmental area of the sow like the floor, the trough and the pen wall, and characterize their microbial profile to use them as negative controls. Vaginal mucosa can be easily sampled before farrowing using a sterile cotton swab (Wang et al., 2017). Similarly, Chen et al. (2018) described a procedure to sample the environmental area by scrubbing the slatted floor of the nursing pen with sterile water. One cannot exclude the possibility, that a small portion of the bacteria population isolated in the present samples (such as those belonging to the

Lactobacillus genus) originated from live bacteria or DNA fragments of the intestinal environment and transported through the bloodstream of the sow. In a study performed on mice, Macpherson and Uhr (2004) showed that intestinal dendritic cells could retain and transport a limited number of commensal bacteria for several days, although the mechanism underlying a possible interaction between dendritic cells from the intestinal environment of the mother and a pre-birth transfer of microbiota in the offspring requires further investigation.

In conclusion, the present study has highlighted the complexity of analysing microbial taxonomy data on low microbial biomass, where the concentrations of the contaminants may be higher than that of the target DNA. A few amendments are still needed, however, such as the use of negative control samples from the surroundings of the samplings and the farm environment, such as vaginal and/or environmental swabs (floor, faeces, etc.) of the farm, respectively. Although, the present workflow can be used as reference to deal with low microbial biomass and in particular with the blood from umbilical cord.

Ethics approval

This experiment was conducted in accordance with the Swiss Guidelines for Animal Welfare, and the Swiss Cantonal Committee for Animal Care and Use approved all procedures involving animals (approval number 2020_46_FR).

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Author contributions

FP and FC validated the data and conducted the main statistical and bioinformatics analyses. MG conceived the study, and MG and GB secured substantial funding. FP, FC and MG performed the animal experiments, recorded the data, and collected and processed the umbilical cord blood samples. MG, GB and PT supervised the analyses. FP and FC drafted the manuscript, and MG, GB and PT critically reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of interest

The authors declare they have no conflict of interest relating to the content of this article.

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