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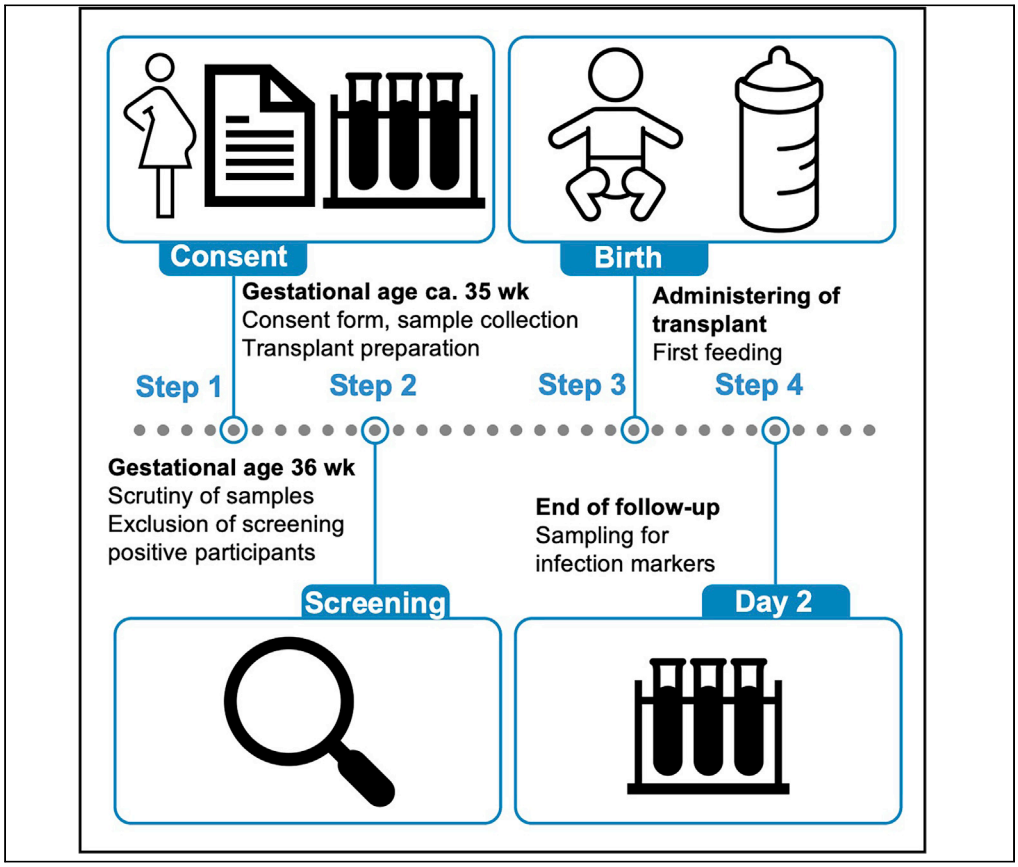
Protocol for oral transplantation of maternal fecal microbiota to newborn infants born by cesarean section



Infants born by cesarean section have an intestinal microbiota that differs from that of infants delivered vaginally. Here, we report a protocol for performing oral transplantation of maternal fecal microbiota to newborn infants born by elective cesarean section. The crucial step of this protocol is the health screening process. This protocol can only be applied to healthy mothers and infants.

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HIGHLIGHTS

Intestinal microbiota differs between infants born vaginally and by cesarean section

Protocol for oral transplantation of maternal fecal microbiota to cesarean-born infants

The crucial step in the protocol is the health screening process

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Protocol for oral transplantation of maternal fecal microbiota to newborn infants born by cesarean section

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SUMMARY

Infants born by cesarean section have an intestinal microbiota that differs from that of infants delivered vaginally. Here, we report a protocol for performing oral transplantation of maternal fecal microbiota to newborn infants born by elective cesarean section. The crucial step of this protocol is the health screening process. This protocol can only be applied to healthy mothers and infants.
For complete details on the use and execution of this protocol, please refer to Korpela et al. (2020).

BEFORE YOU BEGIN

The donors of the fecal microbial transplant need to be screened for the presence of transmittable pathogens according to local routine diagnostics (Cammarota et al., 2017). Besides the screenings suggested here, all other selection processes should take into account local fecal transplantation screening guidelines and adjusted to cover pathogens causing neonatal infections locally. Blood sample screening includes human immunodeficiency virus, human T-cell lymphotrophic virus, Treponema pallidum, hepatitis A, B, C, and E and fecal sample screening protozoa and helminths, *Entamoeba histolytica*, *Clostridioides difficile*, enteric pathogens (*Salmonella, Shigella, Campylobacter, Vibrio cholerae*, pathogenic *Escherichia coli* strains EHEC, ETEC, EPEC, EIEC, EAEC), *Helicobacter pylori*, norovirus, *Giardia lamblia, Listeria monocytogenes*, *Cryptosporidium parvum*, methicillin- resistant *Staphylococcus aureus* (MRSA), Gram-negative multidrug-resistant (MDR) bacteria and vancomycin-resistant enterococci (VRE) (Cammarota et al., 2017). The protocol was initiated before the SARS-CoV-2 pandemic and should now include screening for SARS-CoV-2 in feces. In addition, the presence of group B streptococci (GBS), extended-spectrum beta-lactamase-producing bacteria (ESBL) and methicillin-resistant *Staphylococcus aureus* (MRSA) in perinanal and cervical swabs need to be ruled out. An aliquot of the fecal screening sample is used for preparing the transplant, it is important to time this together with screening, so the transplant sample is not collected too early or too late – in our study we took the sample 3 weeks prior to the expected delivery (Korpela et al 2020).

Note: Apart from reducing the level of pathobionts that are associated with cesarean section delivery (Shao et al 2019), the transplantation procedure has not shown to provide any health benefit to infants and its long-term health consequences have not yet been studied.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fresh fecal samples	From the human subjects	n/a
Chemicals, peptides, and recombinant proteins		
Glycerol pharmaceutical grade (85%)	HUS pharmacy	n/a
Sodium chloride (NaCl), 9 mg/mL	Fresenius Kabi	371807
Experimental models: organisms/strains		
Human subjects (pregnant females, age range 18–40 years)	n/a	n/a

MATERIALS AND EQUIPMENT

Reagent	Final concentration	Amount
Sodium chloride (NaCl) (Fresenius Kabi)	9 mg/mL	13.24 mL
Glycerol pharmaceutical grade (85%)	9.97%	1.76 mL
Total (to be prepared fresh)	**n/a**	**15 mL**

Alternatives: Similar pharmaceutical products are acceptable

STEP-BY-STEP METHOD DETAILS

Recruitment of mothers

Timing: 1–2 days per potential participant

Potential participants are informed about the study and, if wishing to participate, written consent is collected.

1. Contact potential participants at least 2 weeks prior to elective cesarean section.
   a. Information leaflet is given to all interested at maternity outpatient clinic.
   b. Interested subjects are contacted by a healthcare professional associated with the study (study nurse or study physician) who provides information about the study and answers potential questions.
2. Informed consent from the potential participant.
   a. Those who are committed to joining are met by a healthcare professional associated with the study (study nurse or study physician).
   b. If a study physician is not present and a discussion with a physician is required, this is performed through phone or via a face to face appointment.
   c. If study eligibility criteria are met and the mother is willing to participate, written consent is collected.

⚠ CRITICAL: Exclusion criteria include a chronic disease (excluding asthma), known positive GBS status, travel abroad within 3 months of planned delivery or a maternal course of antibiotics within 3 months of planned delivery.

Health screening

Timing: 1–2 weeks

Subjects are screened for potential pathogens. Besides the screenings suggested here, all screening processes should take into account local fecal transplantation screening guidelines and adjusted to cover pathogens causing neonatal infections locally.

3. After written consent, the study participants (mothers) are screened in two phases.
4. First, noninvasive perianal swab samples are gathered either by the study nurse or at a laboratory station for the screening of GBS, ESBL, and MRSA analyzed according to local laboratory protocol.
5. Second, if the study participant is negative for swab-screening she is screened for other pathogens.
   a. A blood sample is collected in a routine laboratory for the screening for human immunodeficiency virus, human T-cell lymphotrophic virus, *Treponema pallidum*, and hepatitis A, B, C, and E.
   b. Fecal samples are collected at home in test tubes (included in the protocol of the laboratory performing the screening analytics) by the participant and taken by the participant or the study nurse to a routine laboratory in room temperature (15°C–25°C) for the screening for protozoa and helminths, *Entamoeba histolytica*, *Clostridioides difficile*, enteric pathogens (*Salmonella*, *Shigella*, *Campylobacter*, *Vibrio cholerae*, pathogenic *Escherichia coli* strains EHEC, ETEC, EPEC, EIEC, EAEC), *Listeria monocytogenes*, *Helicobacter pylori*, norovirus, *Giardia lamblia*, *Cryptosporidium parvum*, MRSA, MDR, and VRE. The protocol was initiated before the SARS-CoV-2 pandemic and should now include screening for SARS-CoV-2.
   c. As a part of the fecal sample screening, a part of the fresh sample taken for screening is collected for preparation of the transplant and taken to the research laboratory and prepared at room temperature (15°C–25°C) (see: Preparing the transplant).
6. If the study participant is positive for any of the screened pathogens she is informed about the specific finding, excluded from the study, and the prepared transplant discarded.

Preparing the transplant

Timing: 30 min

A fresh fecal sample from the mother is processed as soon as possible and within 6 h of donation (at least one week before elective cesarean section), prepared and frozen (“transplant”). The glycerol and NaCl are degassed during autoclavation.

7. Mix 1.76 mL of 85% pharmaceutical grade sterile glycerol and 13.24 mL of sterile NaCl (9 mg/mL) in a sterile tube in the laminar flow cabinet (see Materials and equipment).
8. Weigh the fresh fecal sample.
9. 100 mg of the fresh fecal sample is added to the 15.0 mL NaCl-glycerol mixture with a sterile wooden stick in a hood (anaerobic hood is desired) and mixed thoroughly by vortexing.
10. If any larger fecal particles are present after vortexing they can be left to precipitate. While pipetting the transplant avoid these larger particles.
11. Add 0.5 mL of the mixture to a sterile cryotube and label “transplant” with date and study code of the mother.
12. The transplant (max 3.4 mg of fecal matter) and leftover product are frozen immediately after preparation at −80°C.
13. Store both the leftover unprocessed fresh fecal sample (as a safety reference), the rest of the processed fecal sample and the transplant in −80°C until cesarean section and fecal microbiota transplantation (FMT, within one month of sample collection).

Screening evaluation

Timing: 15 min

When the routine laboratory results for screening are anticipated, they are checked and if positive for any pathogen, the study participant is excluded, and the transplant and other remaining samples discarded. This is double checked **24 h prior** to elective cesarean section.

14. Study physician checks all screening results **24 h prior** to elective cesarean section
    a. If any pathogen tests positive, the FMT is cancelled and the transplant and other remaining samples are discarded.
    b. If any screening test is unanswered, the FMT is cancelled and the transplant and other remaining samples are discarded.
15. Study nurse contacts delivery ward and informs that she (or study physician) will be present at delivery/immediately after delivery to perform the FMT.
16. Study nurse contacts mother to remind her to stimulate lactation by pumping, collecting any milk in a refrigerator and taking it with her (in a cooler) to the delivery the next day (as is customarily in routine protocol of a delivery ward).

Preparing the FMT

Timing: 10 min

On the day of the elective cesarean section, the transplant is thawed and mixed with mother's milk (pasteurized bank milk or mother's own milk).

17. Frozen transplant is collected from the lab and kept frozen while taken to the delivery ward.
    a. The study code of the transplant is checked with the identity of the mother coming to cesarean section.
18. After delivery, as close to first feeding as possible, the transplant (0.5 mL including max 3.4 mg of fecal matter) is thawed immediately by hand-warming before mixing with mother's milk and administering the milk.
19. The mother's own milk is used to which, if needed, pasteurized bank milk is added to reach a volume of 5 mL in a separate sterile test tube.
20. Draw up 0.5 mL of transplant with syringe and add to 5 mL of milk. Mix thoroughly with syringe.

Performing the FMT

Timing: 30 min

Transplant is given to the healthy newborn in mother's milk by an experienced nurse/midwife (study nurse) as a part of the infant's first feeding within 2 h of delivery.

21. Before performing the transplantation make sure that screening results have all been marked **negative** by the study physician.
22. Before feeding (typically 1.5 h after delivery), ask for an assessment of the infant's clinical situation from the midwife treating the infant.
    a. If the infant has any abnormal clinical symptoms (e.g., grunting possibly associated with transient tachypnea of the newborn) the FMT is cancelled and the transplant and other remaining samples are discarded.
23. Milk including the transplant (5.5 mL) is drawn in sterile syringe and warmed in a water bath to 30°C.
24. The infant is swaddled by the study nurse.
25. While carried in an upright position, the newborn is fed according to the normal feeding protocol for first feeding.
26. Ask for the midwife taking care of the infant to make a note of the transplant in the health record of the infant.

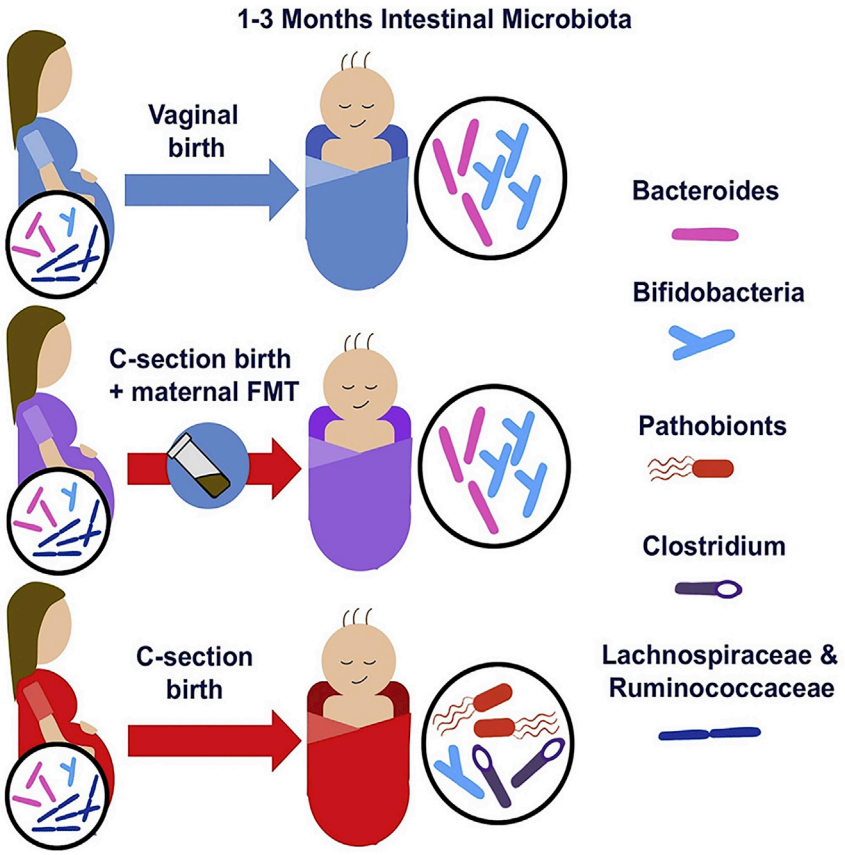

Figure 1. The study description depicting the use of the protocol
Figure reprinted with permission from Korpela et al. (2020).

⚠ CRITICAL: Screening is a crucial part of the protocol. It is imperative that screening results have been evaluated by the study physician. Transplantation shall not proceed if results are pending or positive.

Follow-up after FMT

Timing: 2 days

The infant should be observed for two days on the antenatal care ward.

27. Fever is measured four times a day.
28. A blood sample is taken for the laboratory assessment of routine infection markers (WBC, C-reactive protein) at 2 days of age before discharge.
29. If infection markers are within the normal range, regular follow-up according to the hospital protocol is sufficient.
30. Microbiota development and clinical outcomes are followed up according to the study hypotheses and protocol.

EXPECTED OUTCOMES

The outcomes of this protocol are the safety and feasibility of the transplantation process. Any assessment outcomes are dependent on the evaluation process of the application of this protocol (as an example, Figure 1 depicts a use-case of the protocol). However, as an outcome variable,

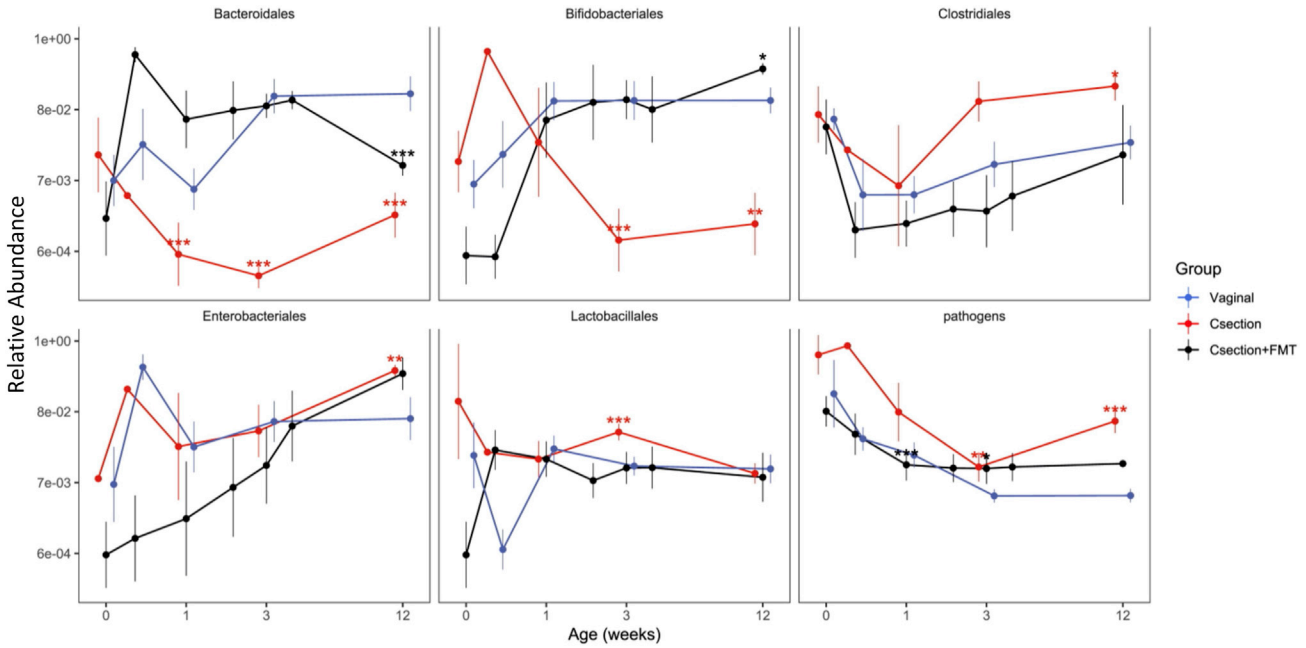

Figure 2. A results pane from the study where the protocol was implemented

Dominant bacterial families and orders are shown as means; also the combined relative abundance of the potential pathogens *Enterococcus faecium*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Haemophilus influenza*, *Campylobacter jejuni*, and *Salmonella enterica* as group means and standard errors of the mean. To prevent overlap of the data points, some small shifts in the time axis were introduced; discrete time points are at 0 (meconium), 2 days, 7 days, 2 weeks, 3 weeks, 4 weeks, and 12 weeks. Significance of the difference between the FMT-treated (black) and non-treated (red) CS groups compared with the vaginally delivered group was tested at 1, 3, and 12 weeks. The significance is shown as asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

Figure reprinted with permission from Korpela et al. (2020).

the abundance of Bacteroidales in fecal samples of FMT-treated infants at 3 weeks should be significantly higher than in samples from infants delivered by cesarean section without FMT intervention (Figure 2).

LIMITATIONS

The screening protocol requirements vary according to local circumstances, for example on drug resistance issues. Therefore, protocol should be adjusted using local FMT guidelines and antenatal practices. This protocol can only be applied to healthy mothers and infants. In addition, besides reducing the level of pathobionts that are associated with cesarean section delivery, the transplantation procedure has not shown to provide any health benefit to infants and its long-term health consequences have not yet been studied.

TROUBLESHOOTING

There is a possibility of aspiration of the milk including the transplant at first feeding.

Problem 1

Possibility of aspiration of the milk including the transplant at first feeding (performing the FMT).

Potential solution

This risk can be minimized, if the feeding is performed by following any local hospital treatment protocol for first feeding. Also, we recommend having an experienced study nurse performing the process to answer any questions that may arise from the parents or the hospital staff. If aspiration does take place, the infant should be followed according to "Follow-up after FMT" and any routine

protocol of the ward on how to proceed after aspiration of non-transplant milk. While it is unlikely, aspiration could cause an infection and septicemia. We suggest that any treatment of neonatal infection suspicion (septicemia) should be initiated according to the routine protocol for suspected neonatal septicemia after vaginal delivery, since due to FMT screening process we expect the pathogens associated with infection in infants subjected to FMT to be similar to those after vaginal delivery.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Otto Helve (otto.helve@helsinki.fi).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

O.H., W.M.d.V., and S.A. conceived the protocol for the study. E.D., A.S., K.L.K., and V.S. made substantial contributions to the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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