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DELIVERABLE

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Executive Summary

The human microbiome plays an important role in human metabolism. It is one of the most important players in the digestive process, and is therefore a key parameter to monitor when developing personalised nutrition. Microbiome analysis will be performed using a new technique developed at EPFL. It relies on cycling temperature capillary electrophoresis (CTCE) integrated with a bioinformatics and biostatistics platform.

For samples to be analysed to maximum effect, they should be fixed within 10 minutes of collection. Fixation is achieved by mixing the samples in a fixing solution. The samples are then stable at room temperature for up to 30 days.

An effective analysis requires at its basis a set of bacterial species defined as “most relevant.” The analysis will then be designed to target those. A list of 230 species has been decided upon.

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Definitions, Acronyms and Abbreviations

Acronym	Title
CTCE	cycling temperature capillary electrophoresis
OTU	operational taxonomic unit
HM	Human Milk

1. Samples requirements

1.1. Sample collection

The human microbiome will be analysed in stool and human milk samples. The bacteria composition in human stool offers a good representation of the microbiome composition in the human gut (which is the largest microbiome niche in the human body). However, as soon as the stool is ejected from the body, it will continue to ferment. The bacteria that will grow outside of the body, will not be representative of those that thrived in the gut. Furthermore, the change in environment changes the delicate balance in ecosystem that is the microbiome of the gut. Due to the speed at which some bacteria divide (as fast as 20 mins), the bacterial composition of the stool can very quickly diverge from the microbiome of the gut which is of interest for the NUTRISHIELD project.
It is therefore of fundamental importance to guarantee that the stool samples will no longer ferment once they are collected. To achieve that, samples need to be fixed immediately upon collection. The fixing procedure should be safe and easy to use by both patients as well as medical doctors (when required). Once fixed, the sample should be stable at room temperature for at least 30 days.
Sampling human stool can sometimes be unpleasant. This means that designing a sampling procedure to minimise unpleasantness for both study subjects, or anyone else collecting the samples, is important. The procedure should be easy, and not require exposure to the stool. Also it should minimise stool manipulations of any kind. A good sampling procedure will guarantee better compliance of the study subjects.
Satisfying the requirements of fixation as well as reduction of unpleasantness related to the sampling necessitates the use of a pre-made kit. This kit should contain the means to collect a small amount (2-4g) of stool and insert it into a tube containing already prepared fixative. It must be possible to introduce the collection device whole into the collection tube and to seal the tube. Simple shaking of the tube should be enough to homogenize the solution of fixative & stool sample.
There exists viable sampling kits on the market. One such example is the OMNIgene gut kit, sold by DNA genotek (Canada) which can be seen in Figure 1.

However, the NUTRISHIELD consortium is still considering the option of devising its own stool sampling kit. The advantage of which would be the full knowledge of the fixative mixture content. Which would remove potential biases.

1.2. Sample preparation

Fixed samples will require DNA extraction. The procedure related to DNA extraction needs to satisfy the following requirements:

- Reproducible
- Easy to automate
- Low cost
- High throughput
- High DNA yield
- Consistent extraction across bacterial species

As of the moment this deliverable was submitted, one of the most promising methods that satisfies all above condition is as follows:

1. Addition of NaOH
2. Microwave boiling of sample
3. Neutralisation with HCL
4. Digestion with Proteinase K for 1h
5. DNA extraction using the MagAttract system from Macherey Nagel

EPFL will however continue to research, and test different DNA extraction techniques to find the optimal strategy.
Once the DNA is extracted, it can be analysed to determine the microbiome composition.

1.3. Logistics

1.3.1. Human Milk study

In the case of the human milk study, some samples will be collected at the hospital, and others will be collected at home of the subjects. Since the study subjects are newborns, it will fall upon mothers to collect stool samples. For that purpose, a kit will be provided to them, that they can use to collect small stool samples when changing diapers. The kit will contain the fixating solution, will be easy to use an intuitive. Once fixed, the samples are stable at room temperature, where they can wait until collection or delivery to the hospital.
In the case mother's milk sample, the procedure will be similar. A kist with fixative will be provided. Mothers will have to place a small amount of milk in the collection vial already containing the fixative.
For stool sample from mothers, a standard stool microbiome sampling kit will be used. It will also rely on the immediate fixing of the sample.

1.3.2. Obesity and diabetes study

Subjects in this study are 8-18-year-old children and the stool collection procedure will be carried out at home by their parents. The parents will be instructed by medical personnel and nurses and will receive informative materials explaining how to collect and store the stool samples. They will be provided a collection kit that will make it as easy and comfortable as possible to collect their samples (for example the kits from DNA genotek). Once collected, they will be asked to keep the samples at room temperature and to deliver it to the hospital within 2 days after collection (possibly during their periodic visit to the hospital).

1.4. Storage & Shipping

In both studies the samples storage at the patient's home and after delivery to the hospital will be at room temperature. Once a sufficient number of samples are grouped, they will be shipped to EPFL for analysis. Shipping will be at room temperature, for greater ease of transport. Once they are received at EPFL they will once again be stored at room temperature until DNA is extracted.

2. Analysis requirement

2.1. Bacteria list

Taking information from the literature on microbiome, as well as the expertise from all members of the consortium, a list of around 300 species has been drafted. These species cover all major families of bacteria likely to be present in the gut of adolescents and young children.
An analytical assay has been developed using these 300 species as basis. The list of species can be found in Supplementary material 1.

2.2. Probe-set

To cover the list of bacteria described in 2.1, a set of between 48 and 96 analytical probes will be selected from the set of probes available for analysis. The aim of the selection will be to maximise resolution across the set of 300 bacteria. The possibility of choosing a different assay in the two studies remains open. Indeed, it is well reported in the literature that the microbiome of newborns has less variety that the microbiome of adolescents[1]. It might therefore be more interesting to reduce the breath of the assay, in favor of having greater resolution around the bacteria families more likely to be found in the microbiome of newborns, and especially preterm infants.
The analysis will be performed at EPFL.

2.3. data analysis

The output of CTCE will be analysed using a statistical pipeline developed at EPFL. It combines signal processing strategies with the output from the bioinformatic platform used for assay design. The output of the data analysis platform is a table containing the relative abundance of each bacterial species separated by the assay used. The segmentation of bacteria is dependent on the assay used, and is optimised according to the sample (children stool, newborn stool, and human milk).

[1] Parra-Llorca, A., Gormaz, M., Alcántara, C., Cernada, M., Nuñez-Ramiro, A., Vento, M., & Collado, M. C. (2018). Preterm Gut Microbiome Depending on Feeding Type: Significance of Donor Human Milk. Frontiers in Microbiology, 9, 1376. http://doi.org/10.3389/fmicb.2018.01376

3. Data storage requirements

3.1. Output

The raw data generated by the analysis using the REM Analytics technology is a set of electropherograms, one for each probe and each sample. Thus, the total number of electropherograms per sample will be between 48 and 96. The analysis of the electropherograms yields between 1 and 16 rational numbers. However, for the sake of completion, all the data should be stored. Each sample requires around 11 Mb of storage space in its raw form. The completely analysed form will double that number to around 24Mb.

4. Supplementary material 1

List of species

Propionibacterium acidipropionici
Propionibacterium acnes
Propionibacterium avidum
Propionibacterium freudenreichii
Propionibacterium propionicum
Bifidobacterium adolescentis
Bifidobacterium animalis
Bifidobacterium asteroides
Bifidobacterium bifidum
Bifidobacterium breve
Bifidobacterium catenulatum
Bifidobacterium dentium
Bifidobacterium gallinarum
Bifidobacterium longum
Bifidobacterium pseudocatenulatum
Bifidobacterium pullorum
Bifidobacterium ruminantium
Bifidobacterium scardovii
Bifidobacterium thermophilum
Gardnerella vaginalis
Bacteroides caccae
Bacteroides cellulosilyticus
Bacteroides clarus
Bacteroides coprocola
Bacteroides eggerthii
Bacteroides faecis
Bacteroides finegoldii
Bacteroides fragilis
Bacteroides graminisolvens
Bacteroides helcogenes
Bacteroides intestinalis
Bacteroides massiliensis
Bacteroides nordii
Bacteroides ovatus
Streptococcus infantis
Streptococcus iniae
Streptococcus intermedius
Streptococcus lutetiensis
Streptococcus macedonicus
Streptococcus mitis
Streptococcus mutans
Streptococcus oligofermentans
Streptococcus oralis
Streptococcus orisuis
Streptococcus parasanguinis
Streptococcus parauberis
Streptococcus pasteurianus
Streptococcus pneumoniae
Streptococcus pseudopneumoniae
Streptococcus pyogenes
Streptococcus ratti
Streptococcus rubneri
Streptococcus salivarius
Streptococcus sanguinis
Streptococcus sobrinus
Streptococcus suis
Streptococcus thermophilus
Streptococcus uberis
Clostridium acetobutylicum
Clostridium aminobutyricum
Clostridium autoethanogenum
Clostridium beijerinckii
Clostridium botulinum
Clostridium butyricum
Clostridium carboxidivorans
Clostridium cellulovorans
Clostridium cylindrosporum
Clostridium diolis

Bacteroides salanitronis
Bacteroides stercoris
Bacteroides thetaiotaomicron
Bacteroides uniformis
Bacteroides vulgatus
Bacteroides xylanisolvens
Dysgonomonas wimpennyi
Odoribacter splanchnicus
Paludibacter propionicigenes
Parabacteroides distasonis
Parabacteroides gordonii
Parabacteroides merdae
Porphyromonas asaccharolytica
Porphyromonas gingivalis
Tannerella forsythia
Bacillus alcalophilus
Bacillus amyloliquefaciens
Bacillus anthracis
Bacillus aquimaris
Bacillus atrophaeus
Bacillus cellulosilyticus
Bacillus cereus
Bacillus circulans
Bacillus clausii
Bacillus coagulans
Bacillus cytotoxicus
Bacillus firmus
Bacillus flavocaldarius
Bacillus halodurans
Bacillus licheniformis
Bacillus megaterium
Bacillus methanolicus
Bacillus mojavensis
Bacillus mycoides
Bacillus novalis
Bacillus pseudofirmus
Bacillus pseudomycoides
Bacillus pumilus
Bacillus smithii
Clostridium histolyticum
Clostridium kluyveri
Clostridium ljungdahlii
Clostridium longisporum
Clostridium novyi
Clostridium paraputrificum
Clostridium pasteurianum
Clostridium perfringens
Clostridium ragsdalei
Clostridium saccharobutylicum
Clostridium saccharoperbutylacetonicum
Clostridium scatologenes
Clostridium sporogenes
Clostridium subterminale
Clostridium taeniosporum
Clostridium tertium
Clostridium tetani
Clostridium tetanomorphum
Acetitomaculum ruminis
Anaerostipes caccae
Blautia coccoides
Blautia hansenii
Blautia hydrogenotrophica
Blautia producta
Butyrivibrio fibrisolvens
Butyrivibrio hungatei
Butyrivibrio proteoclasticus
Cellulosilyticum lentocellum
Cellulosilyticum ruminicola
Coprococcus catus
Lachnoclostridium phytofermentans
Roseburia cecicola
Roseburia hominis
Roseburia intestinalis
Roseburia inulinivorans
Syntrophococcus sucromutans
Tyzzerella nexilis
Acetivibrio cellulolyticus
Ethanoligenens harbinense

Bacillus sonorensis
Bacillus subtilis
Bacillus thermoalkalophilus
Bacillus thuringiensis
Bacillus weihenstephanensis
Brevibacillus brevis
Brevibacillus centrosporus
Brevibacillus choshinensis
Brevibacillus texasporus
Staphylococcus aureus
Staphylococcus caprae
Staphylococcus carnosus
Staphylococcus chromogenes
Staphylococcus epidermidis
Staphylococcus haemolyticus
Staphylococcus hominis
Staphylococcus hyicus
Staphylococcus lentus
Staphylococcus lugdunensis
Staphylococcus pseudintermedius
Staphylococcus saprophyticus
Staphylococcus sciuri
Staphylococcus simulans
Staphylococcus warneri
Staphylococcus xylosus
Enterococcus asini
Enterococcus avium
Enterococcus casseliflavus
Enterococcus columbae
Enterococcus dispar
Enterococcus faecalis
Enterococcus faecium
Enterococcus gallinarum
Enterococcus hermanniensis
Enterococcus hirae
Enterococcus moraviensis
Enterococcus mundtii
Enterococcus pseudoavium
Enterococcus raffinosus
Enterococcus ratti
Faecalibacterium prausnitzii
Ruminiclostridium thermocellum
Ruminococcus albus
Ruminococcus bromii
Ruminococcus champanellensis
Ruminococcus flavefaciens
Ruminococcus gauvreauii
Fusobacterium canifelinum
Fusobacterium mortiferum
Fusobacterium necrophorum
Fusobacterium nucleatum
Fusobacterium varium
Azospira oryzae
Helicobacter pylori
Citrobacter amalonaticus
Citrobacter braakii
Citrobacter freundii
Citrobacter gillenii
Citrobacter koseri
Citrobacter rodentium
Citrobacter werkmanii
Cronobacter helveticus
Cronobacter malonaticus
Cronobacter muytjensii
Cronobacter pulveris
Cronobacter sakazakii
Cronobacter turicensis
Cronobacter universalis
Cronobacter zurichensis
Enterobacter aerogenes
Enterobacter asburiae
Enterobacter cloacae
Enterobacter hormaechei
Enterobacter lignolyticus
Erwinia amylovora
Erwinia billingiae
Erwinia pyrifoliae
Erwinia rhapontici
Erwinia tasmaniensis
Escherichia coli

Lactobacillus acidophilus
Lactobacillus amylovorus
Lactobacillus backii
Lactobacillus brevis
Lactobacillus buchneri
Lactobacillus casei
Lactobacillus crispatus
Lactobacillus curvatus
Lactobacillus delbrueckii
Lactobacillus farraginis
Lactobacillus fermentum
Lactobacillus gasseri
Lactobacillus helveticus
Lactobacillus johnsonii
Lactobacillus kefiranofaciens
Lactobacillus paracasei
Lactobacillus pentosus
Lactobacillus plantarum
Lactobacillus reuteri
Lactobacillus rhamnosus
Lactobacillus ruminis
Lactobacillus sakei
Lactobacillus salivarius
Lactobacillus sanfranciscensis
Pediococcus claussenii
Pediococcus parvulus
Pediococcus pentosaceus
Pediococcus siamensis
Leuconostoc carnosum
Leuconostoc citreum
Leuconostoc gasicomitatum
Leuconostoc gelidum
Leuconostoc kimchii
Leuconostoc lactis
Leuconostoc mesenteroides
Oenococcus oeni
Weissella cibaria
Weissella koreensis
Weissella thailandensis
Lactococcus garvieae

Escherichia fergusonii
Escherichia hermannii
Klebsiella oxytoca
Klebsiella pneumoniae
Klebsiella variicola
Kluyvera ascorbata
Kluyvera cryocrescens
Pantoea agglomerans
Pantoea ananatis
Pantoea stewartii
Pantoea vagans
Proteus mirabilis
Proteus vulgaris
Salmonella bongori
Salmonella enterica
Serratia entomophila
Serratia liquefaciens
Serratia marcescens
Serratia proteamaculans
Serratia symbiotica
Shigella boydii
Shigella dysenteriae
Shigella flexneri
Shigella sonnei
Shimwellia blattae
Yersinia enterocolitica
Yersinia entomophaga
Yersinia intermedia
Yersinia kristensenii
Yersinia pestis
Yersinia pseudotuberculosis
Yersinia ruckeri
Yersinia similis
Pseudomonas aeruginosa
Pseudomonas alcaligenes
Pseudomonas alkylphenolia
Pseudomonas brassicacearum
Pseudomonas chlororaphis
Pseudomonas denitrificans
Pseudomonas entomophila

Lactococcus lactis	Pseudomonas extremaustralis
Lactococcus piscium	Pseudomonas fluorescens
Lactococcus raffinolactis	Pseudomonas fulva
Streptococcus agalactiae	Pseudomonas mendocina
Streptococcus anginosus	Pseudomonas mucidolens
Streptococcus australis	Pseudomonas nitroreducens
Streptococcus canis	Pseudomonas poae
Streptococcus downei	Pseudomonas protegens
Streptococcus dysgalactiae	Pseudomonas putida
Streptococcus equi	Pseudomonas savastanoi
Streptococcus equinus	Pseudomonas stutzeri
Streptococcus gallolyticus	Pseudomonas syringae
Streptococcus gordonii	Pseudomonas taetrolens
Streptococcus hyovaginalis	Pseudomonas viridiflava
Streptococcus infantarius	Akkermansia muciniphila